# Development of innovative PCR and fluorescence activated cell sorting methodologies for the detection of a range of foodborne pathogens.

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# Development of innovative PCR and fluorescence activated cell sorting methodologies for the detection of a range of foodborne pathogens

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UNIVERSITY of LIMERICK

OLLSCOIL LUIMNIGH

Department of Life Sciences

# Development of innovative PCR and fluorescence activated cell sorting methodologies for the detection of a range of foodborne pathogens

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for the degree of

**Doctor of Philosophy**

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*To Maman, Papa, Sophie, Lucie & Mamie*
*for supporting me all the way since the beginning,*
*this PhD thesis is also your success.*

*& to Francisco*
*For providing me a great source*
*of motivation and inspiration since I met you.*

*In Memory of Jean Blondeau,*
*André & Yvette Martinon*

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# Abstract


**Development of innovative PCR and fluorescence activated cell sorting methodologies for the detection of a range of foodborne pathogens**

**Alice Martinon**

The early and rapid detection of the Enterobacteriaceae, *Staphylococcus aureus* and *Listeria monocytogenes* is desirable to prevent foodborne outbreaks. In this thesis, novel molecular and flow cytometric based methods were developed for their qualitative and quantitative detection. For *S. aureus* and *L. monocytogenes*, two highly specific primer sets were selected for application in simplex and duplex SYBR Green-based real-time PCR assays. Melting curve analysis confirmed the absence of unspecific amplifications and the generation of two PCR products with distinct melting temperatures. The duplex method was successfully applied to spiked food samples after 18 h Buffered Peptone Water enrichment. For the Enterobacteriaceae, a screening real-time PCR protocol using LUX™ primers was developed after primer selection to optimise specificity. This method provided comparable data with a commercial molecular detection system when analysing infant formula milk samples. A novel strategy was developed to improve real-time PCR detection of contaminants based on quantitative standards generated by Fluorescence Activated Cell Sorting (FACS), with *S. aureus* as the reference microorganism. Optimal PCR efficiency was observed with these standards compared with calibrants prepared with genomic or plasmid DNA. Once storage and temperature conditions are further optimised, FACS generated standards may provide an alternative method to improve accuracy of PCR assays for bacterial quantification. PCR and FACS-based methods were then applied for enumeration of viable foodborne pathogens swabbed from artificially contaminated food-processed surfaces. Sample preparation used Swab Extraction Tube Systems (SETS) for bacterial recovery and Propidium Monoazide (PMA) to remove DNA from dead cells. No significant difference was found between PMA real-time PCR and plate counts. The developed PMA real-time PCR methods may be suitable for samples containing high bacterial loads or for monitoring of disinfection efficacy. Overall, the novel approach of combining PCR and FACS-based methodologies represents valuable progress in the rapid and accurate detection of foodborne pathogens.

# Declaration of Originality

"I hereby certify that the work on this thesis is based on my own independent work except where I have received help as stated in the acknowledgement and text.

All citations and summary of the work of others have been acknowledged where appropriate.

Permission is granted for this work to be copied in whole or in part for the purpose of private study, provided that in each case the user acknowledges the source and his or her indebtedness to the author."

**Alice Martinon**

Signed: _____

Date: _____

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## Publications

Martinon, A., and Wilkinson, M. G. (2011) 'Selection of Optimal Primer sets for use in a duplex SYBR Green-based Real-time PCR Protocol for the Detection of *L. monocytogenes* and *S. aureus* in Foods', *Journal of Food Safety*, DOI 10.1111/j.1745-4565.2011.00301.x.

Martinon, A., Cronin, U. P., and Wilkinson, M. G. (2011) 'Comparison of In-house and Commercial Real-time PCR Systems for the Detection of Enterobacteriaceae and their Evaluation Within an Interlaboratory Study Using Infant Formula Samples', *Food Analytical Methods*, DOI 10.1007/s12161-12010-19188-12167.

Martinon, A., Cronin, U. P., and Wilkinson, M. G. (2011) 'Development of Defined Microbial Population Standards Using Fluorescence Activated Cell Sorting for the Absolute Quantification of *S. aureus* Using Real-time PCR', *Molecular Biotechnology*, DOI 10.1007/s12033-12011-19417-12033.

## Poster

Martinon, A., Cronin, U.P., and Wilkinson, M. G. (2008) 'Assessment of Real-Time PCR Procedures for the Detection and Quantification of Enterobacteriaceae Using SYBR Green I or LUX™ primers'. 21$^{rst}$ ICFMH Symposium Food Micro 2008, Rapid Methods section, Aberdeen, Scotland.

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# Abbreviations

| Abbreviation | Full word |
|---|---|
| A | Adenine |
| AFNOR | Association Française de Normalisation |
| ALOA | Agar *Listeria* according to Ottaviani and Agosti |
| ANOVA | ANalysis Of Variance |
| AOAC | Association of Official Agricultural Chemists |
| ATCC | American Type Culture Collection |
| ATP | Adenosine triphosphate |
| ATR | Acid tolerance response |
| $a_w$ | Water activity |
| BLAST | Basic Local Alignment Search Tool |
| BHI | Brain Heart Infusion |
| BPW | Buffered Peptone Water |
| BSA | Bovine Serum Albumine |
| CFU | Colony Forming Unit |
| DAEC | Diffusely adherent *Escherichia coli* |
| dsDNA | double stranded DNA |
| C | Cytosine |
| cDNA | copy DNA |
| Cp | Crossing point |
| csv | Comma Separated Values |
| CSIM | *Cronobacter sakazakii* Isolation Medium |
| CTC | 5-cyano-2,3-ditolyltetrazolium chloride |
| Ct | Threshold Cycle |
| CV | Coefficient of variation |
| DGGE | Denaturing Gradient Gel Electrophoresis |
| DMSO | Dimethyl sulfoxide |
| d(NTP) | Deoxynucleotide triphosphate |
| DNA | Deoxyribonucleic Acid |
| DNase | Deoxiribonuclease |
| DST® | Defined Substrate Technology® |
| dUTP | Deoxi-uridine-triphosphate |
| E | Efficiency |
| EAEC | Enteroaggregative *Escherichia coli* |
| EC | European Community |
| EDTA | Ethylenediaminetetraacetic acid |
| EE | Enterobacteriaceae Enrichment |
| EHEC | Enterohaemorrhagic *Escherichia coli* |
| EIEC | Enteroinvasive *Escherichia coli* |
| ELISA | Enzyme-linked immunosorbent assay |
| EMA | Ethidium monoazide |
| EPEC | Enteropathogenic *Escherichia coli* |
| eps | Event per second |
| ETEC | Enterotoxigenic *Escherichia coli* |
| FACS | Fluorescence Activated Cell Sorting |
| FAM | 5-carboxyfluorescein |
| FCI | Fieller's Confidence Interval |
| FCM | Flow Cytometry |
| FISH | Fluorescence *in situ* hybridization |
| FITC | Fluorescein isothiocyanate |

| | |
|---|---|
| FL | Fluorescence |
| FRET | Fluorescence Resonance Energy Transfer |
| FSC | Forward Scatter |
| G | Guanine |
| GIT | Gastro-intestinal tract |
| GMP | Good manufacturing practices |
| HACCP | Hazard analysis and critical control points |
| HEX | 6-carboxy-2´ ,4,4´ ,5´ ,7,7´ -hexachlorofluorescein succinimidyl ester |
| HI | Hexidium iodide |
| HRM | High Resolution Melting |
| IC | Internal Control |
| IMS | Immunomagnetic separation |
| ISO | International Standard for Organization |
| ISPCR | *in-situ* PCR |
| ITS | Transcribed spacer region |
| JOE | 6-carboxy-4,5-dichloro-2,7-dimethoxyfluorescein |
| LB | Luria Bertani |
| LSA | *Listeria* Selective Agar |
| LUX™ | Light Upon eXtension™ |
| MIS | Microbial Identification System |
| mLST | modified Lauryl Sulphate Tryptose |
| *mpl* | Metalloprotease |
| MPN | Most Probable Number |
| mRNA | messenger Ribonucleic Acid |
| MUG | 4-methyl-umbelliferyl-ß-D-glucuronide |
| NA | Nutrient agar |
| NB | Nutrient broth |
| NCTC | National Collection of Type Cultures |
| NADPH | Nicotinamide adenosine dinucleotide phosphate |
| NCBI | National Center for Biotechnology Information |
| *nuc* | thermonuclease |
| ONPG | Ortho-nitrophenyl-ß-galactopyranoside |
| ORF | Open Reading Frame |
| PALCAM | Polymyxin-acriflavine-LiCl-ceftazidime-aesculin-mannitol |
| PBS | Phosphate Buffer Saline |
| PCA | Plate Count Agar |
| PCR | Polymerase Chain Reaction |
| PEG | Polyethylene glycol |
| PFZ | Peptone Physiological Salt |
| PI | Propidium iodide |
| PMA | Propidium azide or phenanthidium, 3-amino-8-azido-5[3-(diethylmethylammonio)]-6-phenyl dichloride |
| PMT | Photomultiplier |
| $R^2$ | Correlation coefficient |
| RT PCR | reverse transcriptase |
| SD | Standard deviation |
| SFP | Staphyloccocal food poisoning |
| SE | Staphylococcal enterotoxin |
| SETS | Swab Extraction Tube System |
| SGU | Signal Generating Unit |
| SSC | Side Scatter |
| STEC | Shiga toxin-producing *Escherichia coli* |
| T | Thymine |

| | |
|---|---|
| $T_A$ | Annealing temperature |
| TET | 6-carboxy-2´ ,4,7,7´ -tetrachlorofluorescein succinimidyl ester |
| TGGE | Temperature Gradient Gel Electrophoresis |
| $T_M$ | Melting temperature |
| TRFLP | Terminal Restriction Fragment Length Polymorphism |
| TSA | Tryptic Soya Agar |
| TSB | Tryptic Soya Broth |
| TSBS | Tryptic Soy Broth Salt |
| TSS | Toxic Shock Syndrome |
| TSST | Toxin Shock Syndrome Toxin |
| TVC | Total Viable Count |
| USDA | United States Department of Agriculture |
| UVM | University of Vermont Media |
| vol/vol | Volume for volume |
| VRBGA | Violet Red Bile Glucose Agar |
| w/w | Weight for weight |
| WGA | Wheat germ agglutinin |
| WHO | World Health Organization |
| YPCE | *Yersinia*-PCR-Compatible-Enrichment |

*La recherche comporte et comportera*

*toujours une part importante d'activité créatrice.*

*Pierre Joliot*

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# CHAPTER ONE

## Literature Review:

## An overview of PCR detection systems for Foodborne Pathogens and their Potential Improvement using Flow Cytometry

# 1. INTRODUCTION

Bacterial pathogens are of major concern for the food industry that may face litigation, adverse publicity and food recalls as consequences of foodborne outbreaks of disease. Microbial analysis is an essential contributor to assuring the safety of foods (Kennedy and Wall, 2007). Conventional or routine bacterial testing is mainly based on ISO (International Organization for Standardization) agar-based methods that are acknowledged as the reference analytical methods by internal, external and government laboratories. Although such methods are recognized as being relatively cheap, they are laborious, time consuming and require large volumes of media and reagents (Jasson *et al.*, 2010). Alternative rapid methods are a potential opportunity for food industry as they may allow reliable data to be obtained more efficiently with improved sensitivity within a shorter analytical time. Numerous alternative methods are emerging in food diagnostics including Polymerase Chain Reaction (PCR) and flow cytometry (FCM) technologies. Indeed conventional PCR is now a familiar analytical tool in food microbiology. However, real-time PCR using a range of detection formats may allow wider acceptance of DNA (Deoxyribonucleic Acid)-based molecular technologies as it is more rapid, sensitive and reproducible with minimal carry-over contamination (Mackay, 2004). Various real-time PCR systems have been developed in-house or are commercially available but with high associated costs per reaction. Nevertheless quantitative real-time PCR creates new possibilities in microbial enumeration methods. FCM is an emerging topic of research and also promises to improve on existing microbial molecular detection techniques. Therefore it is of benefit to investigate these new trends in microbial enumeration by the improvement of real-time PCR quantification with FCM techniques.

# 2. FOODBORNE BACTERIA

## 2.1. Microbial quality and safety of foods

The quality and safety of a food product depends on the presence of foodborne microorganisms, such as bacteria (*Bacillus*, *Pseudomonas* spp., some Enterobacteriaceae), that may induce spoilage or food contamination. Spoilage is due to undesired changes in the presentation of foodstuffs such as: appearance or colour, taste (acid, sulphurous, rancid), odour, texture or composition (gas formation or transformation of the alcohol into vinegar). *Bacillus*, *Pseudomonas* spp. or some Enterobacteriaceae have been reported to be involved in spoilage processes (Gram *et al.*, 2002). In the case of a food poisoning infection, the incriminated microorganisms, for instance members of the Enterobacteriaceae or *Listeria monocytogenes*, are pathogens as they can proliferate (Brown, 2008) and produce widespread inflammation of the gastrointestinal tract depending on factors such as the initial bacterial number ingested and the incubation period. Food intoxications or poisonings are caused by pathogenic microorganisms for example some Enterobacteriaceae, *S. aureus* or *Clostridium botulinum* that produce toxins in foods and / or the digestive system (Brown, 2008). Toxi-infections can be caused by various factors where pathogens are exposed to suitable temperatures of growth for long periods during storage of the food product. The integrity of the cold chain is therefore essential to ensure the microbial safety of the foodstuffs. Defective heat treatments can be the primary origin of food contamination. Moreover, cross contaminations from raw to cooked food and poor hygiene practices further increase the risk of contamination.

European Commission legislation (Anonymous, 2005a) has implemented microbiological criteria for certain important foodborne bacteria, their toxins and metabolites, such as *Salmonella*, *Listeria*, *Cronobacter sakazakii*, staphylococcocal enterotoxins and histamine in specific foodstuffs. These food safety criteria define the acceptable concentration limits of foodborne pathogens in foodstuffs released on the market throughout their entire shelf-life. Moreover, these criteria can be used as reference points in the validation of hazard analysis and critical control points (HACCP) principles applied to all food production post-farm (Gaynor, 2007). This literature review focuses particularly on properties of the Enterobacteriaceae, *S. aureus* and *L. monocytogenes* that are used as reference microorganisms for the research aspect of this thesis.

## 2.2. The Enterobacteriaceae

### 2.2.1. Properties of the Enterobacteriaceae

As described by Janda (2006b), the Enterobacteriaceae family includes more than 40 genera, and almost 200 species. Members of this family are of importance in food microbiology as they include intestinal pathogens, indicators of hygiene, sanitation, and food safety as well as agents of food spoilage (Stiles, 2000). Typically 0.3 to 1.8 µm-lengths, the cells are straight, rod-shaped although longer filamentous forms are present in many genera or if the bacterium is exposed to certain environmental conditions. Cells are motile or non-motile, and often have peritrichous flagella (Baylis, 2006). Some Enterobacteriaceae grow better or are more metabolically active at 25-30°C while most of the species grow well at 37°C; five representative species (*Proteus mirabilis*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Enterobacter cloacae* and *Hafnia alvei*) are considered to grow between 0 to 30°C, within a percentage of aqueous NaCl ranging from 0.5 to 10%, a pH between 4 and 7 and a water activity ($a_w$) from 0.94 to 1% (Baylis, 2006). Gram negative and facultatively anaerobic, the Enterobacteriaceae ferment D-glucose, often with gas production. The "coliform" bacteria are the lactose fermenting members of the Enterobacteriaceae that include *Enterobacter*, *Escherichia*, *Citrobacter* and *Klebsiella* species characterised by their ß-galactosidase activity (Baylis, 2006; Stiles, 2000). Another characteristic is the absence of the cytochrome oxidase activity (Baylis, 2006). Overall, most of the Enterobacteriaceae are catalase positive and reduce nitrate.

### 2.2.2. Pathogenic Enterobacteriaceae

Among the *Escherichia* species, only one can cause food poisoning: *E. coli* (Forsythe and Hayes, 2000). This highly studied bacterial species is mainly present in human and animal faeces and is therefore refers to as faecal coliform. It can be an indicator of faecal pollution but can also cause food poisoning. As stated by Janda (2006a), when present within the gastro-intestinal tract (GIT), commensal *E. coli* is beneficial to the host as it does not have self virulence factor such as for pathogenic *E. coli* which can affect immuno-compromised individuals or persons who receive a heavy inoculum. The diarrheagenic *E. coli* strains can be enteroinvasive (EIEC), enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroaggregative (EAEC), diffusely adherent (DAEC) or enterohaemorrhagic (EHEC). The latter corresponds to a subgroup of a much larger group of *E. coli* called Shiga toxin-producing *E. coli*

(STEC) producing verocytotoxins or Shiga toxins that have similarities in structure with Shiga toxin from *Shigella dysenteriae* I (Baylis, 2006; Janda, 2006a). Among the EHEC strains, the one of most concern in Europe to date is *E. coli* 0157:H7 that causes hemolytic colitis and hemolytic uremic syndrome at the low infectious dose of a fewer than 50 organisms and can induce death (Duffy *et al.*, 2000; Shinoda *et al.*, 1997; Tilden *et al.*, 1996). *Cronobacter sakazakii* is a pathogen of concern in infant nutrition. Infant formula milks occasionally get contaminated and represent a significant health risk to new-borns (Chenu and Cox 2009; Drudy *et al.* 2006; Friedemann 2008; Giovannini *et al.* 2008). It can provoke severe neonatal sepsis and meningitis. The genus *Salmonella* includes 2000 serotypes or serovars involved in food poisoning also known as salmonellosis. The most known are *S.* Typhi and *S.* Paratyphi, responsible for typhoid and paratyphoid fever (Baylis, 2006). *Shigella* spp. are related to shigellosis. The symptoms include diarrhoea and/or dysentery with frequent mucoid bloody stools, abdominal cramps and tenesmus (Niyogi, 2005). Commonly associated with pork and meats, *Yersinia enterocolitica* is responsible for yersinosis. This pathogen can cause various symptoms according to the age of the person infected. In children, fever, abdominal pain, and diarrhoea which is often bloody in appearance (Anonymous, 2005b). A closely related species, *Y. pestis* is the causative agent of the bubonic plague. *Klebsiella pneumoniae* is a common hospital-acquired pathogen, causing urinary tract infections, nosocomial pneumonia, and intra-abdominal infections (Ko *et al.*, 2002).

### 2.2.3. Enterobacteriaceae in foods

Foodstuffs can be contaminated directly or indirectly by Enterobacteriaceae as such microorganisms may be present as contaminants from different sources or may be present within the indigenous flora of the food. This is the case for unprocessed or raw foods (Baylis, 2006). The Enterobacteriaceae like *E. coli*, *Salmonella* or *Yersinia* are present in milk and dairy products, particularly in raw milk. Raw meat, poultry and other associated products may be contaminated particularly by *E. coli* or *Salmonella*. During slaughtering, evisceration and cutting operations of the carcasses, the Enterobacteriaceae may infect the food chain despite good hygiene procedures. Enteric organisms may be transferred to carcasses from the intestines, stomach, oesophagus or stomach contents (Hocquette *et al.*, 2005). Indeed, freshly slaughtered meats are normally contaminated by low levels of Enterobacteriaceae such as *E. coli*,

*Salmonella* and *Y. enterocolitica*. Crowley *et al.* (2005) found at least 3 log more Enterobacteriaceae in fresh, unpackaged minced beef than in pre-prepared or pre-packaged minced beef. Fish and other seafood can be contaminated by Enterobacteriaceae which may be present in their GIT, in polluted waters or even from the processing environment. Fresh fruit and vegetables are also good media for the growth of Enterobacteriaceae. Faecal contamination from contaminated soil and irrigation water can be added to the flora on fruit and vegetables. Some examples of outbreaks are described in Table 1.1.

**Table 1.1.** Some recent foodborne outbreaks linked to Enterobacteriaceae.

| No of cases | Microorganism | Consumed food | Place and year of the outbreak | Source of the outbreak | Reference |
|---|---|---|---|---|---|
| 103 | *Shigella spp* or *Shigella* + *Salmonella spp* | Mixed chicken and rice dish | Thailand, 2005 | Inadequate hygienic conditions | Chanachai *et al.* (2008) |
| 9 | *Escherichia coli* 0157:H7 | Steaks injected with marinade | USA, 2003 | Transfer of the pathogen likely by the tenderizing and injection processes / Safety issues presented by non-intact steak products | Laine *et al.* (2005) |
| 77 | *Escherichia coli* 0157:H7 | Shredded iceberg lettuce | USA, 2006 | NS[a] | Sodha *et al.* (2011) |
| 33 | *Salmonella Panama* | Fresh fruit juice | The Netherlands, 2008 | Unpasteurized fruit juice | Noel *et al.* (2010) |
| 4 | *Escherichia coli* 0157:H7 | Different products from a fast food outlet | UK, 2009 | NS | Hart *et al.* (2009) |
| 17 | Shiga toxin-producing *Escherichia coli* (STEC) O103:H25 | Fermented sausage | Norway, 2006 | NS | Sekse *et al.* (2009) |
| 47 | *Shigella dysenteriae* type 2 | Sugar snaps | Sweden, 2009 | Insufficient international certification and quality standards? | Lofdahl *et al.* (2009) |
| 1500, 2 deaths | *Salmonella enterica* serotype Saintpaul | Mexican style food | USA, 2008 | Contaminated Jalapeno and Serrano raw peppers | Behravesh *et al.* (2011) |
| 2469 | *S. enteritidis* | Mashed potatoes with egg | Turkey, 2008 | Undercooked mashed potatoes and eggs | Kilic *et al.* (2010) |
| 130 | *Salmonella enterica serotype Newport* | Lettuce | UK, 2004 | Impossible to find the source | Irvine *et al.* (2009) |
| 53 | *Salmonella enterica* Serovar Typhimurium | Marlin mousse | Turkey, 2008 | NS | Issack *et al.* (2009) |
| 17, 3 deaths | *Cronobacter sakazakii* | Infant formula milk | France, 1994 | NS | Townsend *et al.* (2008) |
| 12 | *Cronobacter sakazakii* | Infant formula milk | Belgium, 1998 | Insufficient norms regarding microbial contamination of powdered infant milk formula | Van Acker *et al.* (2001) |
| 10 | *Yersinia enterocolitica* | Pasteurized milk | USA, 1995 | Postpasteurization contamination of milk. | Ackers *et al.* (2000) |
| 116 | *Shigella sonnei* | Raw carrot | Hawaii, Japan, Australia, 22 US states, and American Samoa, 2004 | Food hygiene deficiencies | Gaynor *et al.* (2009) |

[a] Not specified

## 2.3. *Staphylococcus aureus*

### 2.3.1. General characteristics

The genus *Staphylococcus* is included in the Micrococcaceae family. *S. aureus* is a Gram-positive and catalase positive coccus. Cells are spherical single or paired cocci, but mainly form grape-like clusters and are approximately 0.5 to 1 μm diameter. *S. aureus* is non-motile, aerobic or a facultative anaerobe with both respiratory and fermentative metabolism. In aerobiosis, colonies on non-selective media are creamy with a golden yellow pigment. *S. aureus* can grow over a wide range of temperatures (7-48°C) with an optimum temperature between 30-37°C (Schmitt *et al.*, 1990). It is resistant to freezing and thawing and survives well in foods stored at -20°C. The pathogen can grow over a pH range of 4.2 to 9.3, with its optimum range being 7 to 7.5 (Bergdoll, 1989). Concerning the water activity $a_w$, staphylococci can grow at values lower than non-halophilic bacteria and growth has been shown as low as $a_w$ 0.83 but the minimum $a_w$ is 0.86 (McClure, 2007). *S. aureus* survives in up to 15% NaCl concentrations (Le Loir *et al.*, 2003). *S. aureus* is also characterised by the synthesis of coagulase and deoxiribonuclease (DNase) or thermostable nucleases (Chang and Huang 1995; Tang *et al.* 2008). The staphylococcal cell wall is resistant to lysosyme digestion but sensitive to lysostaphin that cleaves the pentaglycin bridges (Ablain *et al.*, 2009). Some *S. aureus* strains produce staphylococcal enterotoxins (SEs) causing staphylococcal poisonings. Colombari (2007) described 18 serological SEs but more than 20 exist (Larkin *et al.* 2009). Parts of the pyrogenic toxins, the SEs are short and highly stable proteins that are soluble in water and saline solutions (Le Loir *et al.*, 2003). The quantity of SE synthesised depends on the staphylococcal strain. However, the toxins are produced at extremely low levels during exponential phase but their concentration increases largely during late exponential or early stationary phase of growth (McClure, 2007). Enterotoxins are classified by serological types (Le Loir *et al.*, 2003). SE A to E and SE H have an emetic activity depending of the SE type. The newly discovered SE are classified with existing ones on the basis of partial or complete genome sequences analyses and structural comparisons.

### 2.3.2. Staphyloccocal food poisoning (SFP)

Also called staphylococcal enterointoxication, SFP is the result of ingestion of enterotoxins produced during the growth of staphylococci (McClure, 2007) and is a gastrointestinal disease. Little is known about the mechanisms of SEs but they may have a direct impact on the intestinal epithelium and on the vagus nerve, which may stimulate the emetic centre and affect gut transit times (Arbuthnott *et al.*, 1990). Food poisoning symptoms can include nausea, vomiting, retching, abdominal cramps and diarrhoea, sometimes with headaches, general weakness, dizziness, chills and perspiration. Symptoms usually occur between 2 and 4 hours after ingestion. The illness is self-limiting and recovery is rapid. *S. aureus* enterotoxins along with Toxin Shock Syndrome Toxin-1 (TSST-1) are often referred to as superantigens that can initiate the immune system and create Toxic Shock Syndrome (TSS). As described by Issa and Thompson (2001), these proteins bind directly to molecules of the major histocompatibility complex, class II to enhance a massive T-cell activation. Between 5% and 30% of the entire T-cell population may be activated in contrast to conventional antigens that only activate about 0.01% to 0.1% of the T-cell population. A massive release of cytokines is responsible for many of the clinical signs of TSS: malaise, myalgias, diarrhoea, and chills often preceded by fever, confusion, lethargy with hyperventilation, hypotension, tachycardia, and erythematous rash. Complications may also occur.

### 2.3.3. SFP outbreaks

*S. aureus* is ubiquitous and survives on the body surfaces of warm-blooded animals. Usually a commensal, the microorganism is also opportunistic and causes infection *via* an open wound (McClure, 2007). Humans are overall the main source of contamination to others or to foods. Animals are also a significant reservoir of *S. aureus*. For instance, mastitis may cause milk contamination before and during processing (Akineden *et al.* 2011). Many foods have been implicated in SFP: ground beef, seafoods, delicatessen salads, cream-filled bakery products, fermented foods, dried milk powder and pasta (McClure, 2007). Transmission into foods can occur by direct or indirect contact, or else *via* the respiratory tract. Contamination of foodstuffs may be the result of poor hygienic practices. Some recent outbreaks involving SE-poisoning are described in Table 1.2.

**Table 1.2.** Examples of some recent foodborne outbreaks linked to *S. aureus.*

| No of cases of SFP | Consumed food | Place and year of the outbreak | Source of the outbreak | Reference |
|---|---|---|---|---|
| 10,000 | Traditional-style Japanese deserts | Japan, 2000 | Contaminated powdered low fat milk | Asao *et al.* (2003) |
| 42 | Chicken pancake | Brazil, NS[a] | Food handlers | do Carmo *et al.* (2003) |
| 40 | Milk products | Austria, 2007 | Cows | Schmid *et al.* (2009) |
| 150 | Cake | Argentina, 2006 | Inadequate hygienic conditions, lack of refrigeration and cold chain disruption | Lopez *et al.* (2008) |
| > 100 | Potato balls fried in vegetable oil | India, 2005 | Food handler himself | Nema *et al.* (2007) |
| 8 | Mashed potatoes | Norway, 2003 | Contaminated raw bovine milk used in the preparation of mashed potato | Jorgensen *et al.* (2005) |
| 50, 328 | Minas Cheese | Brazil, 1999 | Food handlers, cattle mastitis | Do Carmo *et al.* (2002) |
| 21 | Scrambled egg | Japan, 1999 | Food handlers? | Miwa *et al.* (Miwa *et al.*, 2001) |

[a] Not specified

In every case of SFP, the foodstuff or one of its ingredients had been contaminated with SE-producing *S. aureus* that were allowed to grow at adequate temperatures for time (Le Loir *et al.*, 2003). The infective dose purported to induce SFP in humans is estimated to be around 0.1 µg but it may vary according to the patient sensitivity. While heat treatment kills the bacteria; SEs however remain intact and active as they are thermostable (Cunha *et al.*, 1996). Therefore, it is important to confirm an outbreak by detecting the toxin in the implicated food.

## 2.4. *Listeria monocytogenes*

### 2.4.1. Taxonomy and description

Firstly described by Murray *et al.* (1926) and Pirie (1927), the bacterium was known under the name *Bacterium monocytogenes* after isolation from dead rabbits where the immune response showed an abnormal increase of monocytes in blood. In 1940, Pirie modified the genus, attributing it to *Listeria*, as a dedication to the English surgeon Sir Joseph Lister (Giaccone and Ottaviani, 2007). The genus *Listeria* is composed of seven species: *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. ivanovii*, *L. grayi* and *L. marthii*. *L. monocytogenes* is taxonomically close to the

*Lactobacillaceae* (Giaccone and Ottaviani, 2007) and is "a small, Gram-positive rod, 1-2 μm in length and 0.5 μm wide, with bacteria often lying parallel to each other in palisades, and thread-like forms present in some cultures" (Low and Donachie, 1997). *L. monocytogenes* colonies are small, smooth, slightly flattened and milky white by reflected light on nutrient agar (Gray and Killinge.Ah, 1966). When cultured on Polymyxin-acriflavine-LiCl-ceftazidime-aesculin-mannitol (PALCAM) or Oxford media, *Listeria* colonies are black. Among its biochemical properties confirming identification, it produces catalase, is positive in the Voges-Proskauer reaction and hydrolyses aesculin. It is indole and oxydase negative, does not hydrolyse urea or reduce nitrates, and there is no liquefaction of gelatine.

*L. monocytogenes* is a psychrotrophic bacterium as it can grow at temperatures below 4°C and even close to 0°C (Gandhi and Chikindas, 2007; Junttila *et al.*, 1988). The optimum temperature range is 30-37°C but growth has been observed at 45°C (Jemmi and Stephan, 2006). Cultures incubated at room temperature showed motile bacteria with many peritrichious flagellae (Peel *et al.*, 1988). Survival of *L. monocytogenes* after heat shock at 60°C is induced by the synthesis of cold shock proteins (Agoston *et al.* 2009). Chan and Wiedmann (2009) highlighted the risk of infection of *L. monocytogenes* in refrigerated ready-to-eat foods stored for extended times.

Before the infection stage in mammalians, *L. monocytogenes* has to respond to acid stress in environments such as acidic foods (Beales 2004; Gahan *et al.* 1996), the stomach before reaching the intestines (Sleator *et al.* 2009) and the phagosome of human cells (Fleming and Campbell, 1997). The acid tolerance response (ATR) *of L. monocytogenes* is an abnormal resistance to lethal acid after an exposure to mild acidic conditions (Davis *et al.* 1996; Kroll and Patchett 1992). It is activated by a series of physiological and biochemical strategies (Cotter *et al.*, 2000, 2001; Cotter *et al.*, 2005; Gahan *et al.*, 1996).

*L. monocytogenes* responds to osmotic stress by physiological changes and modification of gene expression for increase or decrease of different proteins. The induction of protein following salt stress has been extensively studied by Duche *et al*. (2003; 2002(a); 2002(b)).

### 2.4.2. Foodborne listeriosis: clinical aspects

Since the nineteen fifties, following an increase in descriptions of cases in humans with subsequent isolation, *L. monocytogenes* has been considered pathogenic. Gray and Killinger (1966) and Seeliger (1975) showed the importance of infections by *L. monocytogenes* in human pathology. Listeriosis reaches essentially more at-risk populations, including: pregnant women, infants, elderly people, immuno compromised subjects or individuals suffering from debilitating pathologies. However, the bacterium can also target normally immunocompetent people without any identified risk factor (Goulet and Marchetti, 1996).

The disease is commonly a fatal infection of the blood stream and the Central Nervous System (meningitis or meningoencephalitis). In the context of a pregnancy, miscarriages, *in utero* foetal deaths, premature births or severe neonatal infections associated to a high rate of neonatal mortality have described following infection (Mylonakis *et al.*, 2002). The non-invasive forms basically include febrile gastroenteritis cases following ingestion of massively contaminated foods for all age categories concerned (Ooi and Lorber, 2005).

Listeriosis diagnosis is performed using microbiological tests that isolate *L. monocytogenes* from normally sterile samples (blood, cephalorachidian fluids, placenta, etc.) or perinatanal samples. Additional tests like serologic or molecular diagnosis may be performed (Rocourt and Jacquet, 1995). Despite its prevelance in the environment, vegetables, animals and foods, systemic listeriosis is fortunately rare (Berche *et al.*, 1990). Infections caused by *L. monocytogenes* are rare, but serious, and sometimes fatal. The prognosis depends on the rapid prescription of antibiotics active against *L. monocytogenes* (Goulet and Marchetti, 1996; Hof, 2004). *L. monocytogenes* is normally sensitive to all common antibiotics, except cephalosporins (Hof, 1991). The reference treatment is based on the synergic activity of strong doses of amoxicillin and gentamicin (Hof, 2004). However, the horizontal transfer of resistance genes has been described among the different *Listeria* species and also with other microorganisms like *Staphylococcus*, *Streptococcus* and *Enterococcus spp*. (Charpentier and Courvalin, 1997; Poyart-Salmeron *et al.*, 1990). Various studies have shown the emergence of resistant strains to one or several major antibiotics used in the treatment against listeriosis, including ampicillin (Li *et al.* 2007; Morvan *et al.* ; Srinivasan *et al.* 2005; Walsh *et al.* 2001).

### 2.4.3. Epidemiology

*L. monocytogenes* is ubiquitous in the environment; it survives in soils (Dowe *et al.*, 1997) notably in cultivated fields from which it contaminates fruits, vegetables and animal feed. With forage it passes to livestock and therafter to humans (Driehuis and Elferink 2000; Hinton 2000). For this reason, the microorganism is often isolated from unpasteurised milk, fresh and processed meat and fish products. *L. monocytogenes* can survive in manure for more than 90 days (Nicholson *et al.*, 2005) and then contaminate fields. In industry, the pathogen originating from the environment and raw or pre-transformed material can easily settle on working surfaces and equipment by creating a biofilm, with a subsequent risk of contamination of ready-to-eat foods (Chung *et al.* ; Lianou and Sofos 2007; Little *et al.* 2009). In addition, *L. monocytogenes* can survive in closed environments such as packed foods (McLauchlin *et al.*, 2004). Foodborne listeriosis outbreaks tend to occur in cheeses and pre-cooked food eaten without further cooking or heating, or processed by hand (Table 1.3).

**Table 1.3.** Some recent foodborne outbreaks linked to *L. monocytogenes.*

| No of cases of listeriosis | Consumed food | Place and year of the outbreak | Source of the outbreak | Reference |
|---|---|---|---|---|
| 5 (3 deaths) | Pasteurised milk | Massachussetts, USA, 2007 | Poor physical facility design, product flow, and maintenance procedures | Cumming *et al.* (2009) |
| 12 | Jellied pork | Austria, 2008 | Contaminated jellied pork prepared by the restaurant | Pichler *et al.* (2009) |
| 30 (4 deaths, 3 miscarriages) | Sandwich | USA, 2000 | Contaminated sliced turkey | Olsen *et al.* (2005) |
| 2 | Sandwich | Hospital, UK, 2003 | Floor of the factory supplying the sandwiches, very poor hygiene<br>Insufficient cooling | Shetty *et al.* (2009) |
| 14 (5 deaths)<br>20 (3 deaths) | Sour milk curd cheese | Austria and Germany, 2009-2010<br>Austria, Germany, Czech Republic (2009-2010) | Not specified | Fretz *et al.* (2010) |
| 54 (8 deaths, 3 miscarriages) | Turkey deli meat | USA, 2002 | Contaminated turkey | Gottlieb *et al.* (2006) |
| 19 | Cheeses | Japan, 2001 | Extensive contamination within the plant | Makino *et al.* (2005) |
| 13 | Mexican style cheese | USA, 2000 | Contaminated raw milk | MacDonald *et al.* (2005) |
| 28 | Delicatessen meat | USA, 2001 | Insufficient cooling | Frye *et al.* (2002) |
| 10 (1 death)<br>32 (9 deaths, 1 miscarriage) | Rillettes and jellied pork tongue | France, 1999-2000 | Not identified | de Valk *et al.* (2001) |

For *L. monocytogenes*, the infectious dose is estimated *a posteriori* on the remains of the food at the origin of the infection and the specific microbial dose is then quantified (Kothary and Babu, 2001). According to epidemiological data from outbreaks, *L. monocytogenes* is estimated to cause an epidemic or sporadic episode of listeriosis when foods are eaten containing doses ranging from a minimum of $10^2$-$10^3$ CFU/g of food to more than $10^8$ CFU/g (Giaccone and Ottaviani, 2007). The probability that a person will develop the disease increases with the quantity of bacteria ingested.

## 2.5. Sample collection and preparation from foods and surfaces

For the analysis of foodstuffs, the sample can be dry, semisolid, liquid, even viscous or frozen. It can be sampled from the entire processing line: from raw materials to the finished product in their original unopened containers (Thaddeus and Bryant, 2001). The means of sample collection are varied, but samples must be representative of the food being analysed. For instance, aliquots from several areas of the food should be taken or several samples may be required from a large volume of product after mixing of the product to ensure that the product sample is as homogeneous as possible. The instruments of collection (pipets, spatulas, spoons...) must be sterile and samples must be delivered to the laboratory as soon as possible for analysis. The condition, time, and date of the arrival should be recorded and samples should be packed to prevent breakage, spillage, or change in temperature. Refrigerated foods must be transported in an insulated shipping container with sufficient refrigerant to maintain the samples at 0ºC to 4.4ºC until arrival at the laboratory, as required by the International Standard Organisation ISO 6887 standards (Anonymous, 1999b, 2003c, 2003e, 2003f). Frozen samples can be kept frozen on condition that they are shipped with dry ice. In general, 25 ±1 g of food sample are analysed for presence / absence of the pathogenic microorganism using classical cultural methods. Such conventional methods for the enumeration of bacteria are typically colony count methods. Usually, the food sample is prepared by performing a one in ten dilution, in Buffered Peptone Water (BPW) for instance, and homogenized using a Stomacher™ or a Pulsifier™ (de Boer and Beumer, 1999).

The food processing environment can also be analysed including process surfaces and air sampling. However, air sampling will not be described in this review. Verification of cleanliness and microbiological quality acceptability requires analysis of surfaces and various surface contact sampling methods are available (Evancho *et al.*, 2001). The sponge contact method includes a compressed sterile sponge and glove to manipulate the sponge. The cellulose or polyurethane sponge is then vigorously rubbed onto the designated surface using up and down movements. Swab Contact methods are suitable for sampling of surfaces with cracks, corners, or crevices and cotton, alginate, dacron or rayon swabs may be used. Sponge / swab procedures are useful for sampling large areas of food processing equipment and environmental surfaces. Moore and Griffith compared surface sampling methods (swabs, Petrifilms™, dispslides, sponges) for detecting coliforms on food contact surfaces.

The sampling sponge was the least effective means of detecting coliforms on a wet surface, having a minimum detection limit of approximately 100 CFU.cm$^{-2}$, whereas all other test methods were able to detect the presence of <3-5 CFU.cm$^{-2}$. The SETS kit (Swab Extraction Tube System, Roche Diagnostics GmbH, Mannheim, Germany) is a simple swab processing system that allows the recovery of organisms by collection on swab fibres (Nolan *et al.*, 2007). To date, the system has been combined with real-time PCR analysis in virology, for the detection of Herpes Simplex virus (Issa *et al.*, 2005) and in microbiology, for the detection of *Streptococci* (Uhl *et al.*, 2003) and *Francisella tularensis* (Walker *et al.*, 2010). The Replicate Organism Direct Agar Contact (RODAC) plate method uses an agar based contact plate that is applied on flat surfaces for bacterial enumeration. A study on bacterial recoveries showed that the RODAC technique yielded 80% of inoculated *E. coli* cells compared to the swab technique that allowed the enumeration of only 1% of inoculated *E. coli* cells (Foschino *et al.*, 2003). As alternatives to the RODAC plates, commercial medium contact systems such as 3M™ Petrifilm™ (3M, Saint Paul, USA) and Con-Tact-It® (Birko Chemical Corp., Denver, USA) or mylar adhesive tape (Dyna-Tech, Inc., Grafton, USA) are used to transfer a sample to an agar plate for culture. Dipslides are also very useful for cell enumeration on surfaces. These agar slides contain selective or non-selective media and are pressed onto the surface to be tested (de Boer and Beumer, 1999).

## 2.6. The requirement for enrichment

Before plating out some food samples, enrichment methods may be needed to grow cell population present in very low initial levels in foods to a detectable level (Sperber *et al.*, 2001). Enrichment steps have allowed the detection of one cell per 500 g of food (Silliker and Gabis, 1973). Enrichment techniques are also used to recover injured bacteria as microorganisms in foods are typically in stressed conditions (Sperber *et al.*, 2001). The bacterial recovery involves a period of time in suitable conditions to repair cellular damage and activate metabolic pathways (Sperber *et al.*, 2001). Another advantage of enrichment is the growth inhibition of competing non-target microorganisms; such medium is referred to as selective enrichment medium. Enrichment methods provide qualitative data: presence or absence of the target

microorganism. Such methods may eventually provide quantitative results when the Most Probable Number (MPN) method is performed in parallel (Sperber *et al.*, 2001).

Generally, enrichment includes two separate steps: pre-enrichment and selective enrichment. However, a selective enrichment medium generally requires hazardous supplements and additional cost in consumables (Duffy *et al.*, 2001). The pre-enrichment step allows resuscitation of stressed cells using a non-selective or moderately selective broth whereas the selective enrichment step further enhances growth of the target microorganisms while suppressing and inhibiting growth of competitors (Sperber *et al.*, 2001). However, the pre-enrichment step may not be required since some media are used to support both resuscitation and growth of the target microorganism. For instance, a liquid modification of Baird-Parker's medium has been developed to detect low numbers of *S. aureus* cells (<20 g) in foods (Van Doorne *et al.*, 1981). Trncikova *et al.* (2009) and Peresi *et al.* (1996) demonstrated that Giolitti and Cantoni based media allowed the best enrichments in *S. aureus* cells from foods compared with Baird Parker media or Tryptic Soy Broth containing 10% salt (TSBS). Enrichment steps using BPW and Enterobacteriaceae Enrichment (EE) broth are required by the ISO 21528 standard (Anonymous, 2004e) for the detection of the Enterobacteriaceae in foods. However, the use of such broth has been reported to inhibit the growth of some Enterobacteriaceae such as *C. sakazakii* (Gurtler and Beuchat, 2005; Iversen and Forsythe, 2004). For the detection of *L. monocytogenes* in foods, the use of half Fraser broth as primary enrichment medium and Fraser broth as secondary enrichment medium is included in ISO 11290-based procedures. However, Besse *et al.* (2005) demonstrated that such media allowed greater development of competitors such as *Listeria innocua* when simultaneously present with *L. monocytogenes* in foods. Moreover, Duffy *et al.* (2001) and Donelly (2002) showed that the repair and the detection of sub-lethally injured *Listeria* may be inhibited by the use of selective agents present in the selective enrichment medium. As an alternative to Fraser broths, Oravcova *et al.* (2008) obtained similar recoveries of *L. monocytogenes* with Brain Heart Infusion (BHI). Duffy *et al.* (2001) replaced the University of Vermont Media (UVM) by BPW for the recovery of *L. monocytogenes* in minced meat samples and obtained similar results after 24 h of enrichment. These findings were confirmed by Walsh *et al.* (1998b) for analysis of meat, fish, vegetables, salads and dairy products for the presence of *Listeria* spp. Therefore, a simple enrichment with a non-selective medium may be sufficient.

To improve bacterial recoveries, enrichment can be coupled to immunomagnetic separation (IMS) where polystyrene microscopic beads with adsorbed antibodies are usually used to concentrate the target microorganism. O'Brien *et al.* (2005) enriched minced meat samples spiked at contamination levels from $\log_{10}$ 1.58 to 2.58 CFU.g$^{-1}$ in *Escherichia coli* for 6 and 24h at 37°C. The cells were then concentrated using Dynabeads anti-*E. coli* O157 immunomagnetic beads (Dynal Biotech A.S., Oslo, Norway). Bacterial recoveries were found to be higher than those obtained using the direct count enumeration method.

## 2.7. Detection methods in food diagnostics

### 2.7.1. Plating methods

The total bacterial number in a food or a swab sample is typically determined by spreading diluted suspensions of the sample onto the surface of an agar-based growth medium or mixing the test volume with a liquefied agar medium in Petri dishes (Jasson *et al.*, 2010). Plate counts or colony growth detection are performed after incubation at temperatures depending on the protocol. Chromogenic or fluorogenic substrates present in selective media detection can allow direct identification and enumeration, which eliminates the need for subcultures or further biochemical tests (Manafi, 1996). However, colony counting remains time consuming and novel counting techniques using various image analysis for plate counting have been developed to improve efficiency and reduce labour costs (de Boer and Beumer, 1999). ISO methods for the detection and enumeration of microorganisms in foodstuffs are mainly based on plating techniques. The Enterobacteriaceae are detected using Violet Red Bile Glucose Agar (VRBGA) following the ISO 21528 (Anonymous, 2004e, 2004g). However, bacteria other than Enterobacteriaceae can grow on such medium. This is the case for *Aeromonas* spp., although these bacteria are oxidase positive, which allows distinction from the Enterobacteriaceae (Baylis, 2006). The detection and enumeration of *S. aureus* by the ISO 6888 standard (Anonymous, 1999c, 1999d, 2003h) requires the use of Baird Parker medium. Such medium remains the most commonly used for the enumeration of *S. aureus* in foods. *L. monocytogenes* is detected and enumerated in food and animal feeding stuffs by referring to the ISO 11290 standards (Anonymous, 1996, 1998, 2004a, 2004c) that

require the use of Agar *Listeria* according to Ottaviani and Agosti (ALOA), or, alternatively, other solid media such as Oxford or PALCAM.

### 2.7.2. MPN Method

The MPN method is a statistical estimation of the number of viable organisms in a sample (Betts, 2000). After preparing decimal (tenfold) dilutions of a sample, each dilution is usually transferred to three tubes containing identical volumes of broth medium. After incubation, the tubes that show any growth or turbidity are reported over a standard table of data which provides data on the contamination level of the sample. However, this method is dedicated to particular types of testing and remains more labour and materials intensive than plate count methods. Despite the use of replicates for each dilution level, the confidence limits are large and consequently the MPN method is less accurate than plate counting methods but with a greater sensitivity.

### 2.7.3. Modified culturing and identification methods

The 3M™ Petrifilm™ plates or Compact Dry (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) are alternatives to agar-poured plates. These all-in-one plating systems comprise of a film including a cold-water-soluble gelling agent, nutrients and indicators for activity and enumeration (Jasson *et al.*, 2010). These systems generally allow significant space saving in the incubator. No significant difference was obtained between the 3M™ Petrifilm™ Staph Express Count System and direct or pour plate count methods for the enumeration of *S. aureus* in cheese (Fedio *et al.*, 2008) or the Enterobacteriaceae in powdered milk (Ferraz *et al.*, 2010). Comparable results were obtained with the Compact Dry TC method and the standard pour plate method when enumerating the total aerobic count of five different raw meats (Kodaka *et al.*, 2005).

SimPlate® (BioControl Systems Inc., Washington, USA) includes fluorogenic and chromogenic substrates depending on the test, where the sample is distributed among 100 individual plates. After incubation, positive wells are counted and the bacterial number is calculated by using a conversion table under a MPN principle (Baylis, 2006). According to Vulindlu *et al.* (2004), on average, SimPlate® gave a significantly higher count than the conventional plating methods, with reduced labour, and greater ease of determining results. However, correlation indices between conventional plate count and SimPlate® were reported to be poor for the enumeration

of mesophilic aerobic counts. In contrast correlation indices were found to be strong between 3M™ Petrifilm™ Aerobic Count and conventional plate counting (Tavolaro *et al.*, 2005).

TEMPO® (bioMerieux, Craponne, France) is a system which incorporates a dehydrated culture medium and an enumeration card containing wells across different dilutions for the automatic determination of MPN (Jasson *et al.*, 2010). Kunika (2007) demonstrated a good correlation between data obtained for enumeration of total viable count mesophilic and coliforms as *E. coli* in foods, using the user-friendly TEMPO® system and the standard ISO-based plate count methods.

Colilert® (IDEXX Laboratories, Eragny sur Oise, France) is used for the simultaneous detection and enumeration of total *E. coli* in water and wastewater by the MPN principle (Jasson *et al.*, 2010). The presence of the coliform enzyme ß-galactosidase and the *E. coli* enzyme ß-glucuronidase is revealed using the patented Defined Substrate Technology® (DST®) which includes two chromogenic nutrient-indicators, ortho-nitrophenyl-ß-galactopyranoside (ONPG) and 4-methyl-umbelliferyl-ß-D-glucuronide (MUG) which are used as the major carbon sources in Colilert®. Coliforms metabolise the ONPG and the colour of the medium subsequently becomes yellow. *E. coli* can metabolize the MUG substrate allowing its particular detection. Colilert® has been recognised to detect qualitatively and quantitatively a higher proportion of *E. coli* than the ISO 9308-1 reference method (Bonadonna *et al.*, 2007).

The Soleris™ test (Neogen, Auchincruive, UK) observes modifications in the chemical properties of liquid growth medium and detects microorganisms with pH and other sensitive reagents following a change in their spectral patterns. Data are obtained photometrically within a semi-fluid zone of the patented organism-specific vial (Jasson *et al.*, 2010). The time at which growth is first detected is inversely proportional to the bacterial log number. It has been reported that *Listeria* was detected with the CT-Soleris® 2 to 3 days earlier than with the United States Department of Agriculture (USDA) culture method (Yan *et al.*, 2007).

Identification systems mainly include a range of morphological, physiological and biochemical tests and a number are commercially available for a simplified automated identification of microorganisms (de Boer and Beumer, 1999). Various miniaturised methods have been developed to reduce the volume of reagents and

media, save time, labour and space (Fung, 2000). A non-exhaustive list of some current identification systems is shown in Table 1.4.

**Table 1.4.** Non-exhaustive list of identification systems for foodborne pathogens, updated table from Feng (2001).

| Identification system | Manufacturer | Target microorganism |
|---|---|---|
| API® and ID® | bioMerieux, Craponne, France | Enterobacteriaceae*, Listeria*, *Staphylococcus, Campylobacter,* Non-fermenters, anaerobes |
| Micro-ID® | REMEL, Lenexa, USA | *Enterobacteriaceae, Listeria* |
| Enterotube™ II | Becton Dickinson GmbH, Heidelberg, Germany | Enterobacteriaceae |
| BBL™ Crystal™ | Becton Dickinson, Oxford, UK | *Enterobacteriaceae, Vibrionaceae*, Non-fermenters, anaerobes |
| Microgen™ Biochemical ID | Microgen Bioproducts Ltd, Camberley, UK | Gram negatives, *Listeria*, *Bacillus*, *Staphylococcus*, *Streptococcus* |
| Vitek™ | bioMerieux | *Enterobacteriaceae*, Gram negatives, Gram positives |
| Microlog | Biolog, Hayward, USA | *Enterobacteriaceae*, Gram negatives, Gram positives |
| Sherlock® Microbial Identification System (MIS) | MIDI, Inc, Newark, USA | *Enterobacteriaceae, Listeria, Bacillus, Staphylococcus, Campylobacter* |

### 2.7.4. Upcoming developments towards alternative and rapid methods

Alternative methods may considerably reduce time and labour for microbial detection and identification. Applications in food diagnostics are listed in Table 1.5 and this literature review will now focus on PCR and FCM methodologies.

**Table 1.5.** Diagnostic applications relevant to foodborne pathogen detection (sources: Johnson-Green (2002), Jasson (2010)).

| Goal of diagnostic | Example | Current technology | Upcoming developments |
|---|---|---|---|
| Assess microbial contamination | Bacteria on carcasses | Plate counts | FCM[a], impedimetry, biosensors, PCR[b], ELISA[c], ELFA[d] |
| Identify pathogen | *E. coli* O157:H7 | Selective media, immuno-assay, biochemical, DNA tests | Bioluminescence, biosensors, PCR, LFD[e], ELISA, ELFA, FISH[f], microarrays |
| Investigation of outbreak | Tracing pathogen strains to source | Biochemical tests, immuno-assay | PCR, RAPD[g], RFLP[h], pulse-field electrophoresis, 16S rRNA[i] sequence analysis |
| Monitor hygiene | Work surfaces in processing plant | Plate counts / ATP using bioluminescence | Increased specificity (combining hygiene tests with pathogen identification), PCR |

[a]FCM

[b]PCR, Polymerase Chain Reaction

[c]ELISA, Enzyme Linked Immunosorbent Assay

[d]ELFA, Enzyme Linked Fluorescent Assay

[e]LFD, Lateral Flow devices

[f]FISH, Fluorescent *in situ*

[g]RAPD, randomly amplified polymorphic DNA.

[h]RFLP, restriction fragment length polymorphism

[i]rRNA, ribosomal RNA

To satisfy food safety regulations and to be widely accepted by the food industry, all newly developed rapid and alternative methods must be validated. It is essential that these methods provide reliable and equivalent results when compared with reference methods (Jasson *et al.*, 2010).

# 3. COMMON PCR METHODS IN FOOD MICROBIOLOGY

## 3.1. Conventional PCR

PCR is used to amplify target regions of a DNA strand such as a single gene or, just a part of a gene. PCR typically amplifies short DNA fragments less than than 3 kb although larger fragments can be amplified with less efficiency (Sanderson and Nichols, 2003). A PCR reaction usually requires:

- a DNA template containing the region of the DNA fragment to be amplified,
- two primers that anneal to the beginning (forward primer) and the end (reverse primer) of the region to be amplified,
- a DNA polymerase which copies the region to be amplified by polymerisation,
- some deoxynucleotide triphosphates (dNTPs) for synthesis,
- a buffer solution,
- divalent cations such as magnesium or manganese ions
- monovalent cations such as potassium ions.

A thermal cycler is used to carry out the PCR process. The PCR platform heats and cools the reaction tubes adjusting the precise temperature required for each PCR step. The lid of the PCR device is heated to prevent condensation on the inside of the reaction tube caps. Each PCR cycle comprises three steps: denaturation, annealing and extension. The denaturation step allows the separation by heat (usually >90°C) of double-stranded DNA into two single strands, as the weak hydrogen bonds between the bases break at high temperatures. The stronger covalent bonds between the deoxyribose and the phosphate remain intact. During the annealing step, the primers anneal to the target sequence, each primer being complementary to the single DNA strands that were formed during denaturation. Annealing usually occurs between 40°C and 65°C, depending on the length and the sequence of the primers. This allows the primers to anneal to the target sequence with high specificity. The extension step starts at the 3'-end of the annealed primer at approximately 72°C. The replication of the DNA strands by the DNA polymerase begins the synthesis process in the 5' to 3' direction adding free dNTPs present in solution. New double stranded DNA molecules are created, both identical to the original double stranded target DNA region. DNA polymerase synthesizes in the 5' to 3' direction. A complementary strand of the targeted DNA sequence is therefore constructed. At the end of the first PCR cycle, two new DNA strands identical to the original target are then synthesized.

Nevertheless, the DNA polymerase does not recognise the end of the sequence. The newly formed strands have a beginning, which is precisely defined by the 5' end of the primer, but the 3' end is not precisely defined. As the number of cycles increases, a strand with more defined length frequently serves as the template for the newly synthesized sequence. Then, the DNA strand created from such a template has a precisely defined length that is limited at either end by the 5'-end of each of the two annealed primers. These DNA strands are called an amplicon. After only a few cycles, DNA strands which correspond to the target sequence are present in much larger quantity than the variable length sequences. Indeed, the sequence flanked by the two primers is the section that is amplified.

The detection of the Enterobacteriaceae by PCR has been formerly described by Bayardelle and Zafarullah (2002). PCR protocols were developed for the detection of the most frequent members of the Enterobacteriaceae in blood, urine and water samples using primer sets targeting the *wec* gene cluster involved in the synthesis of the enterobacterial common antigen. The detection of Enterobacteriaceae species in foods using PCR has been largely described for *Salmonella* (dos Santos *et al.*, 2001; Radji *et al.*, 2010; Španová *et al.*, 2000; Zahraei Salehi *et al.*, 2005), *Escherichia* (Deng *et al.*, 1996; Ellingson *et al.*, 2005; Huang *et al.*, 2008), *Shigella* (Lampel *et al.*, 1990; Villalobo and Torres, 1998), *Yersinia enterocolitica* (Knutsson and Rådström, 2002; Lambertz *et al.*, 2000; Nilsson *et al.*, 1998) or *Cronobacter sakazakii* (Van Acker *et al.*, 2001; Witthuhn *et al.*, 2007). PCR assays to detect *S. aureus* in foods have been extensively developed (Chen *et al.*, 2001; Kim *et al.*, 2001; Beatriz Pinto *et al.*, 2005) as well as for *L. monocytogenes* (Duffy *et al.*, 1999; Levin, 2003; Niederhauser *et al.*, 1992; Starbuck *et al.*, 1992b).

## 3.2. Design and optimisation of diagnostic PCR

PCR allows the amplification of a target DNA sequence that must be unique to the organism(s) to be detected (Fairchild *et al.*, 2006). However, many genes are shared within the same genus and species of bacteria. The development of a PCR assay involves the identification of a target gene sequence that is strongly associated with the pathogen to be detected (Maurer *et al.*, 1999), such as conserved sequences flanking sequence variable regions (Fischer and Nachamkin, 1991; Schoenhals and Whitfield, 1993; Smith and Selander, 1990), or sequences unique to a serovar

(Gannon *et al.*, 1997; Herrera-Leon *et al.*, 2004) or pathogen (Oyofo *et al.*, 1992). It is preferable to use already developed or commercially available PCR systems rather than starting from "scratch" (Fairchild *et al.*, 2006). PCR protocols for food pathogen detection are regularly described in peer reviewed publications and are a good start for adaptation to "in-house" methods. If starting from "scratch", it is possible to study complete or partial bacterial genomes that are accessible in DNA databases to perform *in silico* PCR analysis. The most important DNA database is GenBank at the National Center for Biotechnology Information (NCBI), National Institutes of Health, Bethesda, USA. The website is www.ncbi.nlm.nih.gov. Every time the sequence of a genome is entered into the database, an accession number is attributed. When accessing genome sequences within the database, the user can find the gene of interest and it sequence using the searching tool of the internet navigator. The first three nucleotides should start with ATG, the start codon or GTG (the rare start codon) and end with TAA, TAG, or TGA, the stop codons (Fairchild *et al.*, 2006). However, the gene may be in the opposite orientation on the chromosome, therefore it is required to invert the DNA sequence and transcribe the opposite DNA strand to identify the start and the end of the Open Reading Frame (ORF). Fairchild *et al.* (2006) advise selecting the ORF where more conserved regions within strains of an organism are likely to be found rather than flanking or intergenic regions. The Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between sequences. BLASTn compares a DNA sequence to other sequences within DNA databases and calculates the statistical significance of matches of strains within the same species as well as matches of species. The algorithm can be used to infer functional and evolutionary relationships between sequences or identify members of gene families (//blast.ncbi.nlm.nih.gov/Blast.cgi). The BLASTn data allows identification of a region of optimal DNA sequence for primer design. However, it is not guaranteed that the selected gene or sequence will provide high specificity to the PCR assay, as the DNA database is non-exhaustive. Therefore, the PCR assay should be experimentally tested. The optimal primer pairs are defined using commercially available software or freeware packages like Primer Premier (PREMIER Biosoft International, Palo Alto, USA), Primer3 (Broad Institute, Cambridge, USA), Oligo (Molecular Biology Insights, Inc., Cascade, USA). Such software provide the user with all the possibilities of primer sets and allows identification of "hairpin" forms or primer-dimers as well as the putative size of the associated PCR product. Indeed, primers should not be

complementary to themselves (forming homodimers) or to each other (forming heterodimers) (Brownie *et al.*, 1997). PCR sensitivity is a function of the size of the amplicon. Fairchild *et al.* (2006) advise targeting small amplicons between 75 and 200 bp. The specificity of the PCR assay depends on the sequences of the primer set used. Once the primers are selected, a search for matches on the GenBank database using Primer-BLAST is carried out. The specificity of the PCR is then verified over genera, species, strains or serovars by performing *in-silico* PCR analysis which will also confirm the size of the PCR product.

Primers are short and single-stranded oligonucleotides usually 20-30 nucleotides in length whose sequence matches the starting and the ending point of the DNA template (Mackay *et al.*, 2007c). Primers that are too short may anneal at several positions on a long DNA template, which can result in non-specific copies. The specificity of a primer set depends on its complete sequence, but most importantly on the 3'-end that is elongated by the DNA polymerase (Rychlik, 1995). Indeed, the 3'-end should ideally include at least five to seven unique nucleotides that are not found elsewhere within the target sequence. However, it is hardly conceivable to find such specific sequence as it may also bind to human genomic DNA (Mackay, 2004). Both primers must be designed to bind with similar or identical efficiency to the target. The adjustment of the annealing temperature ($T_A$) or the concentration of the primer may improve efficiency. Generally, doubling the concentration of one primer increases its' $T_A$ by 1 to 2°C. The melting temperature ($T_M$) is the temperature at which half of the primer binding sites are occupied and increases with the length of the primer (Mackay *et al.*, 2007c). The $T_M$ ranges between 55°C and 65°C. The $T_A$ is usually 5 to 10°C below the $T_M$. To calculate the $T_M$, various methods are available and determine the $T_M$ as a function of primer length, the number of tight-binding Guanine (G) and Cytosine (C) bases compared to weaker binding Adenine (A) and Thymine (T), the distribution of the nucleotides and the conditions of the PCR reaction. However, the most convenient calculation method is given by applying the simple formula:

$$T_M\ (°C) = 4 \times (G + C) + 2 \times (A + T)$$

It is important that the G/C content of both primers should be between 40 and 60% (J. S. Wu *et al.*, 2004), as higher percentages may decrease the PCR efficiency.

To amplify the same gene from different organisms, as the genes themselves are probably similar but not identical, degenerate primers are sometimes used (Nyyssonen *et al.*, 2006). These are mixtures of similar, but not identical, primers. The

other method of obtaining degenerate primers is based on protein sequence (Gorga *et al.*, 2002). As several different codons can code for one amino acid, it is often complicated to deduce which codon is used in a particular case. Therefore primer sequence corresponding to the amino acid isoleucine may be "ATH", where A stands for adenine, T for thymine, and H foradenine, thymine, or cytosine (Hassibi *et al.*, 2008). However, such primers may significantly reduce the specificity of the PCR assay. Touchdown PCR may partially reduce this issue (Piraee and Vining, 2002). It consists of applying a higher annealing temperature than the optimal one during the first cycles to ensure a strong stringency or optimal conditions to ensure DNA matching and thus allowing specific amplification. When the sequence of interest is present in larger amounts than its' competitors, the annealing temperature is gradually decreased, 1°C at every cycle or every 2 cycles for instance, to ensure a better efficiency of PCR.

## 3.3. Post PCR analysis

For the revelation of PCR products, the simplest or most commonly used technique is electrophoresis on an agarose or polyacrylamide gel with visualization by staining with a fluorescent dye that intercalates into the DNA. Ethidium bromide has been firstly used for staining of DNA bands; however, less harmful dyes such as SYBR® Safe, Nancy-520 are available for similar or even more sensitive results. The electrophoresis gel is pre or post-stained with the fluorescent tag and ultraviolet transillumination allows visualization of the DNA. Gel densitometry allows DNA semi-quantification (Freeman and Thompson, 1988; Su *et al.*, 1990) using DNA markers with defined concentrations. Alternatively, the detection of a PCR product is performed using Southern blot that involves hybridization of an immobilised amplicon to a labelled oligonucleotide probe spotted onto a nitrocellulose or nylon membrane (Fach *et al.*, 1993; Wesley *et al.*, 1990). This detection technique is highly specific but it is time and labour intensive and involves multiple steps increasing contamination risks (Mackay *et al.*, 2007c). PCR-ELISA (Enzyme-Linked Immunosorbent Assay) is used to capture PCR products onto a solid phase via biotin or digoxigenin-labeled primers, oligoprobes or by direct capture after incorporation of the biotin or digoxigenin into the amplicon (Daly *et al.*, 2002; Kim and Cho, 2008). After capture, the PCR product is detected using an enzyme-labelled avidin or anti-digoxigenin

reporter molecule in a similar manner to a standard ELISA format. The advantage of this method is that it uses hardware and techniques commonly available for ELISA systems in the laboratory (Mackay *et al.*, 2007c). However, for microbial analysis of food samples ELISA-PCR has been largely overtaken by real-time PCR.

## 3.4. Evolution of PCR methods for food analysis

### 3.4.1. Multiplex PCR

This method is used to simultaneously amplify more than one amplicon in a single PCR reaction. By targeting multiple genes at once, additional information may be provided from a single PCR run that would otherwise require more reagents and technician time to perform. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction and the amplicon sizes should be separated enough in final base pair length to allow differentiation between the PCR products. In a single PCR reaction, one primer set may target the organism for identification (Anbazhagan *et al.*, 2010; Fratamico *et al.*, 2000; Tamarapu *et al.*, 2001) whereas the second set may allow genotyping (Quaglia *et al.*, 2005), as the amplicon size corresponds to a specific organism or gene. Multiplex PCR also allows the simultaneous detection of multiple pathogens in a sample (Kobayashi *et al.*, 2009a; Kumar *et al.*, 2009; Park *et al.*, 2006; Wang *et al.*, 2004; Zhang *et al.*, 2009). However, multiplexing allows less flexibility in primer selection requires significant optimisation and is generally less sensitive and specific than conventional PCR (Sen *et al.* 2004).

### 3.4.2. Terminal Restriction Fragment Length Polymorphism (TRFLP)

Microbial communities may be characterized and identified using a single and universal primer set targeting a single ~1500 bp-length amplicon from the 16S rDNA, a gene that is conserved in all bacterial species (Fairchild *et al.*, 2006). One of the primers is fluorescently tagged with phosphoramidite dyes (Giraffa and Neviani, 2001). After PCR, restriction enzymes are used to recognize restriction sites within genus or species-specific sites within this gene. Bacterial identification profiles are defined by the DNA fragments generated which vary in sizes as a function of the genus or species (Marsh, 1999). The digested amplicons are separated by electrophoresis using either gel- or capillary-based systems (Giraffa and Neviani,

2001). The laser detection of the labelled fragments using an automated analyser resolves minor changes among DNA fragments and measures fluorescence of the eluted DNA fragments. Fluorescently labelled molecular weight standards are used for calibration of the sequencer's capillary column. The DNA fragment size is a function of elution time from the column. The results are analyzed by counting and comparing of peaks position of the TRFLP profile to that which corresponds to a previously identified specific genus/species profile from a database of restriction fragments (Marsh *et al.*, 2000). Such a database is established in-house by cloning and sequencing of 16S rDNA fragments or is available online. TRFLP has been used for identifying signature peaks for microbial pathogens (Christensen *et al.*, 2003; Nilsson and Strom, 2002), or to identify differences in 16S rDNA between them and phylogenetically related commensal organisms (Nordentoft *et al.*, 1997).

The TRFLP technique is similar in some aspects to Denaturing Gradient Gel Electrophoresis (DGGE) or Temperature Gradient Gel Electrophoresis (TGGE). These electrophoresis techniques use either a chemical or temperature gradient to denature the sample as it migrates through an acrylamide gel (Giraffa and Neviani, 2001).

### 3.4.3. Microarrays

Macroarrays, microarrays, high-density oligonucleotide arrays and microelectronic arrays have been developed for gene(s), sequence(s) or specific mRNA (messenger Ribonucleic Acid) screening in a single test (Freeman *et al.*, 2000). An array of thousands of spots of DNA oligonucleotides or fragments unique to a particular gene are immobilised as probes or reporters onto a nylon membrane (macroarray), glass slide or silicon chip (microarray), or electronic microchip (microelectronic array). Such DNA fragments are then hybridized with fluorescently or radioactively labelled nucleic acid material from a test sample. Such a sample may be total genomic DNA for genomic profiling experiments or mRNA for gene expression profiling assays from the test sample (Fairchild *et al.*, 2006; Kostrzynska and Bachand, 2006). Alternatively, the sample DNA can be a labelled PCR product. The DNA microarray technology includes two main types: genomic microarrays and oligonucleotide arrays (Kostrzynska and Bachand, 2006). For pathogen identification and classification, genomic microarrays involve the whole genome, genomic fragments from a copy DNA (cDNA) library, or open reading frames from a strain of

microorganism (Bekal *et al.*, 2003; Taboada *et al.*, 2004). Oligonucleotide arrays comprise 18 to 70 bp-length oligonucleotides and allow pathogen detection and genomic analysis (Call *et al.*, 2003).

## 3.5. DNA sample preparation

### 3.5.1. Impact on DNA polymerase by PCR inhibitors in foods

The choice of DNA polymerase is of importance as its' activity depends on the presence of inhibitors which may be in the sample. Al-Soud and Radstrom (1998) showed that blood, cheese, faeces and meat samples can have an inhibitory effect on nine tested DNA polymerases including:

- the blood-sensitive polymerases (*AmpliTaq* Gold, *Taq*, and *Ultma*),
- the blood-resistant polymerases (Hot*Tub*, *Tfl*, and *Tli*),
- the feces-resistant polymerases (*Pwo* and *rTth*).

The polymerase Expand, made of a mixture of *Pwo* and *Taq*, has a resistance pattern to the inhibitory samples that is different from the other eight enzymes. Moreover, DNA polymerase activity varies with the concentration of ions ($Ca^{2+}$, $Mg^{2+}$, $K^{+}$, $Na^{+}$). Proteinases found to be inhibitory to PCR include proteinases in milk (Powell *et al.*, 1994) and heme in blood (Abu Al-Soud and Radstrom, 1998), as well as collagen .(Kim *et al.*, 2001). Some substances used in pre-processing PCR may have an inhibitory effect on DNA polymerases. Katcher and Schwartz (1994) studied the influence of phenol on DNA polymerases. Among the tested polymerases, *Tth* DNA polymerase displayed the unique property of maintaining both DNA- and RNA-dependent DNA polymerase activities in the presence of 2%-5% (vol/vol) of phenol-saturated PBS buffer.

### 3.5.2. PCR enhancers

According to Abu Al-Soud and Radstrom (2000), bovine serum albumine (BSA), the single-stranded DNA-binding protein gp32, and proteinase inhibitors improve the amplification capacities of *rTth* and *Taq* polymerases in the presence of blood, faeces, and meat. Among the organic solvents, dimethyl sulfoxide (DMSO) and formamide may increase the specificity of amplification (Pavlov *et al.*, 2004; Varadaraj and Skinner, 1994). The non-ionic detergent Tween-20 and Triton X have an enhancing effect on DNA polymerases. Addition of non-ionic detergents (final

concentrations 0.05% Tween 20 and 0.05% Nonidet P-40) stimulates Taq DNA polymerase activity and reduced background created by false terminations with the enzyme (Innis *et al.*, 1988). Betaine and glycerol are the most biologically compatible reagents used to enhance specificity and reduce the formation of secondary structures caused by GC-rich regions (Henke *et al.*, 1997; Varadaraj and Skinner, 1994). Polymers such as polyethylene glycol (PEG) or dextran can also be employed as amplification facilitators (Abu Al-Soud and Radstrom, 2000).

### 3.5.3. DNA extraction from food samples

Pre-processing procedures have been developed to remove or reduce the effects of PCR inhibitors. Their purpose is to convert a complex biological sample with its target nucleic acids/cells into PCR amplifiable samples by combining optimal sample preparation and amplification conditions (Rådström *et al.*, 2004). Previously, the quality of PCR detection depended on the ability to prepare pure, high-molecular-weight DNA. Generally, DNA extraction included lysing of cells, solubilising the DNA and removing proteins, RNA, and other macromolecules by one or more enzymatic or chemical steps. Further purification and concentration of nucleic acids were sometimes required to separate genomic DNA from plasmids or residual debris in the cell lysate. Nowadays, rapid DNA extraction methods have been developed to avoid time consuming purification steps. PCR-inhibitory substances that may reduce the amplification capacity of DNA and the efficiency of amplification are excluded, to concentrate the target organism, reduce the amount of heterogeneous bulk sample and produce a homogeneous DNA sample for amplification with reproducibility and repeatability (Rådström *et al.*, 2004).

DNA extractions rely on biochemical methods and the various commercial DNA extraction kits are available for food analysis as shown in Table 1.6.

**Table 1.6.** Examples of currently available commercial DNA extraction kits.

| DNA extraction kit | Manufacturer |
|---|---|
| BAX® system | DuPont Qualicon, Wilmington, USA |
| PrepMan® Ultra Sample Preparation Reagent | Life Technologies, Carlsbad, USA |
| Genomix, Micromix | Talent, Trieste, Italy |
| DNeasy Blood and Tissue kit | Qiagen, Crawley, UK |
| foodproof® ShortPrep, foodproof® Sample Preparation, foodproof® StartPrep kits | Biotecon Diagnostics GmbH, Potsdam, Germany |
| Universal kit | CapitalBio Corp., Beijing, China |
| QuickExtract™ Bacterial DNA Extraction Kit | Epicentre Biotechnologies, Madison, USA |

DNA extraction provides a homogeneous and purified DNA sample of high quality stored in an appropriate buffer. Nevertheless, this method requires in most cases the food sample to be enriched in liquid medium or cultured on solid medium prior to DNA extraction. Giacomazzi *et al.* (2005) compared three bacterial DNA extraction procedures for analysis of cold-smoked salmon samples and assessed the impact on the efficiency of two physical treatments of the food matrix, ionizing irradiation or freezing. The extraction procedure A included a cell lysis treatment by Proteinase K, SDS and Sarkosyl, a physical treatment by microwave heating. Extraction was performed using a phenol/chloroform/isoamyl alcohol mixture. The extraction procedure B was based on cell lysis with lysosyme and mutanolysine, followed by a phenol/chloroform extraction step. The extraction procedure C used a commercial Qiagen DNeasy tissue kit and best results were obtained using this method. However, data indicated that physical treatments of cold-smoked salmon samples such as freezing and ionizing irradiation had a negative impact on bacterial DNA recovery. Therefore, bacterial detection by PCR could lead to false negative responses depending on physical treatment of the sample prior to analysis.

Immunological methods may also involve use of magnetic beads coated with antibodies. Target cells are captured according to the specificity of the antibody to the antigen and are concentrated directly from a complex food matrix (Jasson *et al.*, 2010). For instance, Pathatrix, is a commercial application that re-circulates automatically the food sample using immunomagnetic separation technology. Liu *et al.* (2006) developed a rapid method based on an immunomagnetic capture-fluorescent PCR assay for *Campylobacter jejuni* in food and water samples. The pathogen was directly detected without the requirement for enrichment and could be

performed within approximately 8 h, thus making it more rapid than other direct PCR methods or by plating. The sensitivity was reported to be very high with a signal from a single cell detectable in 0.1 ml of sample. The assay was purported to be highly specific and was unaffected by the presence of 13 other bacterial species. Compared with conventional methods, immunocapture resulted in a time saving in bacterial isolation and effectively avoided negative results from the presence of viable but not cultivable *Campylobacter jejuni* cells. O'Hanlon *et al.* (2005) compared IMS/real-time PCR and an IMS/culture method to detect *Escherichia coli* O26 and O111 in minced beef and obtained better recoveries by the combined IMS/real-time PCR method.

Various physical methods for DNA sample preparation can be used depending on the physical properties of the target cells: centrifugation (Gerritsen *et al.*, 1991), filtration (Starbuck *et al.*, 1992a), dilution (Al-Soud *et al.*, 1998). Besides these options, aqueous two–phase systems allow separation of PCR inhibitors and target cells into two immiscible phases. Lantz *et al.* (1997) developed a two-phase aqueous polymer system composed of 8% (w/w) polyethylene glycol 4000 and 11% (w/w) dextran 40 to remove PCR-inhibitory substances from human faeces prior to PCR. The majority of the PCR-inhibitory substances, including bile salts, were shown to be distributed in the polyethylene glycol-rich top phase, whereas target bacteria were detected by PCR in the dextran-rich bottom phase.

The buoyant density centrifugation method consists of layering a volume of sample on a low viscosity gradient medium such as Percoll or BactXtractor. After cleaning with NaCl, the pellet is used as the DNA sample without further treatment. Lantz *et al.* (1998) made a comparison between the XTRAX procedure, the aqueous two-phase system and Percoll centrifugation to detect *Yersinia enterocolitica* in minced pork homogenate. Percoll centrifugation was found to be the most efficient sample preparation method for minced pork homogenate. Wang *et al.* (1999) confirmed this work with a study of PCR detection of *Campylobacter jejuni* from chicken rinses.

Physiological methods are based on bacterial growth and biosynthesis of cell components by growing cultures in enrichment broth or agar plates. The purpose is to obtain detectable concentrations of viable target cells prior to PCR. No further lysis steps are required. Knutsson *et al.* (2002) assessed selective enrichment PCR procedures for *Y. enterocolitica*. The data demonstrated that elevating target cell

concentration allowed a rapid detection *Y. enterocolitica* after enrichment in *Yersinia*-PCR-Compatible-Enrichment (YPCE) medium for 3 h at 25 ºC, followed by a centrifugation prior to PCR analysis.

## 3.2. Real-time PCR for food analysis

### 3.2.1. Principle of Real-time PCR in the microbiology laboratory

Real-time PCR is an advanced version of the conventional PCR method allowing both qualitative and quantitative DNA analysis, in addition to highly sensitive DNA amplification (Mackay *et al.*, 2007c). The technique has several important benefits over conventional PCR for qualitative detection and is therefore preferred for routine microbial testing (Table 1.7).

**Table 1.7.** Conventional PCR versus Real-Time PCR (source: Anonymous (2008a)).

| | **Conventional PCR** | **Real-time PCR** |
|---|---|---|
| Labour | Gel electrophoresis required | No post-PCR steps |
| Result interpretation | Subjective | Objective (software based) |
| Reliability | Specificity based on 2 primers | Increased specificity when fluorescent probes are used |
| Skills and infrastructure | Highly demanding to avoid carry-over contamination | Simple use, no risk of carry-over contamination |
| Throughput | Limited throughput due to post-PCR processes | Highly flexible throughput |

The key feature of real-time PCR is that it allows monitoring of the DNA amplification process as it happens. Both enzymatic PCR process and detection of the PCR product occur in the same reaction. Real-time PCR requires suitable PCR platforms that collect data online from every cycle. Indeed, this technique essentially measures the quantity of accumulated PCR product in each cycle using fluorescent labelling technology (DNA-associating dyes, labelled probes or primers). The signal generated is related to the amount of PCR product present during each cycle and increases as the amount of specific amplicon increases. The measurement of fluorescence at each PCR cycle is displayed on a plotted curve. If the target DNA is

present in a sample, the cycle number or time taken to generate a detectable signal depends on its initial concentration in the sample. Mackay (2004) has described a number of reporters available for real-time PCR. In this review, the principle of SYBR Green I, the most used labelled probes and some labelled primers will be described in detail. Real-Time PCR is also called kinetic PCR. The kinetics of PCR has a sigmoid profile when plotted on a graph of number of amplified molecules against cycle number as shown on Figure 1.1.

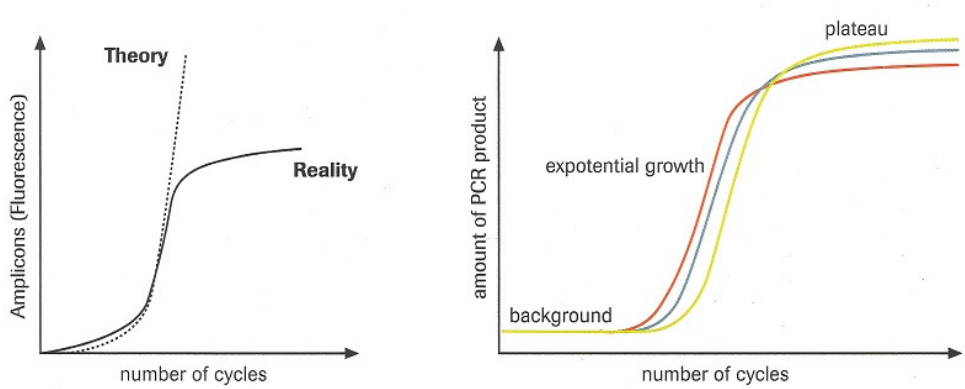


**Figure 1.1.** Sigmoid profile of real-time PCR amplification (source: Obermaier *et al.* (2009)).

As seen in Figure 1.2, the curve is divided into three characteristic phases: the early background phase, the middle exponential growth phase (or log linear phase), and the late plateau phase. The PCR cycle at which the PCR product fluorescence intensity finally overtakes the background and becomes visible is called the crossing point (Cp). Quantification is performed at this stage of the PCR reaction. Then, the exponential phase begins where the PCR rate is maximal. Late in the PCR, the reaction reaches the plateau phase because of a lower PCR product accumulation, as the concentration of substrates decreases, by-products accumulate, and competitor reactions and re-annealing of PCR products increase.

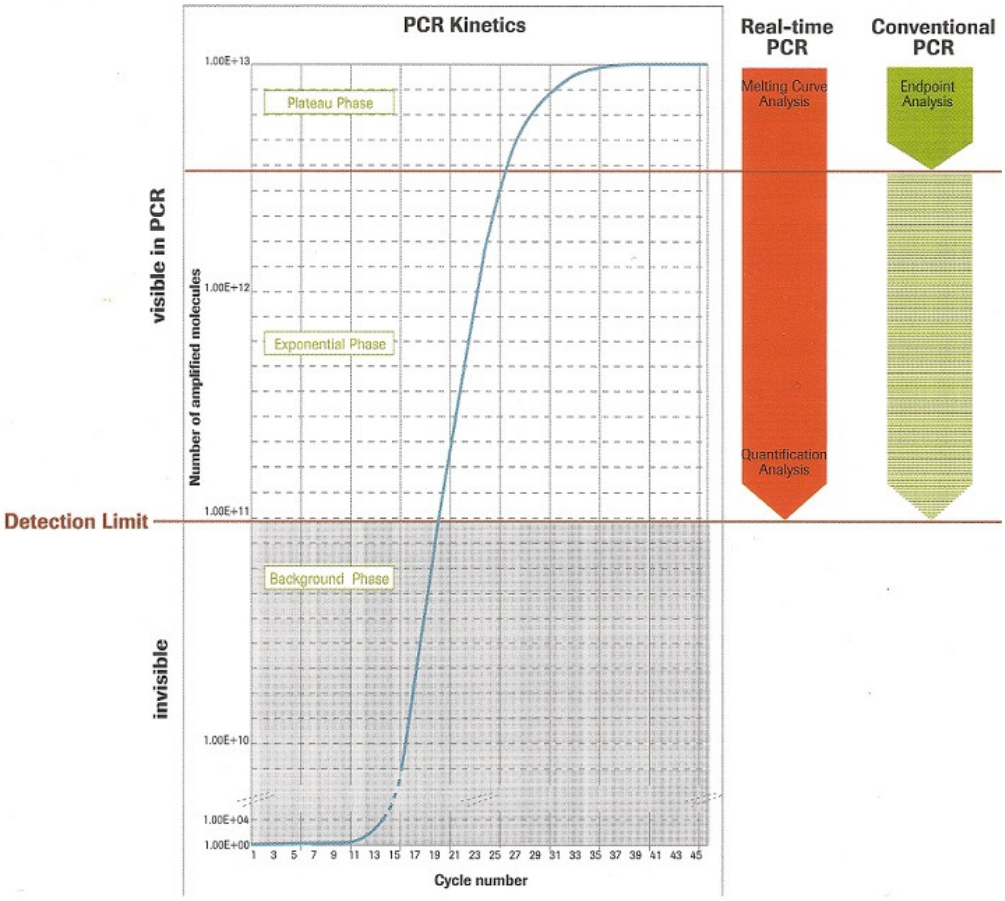


**Figure 1.2.** PCR kinetics (number of amplified molecules function of cycle number) (source: Obermaier *et al.* (2009)).

### 3.2.2. DNA-associated dyes

Classical intercalators, such as ethydium bromide, YO-PRO-I, SYBR Green I or BOXTO, associate with double stranded DNA (dsDNA). Among the double-stranded DNA binding dyes, there are also Hoescht 33258 and BEBO (Martin Bengtsson *et al.*, 2003) which are minor groove binders. These dyes emit virtually no fluorescence when free in solution due to vibrations engaging both aromatic cyclic chemical structures which convert electronic excitation energy into heat that dissipates to the surrounding solvent. The dyes become brightly fluorescent when they bind to dsDNA and are exposed to a wavelength of light capable of exciting these dyes (Mackay *et al.*, 2007c). The fluorescence increases with the amount of dsDNA formed, though not strictly proportionally as the dye fluorescence depends on the dye to base binding ratio, which decreases during the PCR reaction (Kubista *et al.*, 2006). SYBR Green I (Figure 1.3) is commonly used in real-time PCR applications and its' binding is not affected by potential mutations of the target gene (Guilbaud *et al.*,

2005). As dsDNA forms (Figure 1.3A) and is synthesized (Figure 1.3B), SYBR Green I binds the dsDNA and the fluorescent signal from the bound SYBR Green I (green light) increases until the end of the elongation (Figure 1.3C). SYBR Green I binds to any double stranded DNA, notably primer dimers, therefore primer sets used in the PCR assay must be highly specific and generate a single PCR product (Ririe *et al.*, 1997).

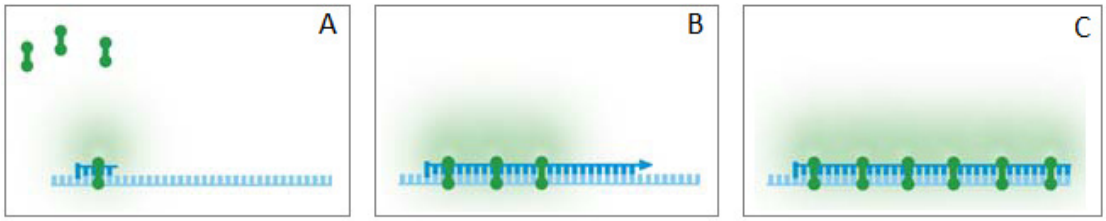


**Figure 1.3.** Mechanism of SYBR Green I activity (source: Obermaier *et al.* (2009)).

SYBR Green I has been used as a detection format in real-time PCR for the detection of a number of foodborne pathogens (Alarcon *et al.*, 2006; Guilbaud *et al.*, 2005; Nam *et al.*, 2005; Oliveira *et al.*, 2005; Tyagi *et al.*, 2009).

### 3.2.3. Labelled probes

#### *3.2.3.1. Hydrolysis probes*

Also known as TaqMan® probes (Figure 1.4), hydrolysis probes are dual labelled probes developed by Applied Biosystems (Life Technologies, Carslbad, USA). These probes rely on the 5' exonuclease activity of the DNA *Taq* polymerase for detecting target DNA in the samples. TaqMan® probes consist of a 18-22 bp oligonucleotide probe which is labelled with a reporter fluorophore derived from fluorescein at the 5' end (5-carboxyfluorescein (FAM); 6-carboxy-2´ ,4,7,7´ -tetrachlorofluorescein succinimidyl ester (TET), 6-carboxy-4,5-dichloro-2,7-dimethoxyfluorescein (JOE), 6-carboxy-2´ ,4,4´ ,5´ ,7,7´ -hexachlorofluorescein succinimidyl ester (HEX), VIC® (Life Technologies)) and a quencher fluorophore derived from rhodamine at the 3' end (TAMRA™, Life Technologies) (Tse and Capeau, 2002). Until the probe is hydrolysed by the DNA polymerase, the quencher and the fluorophore are close to each other, separated only by the length of the probe (Figure 1.4A). However, this does not completely quench the fluorescence of the reporter dye, which provides background fluorescence. When target DNA is amplified, the probe anneals specifically between the forward and reverse primer to an

internal region of the target sequence (Figure 1.4B). DNA polymerase performs an extension of the primer and replicates the template to which the TaqMan® is bound. The 5' exonuclease activity of the DNA polymerase cleaves the probe, which then frees the reporter molecule away from its close proximity to the quencher (Mackay *et al.*, 2007c) (Figure 1.4C). Subsequently, the fluorescence intensity of the reporter dye increases (Figure 1.4D). The process is repeated at each PCR cycle and does not interfere with the accumulation of PCR product.

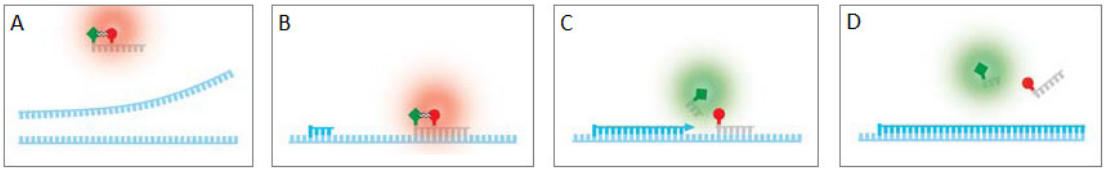


**Figure 1.4.** Mechanism of activity of hydrolysis probes (source: Obermaier *et al.* (2009)).

TaqMan® -based real-time PCR methods have been developed for a number of applications in food microbiology (Nogva *et al.*, 2000; Oravcova *et al.*, 2007; Seo and Brackett, 2005; Yoon *et al.*, 2005)

### *3.2.3.2. Hybridization probes*

The hybridization probes (LightCycler® HybProbes) detection format is based on the principle of Fluorescence Resonance Energy Transfer (FRET) (Figure 1.5). In the PCR mix, two sequence specific oligonucleotide probes are labelled with two dyes: a donor (fluorescein) and an acceptor or quencher (Tse and Capeau, 2002). If the probes are free in the PCR mix, the fluorescence of hydrolysis probes is quenched (Figure 1.5A). When both probes are bound to DNA target, the donor and the acceptor fluorophores are within 1 to 5 nucleotides of each other. Excitation of the donor by blue light at ~470 nm results in an energy transfer to the acceptor which thereafter emits light of longer wavelength (Figure 1.5B). The amount of fluorescence generated is proportional to the amount of PCR product. During the elongation and denaturation steps, HybProbes are displaced, but not cleaved (Figure 1.5C).

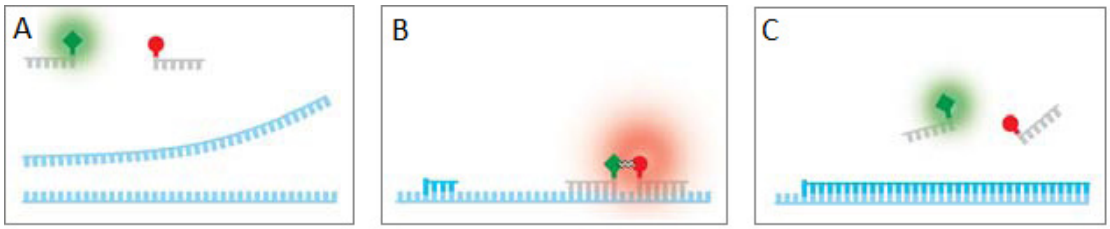


**Figure 1.5.** Mechanism of activity of hybridization probes (source: Obermaier *et al.* (2009)).

Some real-time PCR assays using HybProbes include an internal amplification control that is exogenous control DNA, which is co-amplified with the target DNA in order to detect any PCR inhibition (Muska *et al.*, 2007). Hybridization probes have been applied for the detection of pathogens in a variety of food samples using in-house methods (Berrada *et al.*, 2006a; Boraychuk *et al.*, 2007; Ellingson *et al.*, 2004; O'Hanlon *et al.*, 2004; Yoon *et al.*, 2005) or commercial detection kits (Berrada *et al.*, 2006b; Junge and Berghof-Jager, 2006).

### 3.2.4. Labelled primers: an example using LUX™ (Light Upon eXtension) primers

LUX™ Primers from Invitrogen (Life Technologies) are oligonucleotides labelled with a single fluorophore (FAM or JOE). The D-LUX™ (Light Upon eXtension) detection technology is cheap and includes custom-synthesized primer sets that are easily designed online using D-LUX™ Software. Each primer is typically 20 to 30 bases in length. It is the choice of the user to label either the forward or reverse primer. The labelled primer is designed with the fluorophore close to the 3´-end in a hairpin structure (Figure 1.6). This configuration intrinsically allows fluorescence quenching when free in the PCR mix (Figure 1.6A). The natural quencher is brought into close proximity with the dye via stretches of 5' and 3' complementary sequences (Mackay *et al.*, 2007c). In the presence of target DNA, the primer anneals to the DNA sequence (Figure 1.6B) and the fluorophore is not quenched any more, resulting in a significant increase in fluorescence. A nascent DNA strand is then extended (Figure 1.6C).

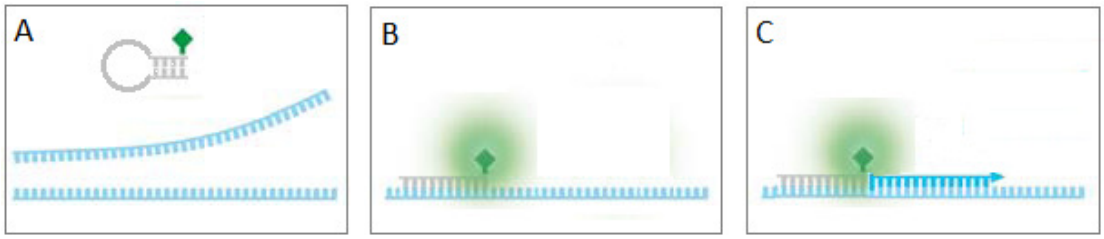


**Figure 1.6.** LUX™ primers (source: modification of Obermaier *et al.* (2009), this thesis).

Real-time PCR assays using LUX™ primers have first been described in virology (Aitichou *et al.*, 2005; Antal *et al.*, 2007; Chen *et al.*, 2004; Nordgren *et al.*, 2008; Slavov *et al.*, 2008), while a few applications in bacteriology have also been reported (Balcazar *et al.*, 2007; Kunchev *et al.*, 2007; McCrea *et al.*, 2007; S. L. Mitchell *et al.*, 2009; R.-s. Xu *et al.*, 2008).

### 3.3.3. Qualitative detection

Microbial analysis of foods using real-time PCR assays is based on the presence / absence of target DNA in the sample and therefore provides data as qualitative detection. In a typical real-time PCR assay of 35 to 40 cycles, the presence of the PCR product is confirmed when some fluorescence is measured by the real-time PCR platform. Fluorescence generated in the late cycle may be considered as non-specific amplifications. This must be checked with end-point melting curve analysis originally used in conjunction with real-time PCR as presumptive identification of the target DNA (Ririe *et al.*, 1997). After PCR, the PCR products are melted at a constant rate (usually 0.1 to 0.3 °C/s) and the decrease in fluorescence is monitored as the strands dissociate (Pryor and Wittwer, 2006). The use of melting curve analysis is an alternative to detection using gel electrophoresis as the $T_M$ of the specific amplicon is analogous to the detection of an electrophoretic band.

When SYBR Green I is used as detection format for real-time PCR multiplex reactions, discrimination of amplicons should be possible, provided the $T_M$ values are sufficiently different (Giglio *et al.*, 2003b) as well as for LUX™ primers applications (Kalvatchev *et al.*, 2010). Melting curve analysis may be performed when using Hybprobes but not *Taqman*® probes. Indeed, the fluorophore is not incorporated in the PCR product in the case of hydrolysis probes. Therefore, there is no variation of fluorescence with melting the dsDNA after PCR. Multiplex real-time PCR assays may involve several primer sets, each set enabling the amplification of a unique DNA

sequence from different bacterial species. A corresponding number of probes specific for one of the amplicons is used and each probe is labelled with a dye of a different colour. The bacterial species is identified by the colour of the resulting fluorescence detected in a specific channel of the real-time PCR platform.

## 3.4. Quantitative detection

### 3.4.1. Quantification strategies

Two main analysis techniques using real-time PCR exist, and depend on the complexity and the type of data to be obtained i.e either relative or absolute quantification. Relative quantification involves the expression of the target concentration as a ratio of target versus reference gene in the same sample as noted in the study of Fitzmaurice *et al.* (2004) of the VT 1 and VT 2 virulence gene expression in *E. coli* O157:H7, rather than an absolute value. However, absolute quantification is truly quantitative as the exact number of nucleic acid targets is determined in the sample t, which is then purported to allow determination of the true bacterial number in a sample. Serial dilutions of an external DNA standard with a predefined known concentration of a gene are used to generate standard curves (Tse and Capeau, 2002). At least five dilution points are prepared and amplified either in duplicate or triplicate. The DNA concentration of the unknown sample is then determined by measuring the Cp value (Figure 1.7).

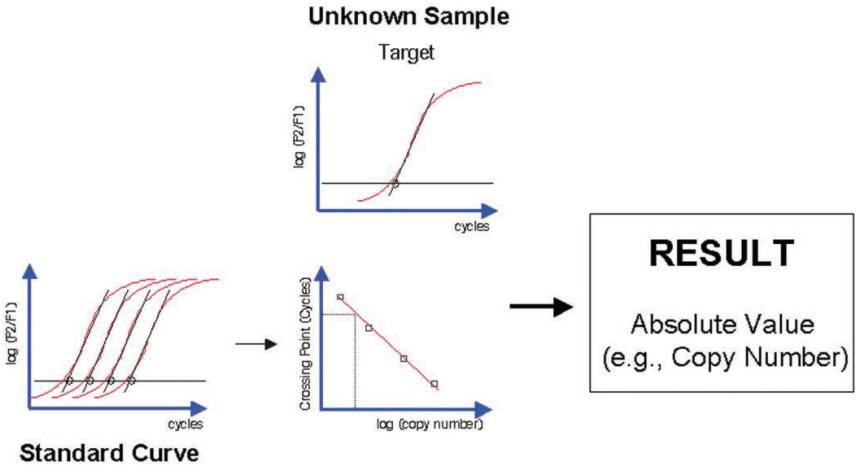


**Figure 1.7.** Mechanism of determination of an unknown sample concentration using real-time PCR (source: (Anonymous, 2003a)).

The concentrations selected to generate the standard curve should fall within the expected concentration range of the DNA target. The standard curve corresponds to the linear regression line through the data points plotted as Cp versus logarithm of standard concentration. Real-time PCR software associated with the platform allows determination of the Cp and displays the concentrations of the unknowns that are calculated from the standard curve. LightCycler® Software provides additional values: the error value and the slope. The error value (mean squared error of the single data points fit to the regression line) provides a measure of the accuracy of the quantification result based on the standard curve, with an acceptable value < 0.2. The slope describes the kinetics of the PCR amplification. The PCR efficiency (E) is calculated using the formula: $E = 10^{-1/slope}$. An efficiency of 2 indicates that the number of target molecules is doubling at each PCR cycle (Anonymous, 2007c).

Absolute quantification strategies are purported to determine the exact number of nucleic acid targets in a sample and data should be expressed in relation to a specific units which are universally interpreted among scientists and laboratories (Mackay *et al.*, 2007b). In food microbiology, examples of denominators include unit mass of foods (Alarcon *et al.* 2006; Berrada *et al.* 2006; Hein *et al.* 2001), unit volume of milk (Lopez-Enriquez *et al.*, 2007), unit volume of enrichment broth (Rossmanith *et al.*, 2006), unit surface of swabbed areas (Guilbaud *et al.*, 2005; Mafu *et al.*, 2009). However, to maintain the relationship between bacterial number in the original food sample and the enumeration by quantitative real-time PCR, prior enrichment is normally proscribed. Therefore, quantification by real-time PCR requires a dedicated sample preparation step for extraction of cells from the food and obtaining highly purified DNA for amplification (Jasson *et al.*, 2010). To date, real-time PCR has been applied for the enumeration of *L. monocytogenes* in meat, *S. aureus* in foods and *Campylobacter* spp. in poultry carcasses rinses. However, the limit of quantification ranges from $10^3$ to $10^4$ cells per g, which is normally above the typical bacterial concentrations found in many commercial food products subjected to good manufacturing practices (GMP) (usually less than 100 CFU / g).

### 3.4.2. Standards used for absolute quantification

Muska *et al.* (2007) employed the term "standard" or "reference standard" to describe "a material suitable for real-time PCR applications which is a specimen derived from a known biological source and whose value has been established by

consensus means". Standards are purported to provide the means by which scientists adhere to a common measurement. Only a few reference standards, notably for quantification diagnostics in virology, are procured by institutions such as the World Health Organization (WHO) or the UK National Institute of Biological Standards and Control. The introduction of quantitative molecular testing has begun to be commercialized with some applications in foods, for instance the foodproof® *Campylobacter* quantification kit (Biotecon Diagnosctics GmbH, Potsdam, Germany) in chicken rinses. Laboratories normally produce their own calibration standards in order to quantify unknown food samples and standards are typically expressed as "copy number" or Colony Forming Unit (CFU).

"Copy number" unit measures the quantity of target molecules initially present in the PCR reaction and are typically prepared using genomic DNA (Alarcon *et al.*, 2006; Guilbaud *et al.*, 2005; Hein *et al.*, 2001; Rossmanith *et al.*, 2006), plasmids containing the target sequence (Chen *et al.*, 2008; Loddenkotter *et al.*, 2005; Sails *et al.*, 2003; W. Xu *et al.*, 2008), or PCR products (Furet *et al.*, 2004). The DNA concentration of a standard is first determined by UV spectrophotometry giving an approximate value (Tse and Capeau, 2002; Vaerman *et al.*, 2004) in µg / µl. Then, the copy number of a DNA target sequence is calculated using the following formula:

$$n = (m \times N_A) / M$$

where $n$ is the number of base pairs, $m$ is the DNA mass, $N_A$ is the Avogadro's number ($6.02 \times 10^{23}$ bp/mol) and $M$ is the average molecular weight of a base pair ($610$ g.mol$^{-1}$).

When using genomic DNA as a standard, the size of the bacterial genome (n) is needed; however, such data is not systematically available for every given microorganism. The preparation of plasmid standards requires the insert to be purified and cloned into a vector, which can be expensive and laborious. Moreover, the efficiencies of cloning and transformation depend on: the nature of the insert (size, toxicity, vector-to-insert ratio, freshness of the PCR products), the plasmid size and the competent state of the cells (Anonymous, 2006b; Siguret *et al.*, 1994; Szostkova and Horakova, 1998). The use of PCR products as standards (Dhanasekaran *et al.*, 2010) may be not advised since errors may be generated during subsequent PCR (Loewen and Switala, 1995).

"CFU per unit volume" of sample is the expression used for DNA standards prepared by serial dilution of a pure bacterial culture followed by DNA extraction (Ott

*et al.*, 2004; Yang *et al.*, 2007) or, alternatively, standard curves can be obtained from serial dilutions of a spiked food sample (Berrada *et al.*, 2006a; Berrada *et al.*, 2006b; Takahashi *et al.*, 2005). In both cases, the concentration of the pure bacterial culture is determined using spectrophotometry or a counting chamber giving an approximate value that must be confirmed by plate counting. Moreover, one CFU may consist of a chain or a cluster of cells.

### 3.4.3. Detection of viable cells

#### *3.4.3.1. Reverse transcription PCR*

An alternative method based on the amplification of messenger RNA (mRNA) by reverse transcription (RT) PCR allows distinguishing between live and dead cells (Betts, 2000). The mRNA strand is first reverse transcribed into its complementary DNA (cDNA). Then, the usual PCR process follows. RT PCR can either be performed in one or two steps. In the first step or the "first strand reaction", complementary DNA is generated from the mRNA template using dNTPs and an RNA-dependent DNA polymerase or reverse transcriptase. Reverse transcription is ensured using a DNA primer in a reverse transcriptase buffer for one hour at 37°C. After completion, the "second strand reaction" or the PCR is initiated. The original RNA template is degraded by RNase H, leaving pure cDNA (plus spare primers). This process can be simplified into a single step process by the use of wax beads containing the required enzymes for the second stage of the process which are melted, releasing their contents, on heating for primer annealing in the second strand reaction.

Various conventional and real-time RT PCR methods have been developed for the detection in foods of viable Enterobacteriaceae (Klein and Juneja, 1997; McIngvale *et al.*, 2002; Szabo and Mackey, 1999; Techathuvanan and D'Souza, 2011; Techatruvanan *et al.*, 2010) or *L. monocytogenes* (Herman, 1997; Klein and Juneja, 1997). However, the use of RT PCR is limited by the complexity of extracting undegraded mRNA within food matrices (Mustapha and Li, 2006) and assays are not very reproducible when using mRNA as a target (McKillip *et al.*, 1998; Sheridan *et al.*, 1998).

### *3.4.3.2. Photoactivable DNA-intercalating dyes*

DNA from dead cells may persist in the environment from a few days to three weeks and therefore total DNA quantification may be overestimated or false positive data may be recorded (Nocker and Camper, 2006). Viable PCR or real-time PCR is becoming more popular by the use of selective nucleic acid intercalating dyes such as ethidium monoazide (EMA) or propidium monoazide (PMA) before DNA extraction. EMA and PMA are purported to reduce PCR signals from DNA originating from dead cells (Fittipaldi *et al.*, 2011) by penetrating into membrane-compromised or dead cells and intercalating into the DNA. Both molecules consist of an azide group allowing covalent binding to DNA or RNA upon exposure to bright visible light (Bolton and Kearns, 1978; Nocker and Camper, 2006). Photolysis converts the azide group into a highly reactive nitrene radical which reacts with the bound DNA (Fittipaldi *et al.*, 2011). Therefore, the DNA can not be amplified by PCR (Nocker and Camper, 2009; Rudi *et al.*, 2005a). Unbound excess dye reacts with water molecules during cross-linking with the DNA, giving hydroxylamine which does not react with the DNA that may be extracted from live cells (Fittipaldi *et al.*, 2011). However, it has been demonstrated that EMA may penetrate cells with intact membranes (Flekna *et al.*, 2007; Nocker and Camper, 2006), the degree of which may also vary according to the bacterial species (Nocker *et al.*, 2006) or the concentration (Wang *et al.*, 2009). PMA is preferred for combination with real-time PCR protocols as it is purported to penetrate membrane-compromised or dead cells with permeabilized cell membranes only (Bae and Wuertz, 2009; Garcia-Cayuela *et al.*, 2009; Josefsen *et al.*, 2010; Kobayashi *et al.*, 2009c; Nocker *et al.*, 2007a; Varma *et al.*, 2009). Another DNA intercalating dye is a commercially available product: Reagent D (Biotecon Diagnostics GmbH) which is purported to eliminate DNA from dead cells according to the same principle as EMA or PMA. However, the reagent has been optimised for elimination of dead Enterobacteriaceae in infant milk formula and therefore may not work with other bacterial species or within other food matrices.

# 4. POTENTIAL IMPROVEMENT OF PCR METHODOLOGY USING FCM IN FOOD MICROBIOLOGY

## 4.1. FCM principles

FCM is a detection technology based on the immediate measurement of isolated cells at a sensing site as they flow past in a liquid stream (Carter and Ormerod, 1990). A pneumatic system distributes the cell suspension into a laminar flow of sheath fluid; hydrodynamic targeting of the sample stream causes cells to be interrogated one by one, by a focus light source (Comas-Riu and Rius, 2009) as shown in Figure 1.8.

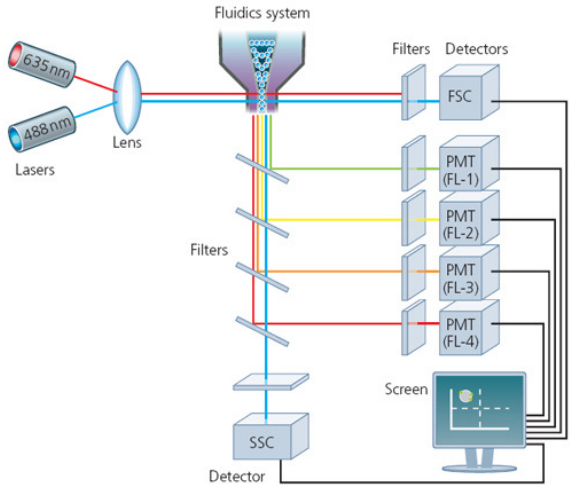


**Figure 1.8.** Principle of FCM (source: (Anonymous, 2011), Abd Serotec)).

FCM provides individual, qualitative and quantitative data for particles in suspension in the flow stream. FCM analyses optical and chemical signals emitted by a particle passing an ultraviolet or laser beam (Ishii *et al.*, 2010). The measured signals are essentially associated with:

- intrinsic optical properties of the particles, corresponding light diffusion related to the dimensions of the particle, its' internal structure and the auto fluorescence generated by some cells. The degree of light refractability from interrogated cells is associated with cell surface properties such as size and internal structures and which affect forward (FSC) and side (SSC) light scattering. The FSC intensity provides information on the particle size and allows cell distinction from cellular debris (Rothe, 2009). However, there is no direct correlation between size and FSC (Julia *et al.*, 2000). The SSC signal corresponds to the light measured at 90° angle to the excitation line providing data about the granular content of the particle and cell morphology

(Comas-Riu and Rius, 2009). Both FSC and SSC signals are unique to each particle and may be used to differentiate cell types within a heterogenous sample.

- induced optical properties of fluorescence (FL) obtained by specific labelling of structures or cellular functions. Intracellular components such as flavin nucleotides pyridine and photosynthetic pigments (Deere *et al.*, 2003) or nicotinamide adenosine dinucleotide phosphate (NADPH) (Comas-Riu and Rius, 2009) emit natural fluorescence (autofluorescence). However, most microorganisms are optically too similar to resolve from each other or from debris by conventional FCM. Therefore, fluorescent labelling using dyes is often used to probe the viability and metabolic status of microorganisms using FCM (Flint *et al.*, 2007; Gunasekera *et al.*, 2003).

The signals are collected by optical filters combined with light detectors such as photomultipliers (PMTs) tubes or photodiodes, then amplified, digitized, treated and data stored in a computer. This cell-by-cell analysis process is a multiparametric process and is able to be performed at a speeds ranging from several thousand to 100,000 events/cells per second (Comas-Riu and Rius, 2009). The computer software of the cytometer calculates statistical data associated with the distribution of parameters measured representing data in the form of histograms (one parameter) or cytograms (two parameters) for one or several cell populations. Software exploiting the data provided by the flow cytometer allows data gating to target particular sub-populations, for the detection of cells of interest (Comas-Riu and Rius, 2009).

Detected particles/cells may be purified in cell sorters (Figure 1.9).

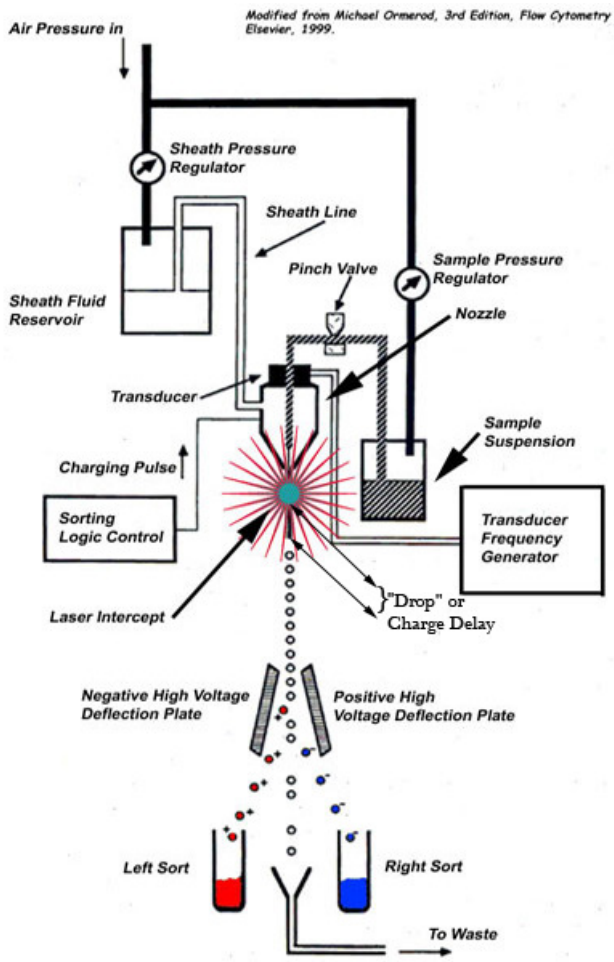


**Figure 1.9.** Principle of cell sorting (source: Carter and Ormerod (2000) modified by (no date), University of Connecticut Health Center).

The hardware and/or software used to make sort decisions may be identical to those used for data analysis for conventional FCM (Shapiro, 2003a). In the droplet formation system of a cell sorter, the sheath fluid containing the cells is broken evenly into droplets by the vibration of the piezoelectric crystal attached to the flow chamber (Comas-Riu and Rius, 2009). At the droplet break off point, a sort decision signal is generated (Shapiro, 2003a). When a cell is about to be sorted, a drop charging circuitry applies a voltage to the droplet containing the cell. The charged and uncharged droplets pass through an electric field maintained between two deflection plates which are highly charged and of opposite polarity. The charged droplets are directed out of the main stream toward the deflection plate of the opposite charge to

be collected in tubes or plates. The uncharged main stream is thereafter rejected into a waste reservoir.

The acronym "Fluorescence Activated Cell Sorting" (FACS), trademarked and owned by Becton Dickinson and Company (Franklin Lakes, USA), associates FCM and sorting as described above. The term is however frequently used by scientists for all types of sorting and even non-sorting applications, while it is not generic for FCM (Jaroszeski and Radcliff, 1999). It has been demonstrated that FACS allows the rapid concentration and enrichment of cell populations present in low abundance (Wallner *et al.*, 1997) or sorting of defined cell numbers into tubes or microplates (Wallner *et al.*, 1997).

## 4.2. Flow cytometric bacterial detection

### 4.2.1. Distinction between Gram-positive and negative bacteria

FCM can be used for the analysis of food products allowing early pathogen detection. Cytometric methods differentiating Gram-positive and Gram negative bacteria have been reported. One method used two fluorescent DNA-binding stains, SYTO 13 and hexidium iodide (HI). SYTO 13 is a membrane permeable stain whereas HI is blocked by the lipopolysaccharide layer of Gram-negative bacteria and is therefore permeable to Gram-positive bacteria and Gram-negative bacteria with a compromised lipopolysaccharide layer (Mason *et al.*, 1998). Another method consists in combining Oregon Green-conjugated wheat germ agglutinin (WGA) with HI. WGA binds to *N*-acetylglucosamine in the peptidoglycan layer of the cell wall of Gram-positive bacteria and HI binds to the DNA of all bacteria after permeabilization using ethylenediaminetetraacetic acid (EDTA) after incubation at 50°C for 15 min (Holm *et al.*, 2004).

### 4.2.2. Bacterial viability

Food microbial analysis can be conducted using FCM for assessment of bacterial viability. The LIVE/DEAD® *Bac*Light™ bacterial viability kit consists of two nucleic acid-binding stains which in combination induces varying degrees of red/green fluorescence depending on cell viability. SYTO 9™ penetrates all bacterial membranes while propidium iodide (PI) only penetrates cells with damaged membranes (Comas-Riu and Rius, 2009). Therefore increasing green fluorescence is

related to live/intact cells while increasing red fluorescence is related to damaged/permeabilised/dead cells within a microbial population. Hence this kit is potentially useful for carrying out rapid viable counts in processed foods (Duffy and Sheridan, 1998) and drinking water (Boulos *et al.*, 1999). However, the application of FCM is possible in case of high bacterial concentrations in a food matrix (Comas-Riu and Rius, 2009); benzalkonium chloride is purported to reduce background signals. Moreover, the gating of bacterial populations on the flow cytometer software may be crucial for samples with unknown bacterial concentrations.

In flow cytoenzymology, it has been demonstrated that dehydrogenase activity could be an indicator of bacterial viability using the substrate 5-cyano-2, 3-ditolyltetrazolium chloride (CTC) as shown by Kaprelvants and Kell (1993) in a suspension of *Micrococcus luteus*. The use of fluorogenic esters monitors esterase activity and therefore the viability of a wide range of bacterial species. The D-count® flow cytometer from AES Chemunex (Paris, France) allows bacterial quantification in water, foods and pharmaceutical products of viable cells using a fluorogenic ester as a reporter of intracellular enzyme activity (Diaper and Edwards, 1994). It is a rapid and automated viable counting technique (Flint *et al.*, 2006). A non-fluorescent substrate is cleaved by the esterase activity of the bacteria, which induces fluorescence at exposure to light of certain wavelengths. The bacterial count is function of the fluorescence generated by the cells and resulting from the enzyme activity. The sample fluid composed of labelled cells is slowly injected into the middle of the sheath fluid. A laminar flow is then created from the speed difference between both fluids, which reduces the diameter of the sample flow to a few μm, and ensures that the cells pass one-by-one through the quartz flow cell. The fluorochrome located in the cells is excited at 488 nm by an argon laser. The fluorescence is emitted at 515 nm and detected by two sensors at red and green wavelengths. The red to green fluorescent intensity ratio discriminates auto-fluorescent particles from cell that display fluorescence. A ratio close to 1 indicates a labelled microorganism that is counted. Flint *et al.* (2006) demonstrated that D-count® could be used to enumerate the total bacteria in whole milk and whey powder samples collected at various stages during manufacture of these products, within 2 h. Another example from Chemunex purported to be suitable for microbial analysis of foods is the BactiFlow ALS® which uses the same technology as the D-count® but is not automated (Flint *et al.*, 2006).

However, both systems are unable to identify different bacterial species and the applications in food analysis are therefore quite limited.

### 4.2.3. The use of antibodies

Bacterial identification in FCM is also possible using monoclonal and polyclonal antibodies conjugated to fluorochromes such as fluorescein isothiocyanate (FITC) (Kusunoki *et al.*, 1996; Raybourne, 2001; Yamaguchi *et al.*, 2003) or phycoerythrin (McClelland and Pinder, 1994). Hibi *et al.* (2006) described an FCM-based method combined with IMS of *L. monocytogenes* cells from a cell suspension containing *Pseudomonas fluorescens*. The method is suitable for the detection of *L. monocytogenes* in suspension in the range $10^2$ to $10^8$ cells per ml.

Regulations for food products specify quite low thresholds in pathogen concentration and the limit may sometimes be above the level of detection possible using FCM and antibody labelling required to satisfy legislation (Comas-Riu and Rius, 2009). Although FCM using labelled antibodies for the detection of foodborne pathogens is a direct technique, false positive results generated by background autofluorescent bacteria may be observed (Phillips and Martin, 1988). Therefore, a prior enrichment of the food sample may be required before FCM analysis. Indeed, *Pseudomonas* species fluoresce at 525 nm in the absence of antibody (Shapiro, 2003d). Another example is the case of *Synechoccus* that produces ample quantities of phycoerythrin (Shapiro, 2003d). Vesey *et al.* (1997) described a procedure of selection of labels for detection of pathogens in water samples. Considering the autofluorescence of the particles present in the water and the background fluorescence in the water itself, the signal-to-noise for targeted specimens was optimized. According to Shapiro (2003a), fluorescent antibodies are mainly available for identifying one species or a strain but not for targeting a large number of species.

### 4.3.4. FLOW-FISH

Fluorescence *in situ* hybridization (FISH) is coupled with FCM (FLOW-FISH) to detect specific food contaminants with high resolution. FISH is a sensitive and robust molecular method that uses sequence-specific rRNA-targeted fluorescently-labelled oligonucleotide probes, also called phylogenetic stains that specifically label permeabilized bacterial cells (Bisha, 2009; Davey, 2002). Such probes can be covalently labelled with FITC, isothiocyanate derivative (CY3). Various probes

anneal to sequences in the 16S rRNA and target primary kingdoms (e.g. Eubacteria), among smaller taxonomic groups, or species and strain level (Giovannoni *et al.*, 1988). Analysis by FCM using probes complementary to group-specific region of 16S RNA sequences was reported to provide satisfactory results (Amann *et al.*, 1990; Wallner *et al.*, 1993). Indeed, target and non-target bacterial populations could be separated. However, in some nutrient-limited media, bacteria may grow slowly or become dormant so that the concentration of ribosomes may be below the threshold of detection (Davey, 2002). Therefore, signal amplification may be convenient (Zarda *et al.*, 1991). As an application to food samples, Bisha and Brehm-Stecher (2009) developed a FLOW-FISH method detecting low levels of *Salmonella* spp. (~$10^3$ cells.mL$^{-1}$ sprout wash) against high levels of naturally occurring sprout flora (~ $10^7$ to $10^8$ CFU/g sprouts). However, to date FLOW-FISH has not been widely used for microbial detection applications.

## 4.3. The potential for combination of PCR and FACS methodologies for bacterial detection

FACS methodologies separate and sort bacterial populations for subsequent analysis for pathogen detection. Morgan *et al.* (2004) introduced the production of microbiological quality control standards for routine use in microbiology by sorting of *L. monocytogenes*, *E. coli* and *Bacillus cereus* cells using a FACSCalibur™ (Becton Dickinson and Company). Such standards allow an assessment of the efficacy of testing methods and culture media. In practice, the concentration of a pure culture is estimated by spectrophotometry and / or plate counting and microbiological standards are then prepared by serial dilutions. To circumvent variability and inaccuracy issues, FCM associated with cell sorting provides with high reproducibility microbiological standards in an easy-to-use format with simple storage conditions. In Morgan *et al.*'s study (2004), a single 25-µl droplet contained a precise number of living cells and, directly after droplet release, the standards were freeze dried into liquid nitrogen. Wohlsen *et al.* (2006) applied Bioball™ cultures for the enumeration of *E. coli* and other coliforms in water samples. Bioball™ systems are 3-mm diameter white beads containing 30 viable organisms produced as described by Morgan *et al*. (2004). *E. coli* and *E. cloacae* Bioball™ cultures were then used as precise reference standards to

compare testing methods including membrane filtration, plate count (pour and spread plate methods), Colilert™ and Colisure™, MPN and 3M™ Petrifilm™. Wohlsen *et al.* (2006) were in agreement with Morgan *et al.* (2004) concluding that these precise microbial standards were a very suitable tool for evaluating the efficiency of methods for bacterial enumeration in water samples. Morgan *et al.* (2004) highlighted the applicability of such standards as controls to measure PCR inhibition. Indeed, a target DNA sequence can be inserted into a bacterial genome to create a PCR internal control. The resulting bacterial cells could be dispensed accurately to provide a quantitative amount of the specific sequence of interest.

Cell sorting has also become a screening method allowing further analysis using PCR; however, to date, very few applications have been developed for detection and characterization of microorganisms. Guillebault *et al.* (2010) combined cell sorting with PCR for the identification of marine bacterial sub-populations. Bowers *et al.* (2010) confirmed by real-time PCR the presence of a specific algal prey inside the vacuoles of sorted algal bloom species. Hoffeman *et al.* (2007) determined gene expression levels in sorted eukaryote cell populations by relative real-time PCR.

Another strategy is combining *in-situ* PCR (ISPCR) with flow cytometry that allows the detection of specific DNA or RNA target sequences and the identification of positive cells in a mixed-cell population. Prior to a FCM procedure, intracellular PCR is carried out where the amplicon is directly labelled by including in the PCR reaction mix a deoxi-uridine-triphosphate (dUTP) instead of thymine usually coupled with digoxygenin (Gibellini *et al.*, 1997) or fluorescein (Sachidanandham and Gin, 2003) and are then detected using FCM. ISPCR has the advantage of maintaining the morphological cell identity while amplifying gene copies within the bacterial cell. ISPCR has been applied in various microbiological studies for gene identification and characterization within bacterial sub-populations (Chen *et al.*, 2000; Sachidanandham and Gin, 2003; C. Wu *et al.*, 2004).

# 5. VALIDATION OF AN ALTERNATIVE MICROBIAL DETECTION METHOD FOR POTENTIAL USE IN THE FOOD INDUSTRY

EU regulations 2073/2005 require that alternative analytical methods are validated following ISO 16140 (Anonymous, 2003j) in the field of microbial analysis of food, animal feeds, environmental and veterinary samples (Betts and Rentenaar, 1998; Rentenaar, 1996). An expert laboratory accredited ISO 17025 is generally in charge of the co-ordination and supervision of the studies in the framework of the certification procedure. Several European validation bodies following the ISO 16140 provide validation certificates for alternative analytical methods: AFNOR (Association Française de Normalisation), NordVal (part of the Nordic Committee on food analysis, Norway) and MicroVal (European Validation and Certification Organisation, Europe). The AOAC (Association of Official Agricultural Chemists) INTERNATIONAL and AOAC Research Institute validate alternative methods in the United States of America according to specific guidelines (Feldsine *et al.*, 2002). Jasson *et al.* (2010) established a list of validated alternative detection methods for the food industry.

For qualitative detection methods, the validation comprises two stages: method comparison study and interlaboratory study. Any method comparison study has to confirm that the alternative method reaches an acceptable degree of correspondence with the reference method using identical samples. 60 samples are generally tested for one matrix. The study evaluates the accuracy, the specificity, the sensitivity, the detection limit, the inclusivity (on more than 50 target strains) and the exclusivity (on more than 30 non-target strains) of the method. The validation process includes inter-laboratory testing to assess the precision and the robustness of the method. If differences are obtained between the alternative and reference method, explanations for the discrepancies may be provided. The inter-laboratory study involves more than 10 laboratories that supply a variability of results using different machines, PCR platforms for instance.

In the case of quantitative methods, a comparison study evaluates the linearity, the accuracy and the bias of the method. Detection and quantification limits are determined as well as relative sensitivity. The inclusivity and exclusivity are evaluated over at least 30 positive strains and at least 20 negative strains. More than 8 laboratories participate to the interlaboratory study.

When validated, a PCR-based method for the detection of foodborne pathogens fulfils criteria as described by Malorny *et al.* (2002). A standardized diagnostic PCR responds to criteria indicated in Table 1.8.

**Table 1.8.** Criteria for standardized diagnostic PCR (source Malorny *et al.* (2002)).

| Criteria | Comment |
|---|---|
| Analytical and diagnostic accuracy | Low false negativeor false positive results |
| Low detection limit | Less than one cell per 25 g |
| High robustness | Inter-lab reproducibility |
| Amplicication controls | Reagent- and positive controls, internal amplication control (list of controls described by Malorny *et al.*, (2002)) |
| Low carry-over contamination risk | Separated working areas, UNG-treatment |
| High speed | At-line or on-line analysis |
| Acceptance | Validation and standardization, nonpatented primer sets |
| Low cost | Cost per analysis |
| Simplicity | User-friendliness and automotion |
| Sample matrix flexibility | No PCR interference |
| Quantitative analysis | Food spoilage microorganisms |

# 6. CONCLUSIONS

The monitoring of foodborne pathogens such as the Enterobacteriaceae, *S. aureus* and *L. monocytogenes* is critical for the food industry to ensure product safety and there is a clear need to detect these microbes more rapidly using novel assays which can provide real-time data. This information can then be used to prevent outbreaks occurring from the consumption of contaminated food products following early and reliable pathogen detection. As earlier stated, detection methods for these microbial groups are multiple including: ISO methods, other modified culturing and identification and emerging alternative rapid methods. Real-time PCR and FCM are two emerging rapid technologies that are becoming more extensively applied in microbial analysis, notably in food products. In food microbiology, real-time PCR allows the detection of a DNA target sequence of microorganisms using a range of detection formats revealing the presence of PCR product as it accumulates in the PCR reaction. *TaqMan*® and LightCycler® HybProbes are today mainly used for microbial analysis. However, the potential of simpler detection formats such as SYBR Green I or LUX™ primers has not been fully exploited in food microbiology for the detection of the Enterobacteriaceae, *S. aureus* or *L. monocytogenes* in simplex or multiplex real-time PCR protocols. FCM is becoming more frequently used for the detection of bacterial populations with the subsequent dispensing of targeted cells by cell sorting. The reliability of the flow cytometric methods relies on the development of suitable cell staining, immunological and molecular methods as well as the use of rapid sample preparation methods. The conjoint use of PCR and FCM has a potential advantage in the detection of bacterial populations as these technologies are complementary from each other. One particular application is sorting of defined bacterial populations for use as standards for PCR. To date, the use of FACS has not been evaluated for the preparation of bacterial standards in absolute quantification by real-time PCR. In food samples, such quantification strategy is restricted arising from detection limits which are normally too high or issues with sample preparation and obtaining a highly purified DNA template. It is clear therefore that the enumeration of viable bacteria from food-processed environments using real-time PCR needs further investigation with the development of a suitable sample preparation where the use of SETS may be of particular interest. Hence, this research project was carried out to assess improvements in PCR and explore its potential combination with FCM for detection of a range of food-borne pathogens.

# 7. REFERENCES

Ablain, W., Soulier, S. H., Causeur, D., Gautier, M. and Baron, F. (2009) 'A simple and rapid method for the disruption of *Staphylococcus aureus*, optimized for quantitative reverse transcriptase applications: Application for the examination of Camembert cheese', *Dairy Science & Technology,* 89(1), 69-81.

Abu Al-Soud, W. and Radstrom, P. (1998) 'Capacity of Nine Thermostable DNA Polymerases To Mediate DNA Amplification in the Presence of PCR-Inhibiting Samples', *Applied and Environmental Microbiology,* 64(10), 3748-3753.

Abu Al-Soud, W. and Radstrom, P. (2000) 'Effects of Amplification Facilitators on Diagnostic PCR in the Presence of Blood, Feces, and Meat', *J. Clin. Microbiol.,* 38(12), 4463-4470.

Ackers, M. L., Schoenfeld, S., Markman, J., Smith, M. G., Nicholson, M. A., DeWitt, W., Cameron, D. N., Griffin, P. M. and Slutsker, L. (2000) 'An outbreak of *Yersinia enterocolitica* O : 8 infections associated with pasteurized milk', *Journal of Infectious Diseases,* 181(5), 1834-1837.

Aitichou, M., Javorschi, S. and Ibrahim, M. S. (2005) 'Two-color multiplex assay for the identification of orthopox viruses with real-time LUX-PCR', *Molecular and Cellular Probes,* 19(5), 323-328.

Al-Soud, W. A., Lantz, P.-G., Backman, A., Olcen, P. and Radstrom, P. (1998) 'A sample preparation method which facilitates detection of bacteria in blood cultures by the polymerase chain reaction', *Journal of Microbiological Methods,* 32(3), 217-224.

Alarcon, B., Vicedo, B. and Aznar, R. (2006) 'PCR-based procedures for detection and quantification of *Staphylococcus aureus* and their application in food', *Journal of Applied Microbiology,* 100(2), 352-364.

Amann, R. I., Binder, B. J., Olson, R. J., Chisholm, S. W., Devereux, R. and Stahl, D. A. (1990) 'Combination of 16S Ribosomal RNA Targeted Oligonucleotide Probes with Flow Cytometry for Analysing Mixed Microbial Populations', *Applied and Environmental Microbiology,* 56(6), 1919-1925.

Anbazhagan, D., Kathirvalu, G. G., Mansor, M., Gracie, O. S. Y., Yusof, M. Y. and Sekaran, S. D. (2010) 'Multiplex polymerase chain reaction (PCR) assays for the detection of Enterobacteriaceae in clinical samples', *African Journal of Microbiology Research,* 4(11), 1186-1191.

Anonymous (1996) 'ISO 11290-1:1996 Microbiology of food and animal feeding stuffs -- Horizontal method for the detection and enumeration of *Listeria monocytogenes* -- Part 1: Detection method',

Anonymous (1998) 'ISO 11290-2:1998 Microbiology of food and animal feeding stuffs -- Horizontal method for the detection and enumeration of *Listeria monocytogenes* -- Part 2: Enumeration method',

Anonymous (1999a) 'ISO 6887-1:1999 Microbiology of food and animal feeding stuffs -- Preparation of test samples, initial suspension and decimal dilutions for microbiological examination -- Part 1: General rules for the preparation of the initial suspension and decimal dilutions',

Anonymous (1999b) 'ISO 6888-1:1999 Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) -- Part 1: Technique using Baird-Parker agar medium',

Anonymous (1999c) 'ISO 6888-2:1999 Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) -- Part 2: Technique using rabbit plasma fibrinogen agar medium',

Anonymous (2003a) 'Absolute Quantification with External Standards', *Roche Applied Science - Part of Roche Diagnostics - Technical Note No. LC11* [online], available: http://www.roche-applied-science.com/sis/rtpcr/lightcycler/lightcycler_docs/technical_notes/lc_11_updated.pdf [accessed

Anonymous (2003b) 'ISO 6887-2:2003 Microbiology of food and animal feeding stuffs -- Preparation of test samples, initial suspension and decimal dilutions for microbiological examination -- Part 2: Specific rules for the preparation of meat and meat products',

Anonymous (2003c) 'ISO 6887-3:2003 Microbiology of food and animal feeding stuffs -- Preparation of test samples, initial suspension and decimal dilutions for microbiological examination -- Part 3: Specific rules for the preparation of fish and fishery products',

Anonymous (2003d) 'ISO 6887-4:2003 Microbiology of food and animal feeding stuffs -- Preparation of test samples, initial suspension and decimal dilutions for microbiological examination -- Part 4: Specific rules

for the preparation of products other than milk and milk products, meat and meat products, and fish and fishery products',

Anonymous (2003e) 'ISO 6888-3:2003 Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) -- Part 3: Detection and MPN technique for low numbers, Geneva, Switzerland',

Anonymous (2003f) *ISO 16140:2003 Microbiology of food and animal feeding stuffs -- Protocol for the validation of alternative methods* Geneva, Switzerland: International Organization for Standardization.

Anonymous (2004a) 'ISO 11290-1:1996/Amd 1:2004 Modification of the isolation media and the haemolysis test, and inclusion of precision data',

Anonymous (2004b) 'ISO 11290-2:1998/Amd 1:2004 Modification of the enumeration medium',

Anonymous (2004c) *ISO 21528-1:2004 Microbiology of food and animal feeding stuffs -- Horizontal methods for the detection and enumeration of Enterobacteriaceae -- Part 1: Detection and enumeration by MPN technique with pre-enrichment. ISO, Geneva, Switzerland*, Geneva, Switzerland: International Organization for Standardization.

Anonymous (2004d) 'ISO 21528-2:2004 Microbiology of food and animal feeding stuffs -- Horizontal methods for the detection and enumeration of Enterobacteriaceae -- Part 2: Colony-count method',

Anonymous (2005a) 'Commission Regulation (EC) No 2073/2005 of 15 November, 2005 on microbiological criteria for foodstuffs',

Anonymous (2005b) '*Yersinia enterocolitica*. Centers for Disease Control and Prevention, Department of Health and Human Services.', [online], available: http://www.cdc.gov/ncidod/dbmd/diseaseinfo/yersinia_g.htm [accessed

Anonymous (2006) 'TOPO TA Cloning® Kit for Sequencing - Five-minute cloning of Taq polymerase-amplified PCR products for sequencing, Invitrogen Corporation ',

Anonymous (2007) 'LightCycler® 2.0 Instrument Operators' Manual - Software version 4.1 - Manual B: for General Laboratory Use - Roche Diagnostics GmbH',

Anonymous (2008) 'Microbiology goes molecular, from DNA to real-time PCR', in Biosystems, A., ed. Dublin,

Anonymous (2011) 'Signal processing', [online], available: http://www.abdserotec.com/support/signal_processing-710.html [accessed

Anonymous (no date) 'How Cytometers Work (Basic Operation of a Cell Sorter)', [online], available: http://flowcytometry.uchc.edu/protocols/cytometers.html [accessed

Antal, M., Farkas, T., German, P., Belak, S. and Kiss, I. (2007) 'Real-time reverse transcription-polymerase chain reaction detection of Newcastle disease virus using light upon extension fluorogenic primers', *Journal of Veterinary Diagnostic Investigation,* 19(4), 400-404.

Arbuthnott, J. P., Coleman, D. C. and Deazavedo, J. S. (1990) 'Staphylococcal Toxins in Human Disease', *Journal of Applied Bacteriology,* 69, S101-S107.

Asao, T., Kumeda, Y., Kawai, T., Shibata, T., Oda, H., Haruki, K., Nakazawa, H. and Kozaki, S. (2003) 'An extensive outbreak of staphylococcal food poisoning due to low-fat milk in Japan: estimation of enterotoxin A in the incriminated milk and powdered skim milk', *Epidemiology and Infection,* 130(1), 33-40.

Bae, S. and Wuertz, S. (2009) 'Rapid decay of host-specific fecal Bacteroidales cells in seawater as measured by quantitative PCR with propidium monoazide', *Water Research,* 43(19), 4850-4859.

Balcazar, J. L., Vendrell, D., de Blas, I., Ruiz-Zarzuela, I., Girones, O. and Muzquiz, J. L. (2007) 'Quantitative detection of *Aeromonas salmonicida* in fish tissue by real-time PCR using self-quenched, fluorogenic primers', *Journal of Medical Microbiology,* 56(3), 323-328.

Bayardelle, P. and Zafarullah, M. (2002) 'Development of oligonucleotide primers for the specific PCR-based detection of the most frequent Enterobacteriaceae species DNA using wec gene templates', *Canadian Journal of Microbiology,* 48(2), 113-122.

Baylis, C. L. (2006) 'Enterobacteriaceae' in Blackburn, C. d. W., ed. *Food Spoilage Microorganisms*, Campden and Chlorleywood Food Research Association, UK: Woodhead Publishing in Food Science, Technology and Nutrition, 624-667.

Bekal, S., Brousseau, R., Masson, L., Prefontaine, G., Fairbrother, J. and Harel, J. (2003) 'Rapid identification of *Escherichia coli* pathotypes by virulence gene detection with DNA microarrays', *Journal of Clinical Microbiology,* 41(5), 2113-2125.

Bengtsson, M., Karlsson, H. J., Westman, G. and Kubista, M. (2003) 'A new minor groove binding asymmetric cyanine reporter dye for real-time PCR', *Nucl. Acids Res.,* 31(8), e45-.

Berche, P., Bonnichon, M., Beretti, J. L., Raveneau, J., Gaillard, J. L., Veron, M., Kreis, H., Reich, K. A., Cossart, P., Geoffroy, C. and Geslin, P. (1990) 'Detection of anti-listeriolysin O for serodiagnosis of human listeriosis', *The Lancet,* 335(8690), 624-627.

Bergdoll, M. S. (1989) '*Staphylococcus aureus*' in Doyle, M. P., ed. *Foodborne Bacterial Pathogens*, New York, NY, USA: Marcel Dekker, 463-523.

Berrada, H., Soriano, J. M., Manes, J. and Pico, Y. (2006a) 'Real-time quantitative PCR of *Staphylococcus aureus* and application in restaurant meals', *Journal of Food Protection,* 69(1), 106-111.

Berrada, H., Soriano, J. M., Pico, Y. and Manes, J. (2006b) 'Quantification of *Listeria monocytogenes* in salads by real-time quantitative PCR', *International Journal of Food Microbiology,* 107(2), 202-206.

Besse, N. G., Audinet, N., Kerouanton, A., Colin, P. and Kalmokoff, M. (2005) 'Evolution of *Listeria* populations in food samples undergoing enrichment culturing', *International Journal of Food Microbiology,* 104(2), 123-134.

Betts, R. (2000) 'Detecting Pathogens in Foods' in Blackburn, C. d. W. and McClure, P., eds., *Foodborne Pathogens*, CRC Press ed., Cambridge, UK: Woodhead Publishing Limited, 13-52.

Betts, R. P. and Rentenaar, I. M. F. (1998) 'MicroVal: A European approach to the certification of new microbiological methods', *Journal of Food Protection,* 61(11), 1579-1582.

Bisha, B. (2009) *Fluorescence in situ hybridization-based detection of Salmonella spp. and Listeria monocytogenes in complex food matrices*, unpublished thesis (Dissertation), Iowa State University

Bisha, B. and Brehm-Stecher, B. F. (2009) *Flow-through imaging cytometry for characterization of Salmonella subpopulations in alfalfa sprouts, a complex food system* WILEY-VCH Verlag.

Bolton, P. H. and Kearns, D. R. (1978) 'Spectroscopic Properties of Ethidium Monoazide - Fluorescent Photoaffinity Label for Nucleic Acids', *Nucleic Acids Research,* 5(12), 4891-4903.

Bonadonna, L., Cataldo, C. and Semproni, M. (2007) 'Comparison of methods and confirmation tests for the recovery *Escherichia coli* in water', *Desalination,* 213(1-3), 18-23.

Boraychuk, V. M., Gensler, G. E., McFall, M. E., King, R. K. and Renter, D. G. (2007) 'A real-time PCR assay for the detection of *Salmonella* in a wide variety of food and food-animal matrices', *Journal of Food Protection,* 70(5), 1080-1087.

Boulos, L., Prevost, M., Barbeau, B., Coallier, J. and Desjardins, R. (1999) 'LIVE/DEAD (R) BacLight (TM): application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water', *Journal of Microbiological Methods,* 37(1), 77-86.

Bowers, H. A., Brutemark, A., Carvalho, W. F. and Graneli, E. (2010) 'Combining Flow Cytometry and Real-Time PCR Methodology to Demonstrate Consumption by *Prymnesium parvum*', *Journal of the American Water Resources Association,* 46(1), 133-143.

Brown, A. (2008) 'Food Safety' in P., A., ed. *Understanding Food - Principles and Preparation*, Belmont, USA: Thomson Learning Inc.

Brownie, J., Shawcross, S., Theaker, J., Whitcombe, D., Ferrie, R., Newton, C. and Little, S. (1997) 'The elimination of primer-dimer accumulation in PCR', *Nucleic Acids Research,* 25(16), 3235-3241.

Call, D. R., Borucki, M. K. and Besser, T. E. (2003) 'Mixed-genome microarrays reveal multiple serotype and lineage-specific differences among strains of *Listeria monocytogenes*', *Journal of Clinical Microbiology,* 41(2), 632-639.

Carter, N. P. and Ormerod, L. G. (1990) 'Introduction to the Principles of Flow Cytometry' in Ormerod, L. G., ed. *Flow Cytometry a Practical Approach*, Oxford, UK: IRL Press, 1-28.

Carter, N. P. and Ormerod, L. G. (2000) 'Introduction to the Principle of Flow Cytometry' in Ormerod, M. G., ed. *Flow Cytometry*, Third ed., Oxford: Oxford University Press, 1-22.

Chan, Y. C. and Wiedmann, M. (2009) 'Physiology and Genetics of Listeria Monocytogenes Survival and Growth at Cold Temperatures', *Critical Reviews in Food Science & Nutrition,* 49(3), 237-253.

Charpentier, E. and Courvalin, P. (1997) 'Emergence of the trimethoprim resistance gene dfrD in *Listeria monocytogenes* BM4293', *Antimicrobial Agents and Chemotherapy,* 41(5), 1134-1136.

Chen, F., Binder, B. and Hodson, R. E. (2000) 'Flow cytometric detection of specific gene expression in prokaryotic cells using in situ RT-PCR', *Fems Microbiology Letters,* 184(2), 291-295.

Chen, R., Huang, W. M., Lin, Z. X., Zhou, Z. F., Yu, H. Q. and Zhu, D. Z. (2004) 'Development of a novel real-time RT-PCR assay with LUX primer for the detection of swine transmissible gastroenteritis virus', *Journal of Virological Methods,* 122(1), 57-61.

Chen, T. R., Hsiao, M. H., Chiou, C. S. and Tsen, H. Y. (2001) 'Development and use of PCR primers for the investigation of C1, C2 and C3 enterotoxin types of *Staphylococcus aureus* strains isolated from food-borne outbreaks', *International Journal of Food Microbiology,* 71(1), 63-70.

Chen, X., Wang, J. and Liu, J. (2008) 'Development of real-time PCR approach for cellulolytic bacteria quantification in the rumen', *Chinese Journal of Animal Science,* 44(11), 36-40.

Christensen, J. E., Stencil, J. A. and Reed, K. D. (2003) 'Rapid identification of bacteria from positive blood cultures by terminal restriction fragment length polymorphism profile analysis of the 16S rRNA gene', *Journal of Clinical Microbiology,* 41(8), 3790-3800.

Colombari, V., Mayer, M. D. B., Laicini, Z. M., Mamizuka, E., Franco, B., Destro, M. T. and Landgraf, M. (2007) 'Foodborne outbreak caused by *Staphylocloccus aureus*: Phenotypic and genotypic characterization of strains of food and human sources', *Journal of Food Protection,* 70(2), 489-493.

Comas-Riu, J. and Rius, N. (2009) 'Flow cytometry applications in the food industry', *Journal of Industrial Microbiology & Biotechnology,* 36(8), 999-1011.

Cotter, P. D., Gahan, C. G. M. and Hill, C. (2000) 'Analysis of the role of the Listeria monocytogenes F0F1-ATPase operon in the acid tolerance response', *International Journal of Food Microbiology,* 60(2-3), 137-146.

Cotter, P. D., Gahan, C. G. M. and Hill, C. (2001) 'A glutamate decarboxylase system protects Listeria monocytogenes in gastric fluid', *Molecular Microbiology,* 40(2), 465-475.

Cotter, P. D., Ryan, S., Gahan, C. G. M. and Hill, C. (2005) 'Presence of GadD1 glutamate decarboxylase in selected *Listeria monocytogenes* strains is associated with an ability to grow at low pH', *Applied and Environmental Microbiology,* 71(6), 2832-2839.

Crowley, H., Cagney, C., Sheridan, J. J., Anderson, W., McDowell, D. A., Blair, I. S., Bishop, R. H. and Duffy, G. (2005) 'Enterobacteriaceae in beef products from retail outlets in the Republic of Ireland and comparison of the presence and counts of E. coli O157 : H7 in these products', *Food Microbiology,* 22(5), 409-414.

Cumming, M., Kludt, P., Matyas, B., DeMaria, A., Stiles, T., Han, L., Gilchrist, M., Neves, P., Fitzgibbons, E. and Condon, S. (2009) 'Outbreak of *Listeria monocytogenes* Infections Associated With Pasteurized Milk From a Local Dairy-Massachusetts, 2007 (Reprinted from MMWR, vol 57, pg 1097-1100, 2008)', *Jama-Journal of the American Medical Association,* 301(8), 820-822.

Cunha, M. D. L. R. D. S. D., Oliveira, T. C. R. M. D. and Hirooka, E. Y. (1996) 'Generality about staphylococci enterotoxin in foods', *Revista de Ciencias Farmaceuticas,* 17(0), 9-22.

Daly, P., Collier, T. and Doyle, S. (2002) 'PCR-ELISA detection of *Escherichia coli* in milk', *Letters in Applied Microbiology,* 34(3), 222-226.

Davey, H. M. (2002) 'Flow cytometric techniques for the detection of microorganisms', *Methods in Cell Science,* 24(1-3), 91-97.

de Boer, E. and Beumer, R. R. (1999) 'Methodology for detection and typing of foodborne microorganisms', *International Journal of Food Microbiology,* 50(1-2), 119-130.

de Valk, H., Valliant, V., Jacquet, C., Rocourt, J., Le Querrec, F., Stainer, F., Quelquejeu, N., Pierre, O., Pierre, V., Desenclos, J. C. and Goulet, V. (2001) 'Two consecutive nationwide outbreaks of listeriosis in France, October 1999-February 2000', *American Journal of Epidemiology,* 154(10), 944-950.

Deere, D., Vesey, G., Gauci, M. and Ashbolt, N. (2003) *Flow Cytometry and Cell Sorting for Monitoring Microbial Cells, Encyclopedia of Environmental Microbiology,* John Wiley & Sons, Inc.

Deng, M. Y., Cliver, D. O., Day, S. P. and Fratamico, P. M. (1996) 'Enterotoxigenic *Escherichia coli* detected in foods by PCR and an enzyme-linked oligonucleotide probe', *International Journal of Food Microbiology,* 30(3), 217-229.

Dhanasekaran, S., Doherty, T. M., Kenneth, J. and Grp, T. B. T. S. (2010) 'Comparison of different standards for real-time PCR-based absolute quantification', *Journal of Immunological Methods,* 354(1-2), 34-39.

Diaper, J. P. and Edwards, C. (1994) 'The use of fluorogenic esters to detect viable bacteria by flow cytometry', *Journal of Applied Microbiology,* 77(2), 221-228.

do Carmo, L. S., Dias, R. S., Linardi, V. R., de Sena, M. J. and dos Santos, D. A. (2003) 'An outbreak of staphylococcal food poisoning in the Municipality of Passos, Mg, Brazil', *Brazilian Archives of Biology and Technology,* 46(4), 581-586.

do Carmo, L. S., Dias, R. S., Linardi, V. R., de Sena, M. J., dos Santos, D. A., de Faria, M. E., Pena, E. C., Jett, M. and Heneine, L. G. (2002) 'Food poisoning due to enterotoxigenic strains of *Staphylococcus* present in Minas cheese and raw milk in Brazil', *Food Microbiology,* 19(1), 9-14.

Donnelly, C. W. (2002) 'Detection and isolation of *Listeria monocytogenes* from food samples: Implications of sublethal injury', *Journal of Aoac International,* 85(2), 495-500.

dos Santos, L. R., do Nascimento, V. P., de Oliveira, S. D., Flores, M. L., Pontes, A. P., Ribeiro, A. R., Salle, C. T. P. and Lopes, R. F. F. (2001) 'Polymerase chain reaction (PCR) for the detection of *Salmonella* in artificially inoculated chicken meat', *Revista do Instituto de Medicina Tropical de Sao Paulo,* 43(5), 247-250.

Dowe, M. J., Jackson, E. D., Mori, J. G. and Bell, C. R. (1997) '*Listeria monocytogenes* survival in soil and incidence in agricultural soils', *Journal of Food Protection,* 60(10), 1201-1207.

Duffy, G., Cloak, O. M., Sheridan, J. J., Blair, I. S. and McDowell, D. A. (1999) 'The development of a combined surface adhesion and polymerase chain reaction technique in the rapid detection of *Listeria monocytogenes* in meat and poultry', *International Journal of Food Microbiology,* 49(3), 151-159.

Duffy, G., Garvey, P. and Sheridan, J. J. (2000) '*E. coli* O157 : H7; A European perspective', *Irish Journal of Agricultural and Food Research,* 39(2), 173-182.

Duffy, G. and Sheridan, J. J. (1998) 'Viability staining in a direct count rapid method for the determination of total viable counts on processed meats', *Journal of Microbiological Methods,* 31(3), 167-174.

Duffy, G., Walsh, D., Sheridan, J. J., Logue, C. M., Harrington, D., Blair, I. S. and McDowell, D. A. (2001) 'Comparison of selective and non-selective enrichment media in the detection of *Listeria monocytogenes* from meat containing *Listeria innocua*', *Journal of Applied Microbiology,* 90(6), 994-999.

Ellingson, J. L. E., Anderson, J. L., Carlson, S. A. and Sharma, V. K. (2004) 'Twelve hour real-time PCR technique for the sensitive and specific detection of *Salmonella* in raw and ready-to-eat meat products', *Molecular and Cellular Probes,* 18(1), 51-57.

Ellingson, J. L. E., Koziczkowski, J. J., Anderson, J. L., Carlson, S. A. and Sharma, V. K. (2005) 'Rapid PCR detection of enterohemorrhagic *Escherichia coli* (EHEC) in bovine food products and feces', *Molecular and Cellular Probes,* 19(3), 213-217.

Evancho, G. M., Sveum, W. H., Moberg, L. J. and Frank, J. F. (2001) 'Microbiological Monitoring of the Food Processing Environment' in Pouch Downes, F. and Ito, K., eds., *Microbiological Examination of Foods*, 4 ed., Washington, USA: American Public Health Association, 25-36.

Fach, P., Hauser, D., Guillou, J. P. and Popoff, M. R. (1993) 'Polymerase Chain Reaction for the Rapid idenfication of *Clostridium botulinum* type A Strains and Detection in Food Samples', *Journal of Applied Bacteriology,* 75(3), 234-239.

Fairchild, A., Lee, M. D. and Maurer, J. (2006) 'PCR Basics' in Maurer, J., ed. *PCR Methods in Foods*, Athens, USA: Springer, 1-26.

Fedio, W. M., Wendakoon, C. N., Zapata, R., Carrillo, C. and Browning, P. (2008) 'Comparison of Petrifilm (TM) Staph Express Count System with the Bacteriological Analytical Manual Direct-Plating Method for Enumeration of *Staphylococcus aureus* in Artificially Contaminated Hard Cheese', *Journal of Aoac International,* 91(5), 1138-1141.

Feldsine, P., Abeyta, C. and Andrews, W. H. (2002) 'AOAC INTERNATIONAL Methods Committee guidelines for validation of qualitative and quantitative food microbiological official methods of analysis', *Journal of Aoac International,* 85(5), 1187-1200.

Feng, P. (2001) 'Bacteriological Analytical Manual -- Appendix 1 -- Rapid Methods for Detecting Foodborne Pathogens ', [online], available: http://www.fda.gov/food/scienceresearch/laboratorymethods/bacteriologicalanalyticalmanualbam/ucm109652.htm#authors [accessed

Ferraz, M. A., Cerqueira, M. and Souza, M. R. (2010) 'Evaluation of Enterobacteriaceae in the powdered milk production chain using both traditional (ISO 21528:2) and rapid (3M (TM) Petrifilm (TM)) methods', *Annals of Microbiology,* 60(2), 373-376.

Fischer, S. H. and Nachamkin, I. (1991) 'Common and variable domains of the flagellin Gene, *FlaA*, in *Campylobacter jejuni*', *Molecular Microbiology,* 5(5), 1151-1158.

Fittipaldi, M., Codony, F., Adrados, B., Camper, A. and Morató, J. (2011) 'Viable Real-Time PCR in Environmental Samples: Can All Data Be Interpreted Directly?', *Microbial Ecology,* 61(1), 7-12.

Fitzmaurice, J., Glennon, M., Duffy, G., Sheridan, J. J., Carroll, C. and Maher, M. (2004) 'Application of real-time PCR and RT-PCR assays for the detection and quantitation of VT 1 and VT 2 toxin genes in *E. coli* O157 : H7', *Molecular and Cellular Probes,* 18(2), 123-132.

Flekna, G., Stefanic, P., Wagner, M., Smulders, F. J. M., Mozina, S. S. and Hein, I. (2007) 'Insufficient differentiation of live and dead *Campylobacter jejuni* and *Listeria monocytogenes* cells by ethidium monoazide (EMA) compromises EMA/real-time PCR', *Research in Microbiology,* 158(5), 405-412.

Fleming, S. D. and Campbell, P. A. (1997) 'Some macrophages kill Listeria monocytogenes while others do not', *Immunological Reviews,* 158, 69-77.

Flint, S., Drocourt, J.-L., Walker, K., Stevenson, B., Dwyer, M., Clarke, I. and McGill, D. (2006) 'A rapid, two-hour method for the enumeration of total viable bacteria in samples from commercial milk powder and whey protein concentrate powder manufacturing plants', *International Dairy Journal,* 16(4), 379-384.

Flint, S., Walker, K., Waters, B. and Crawford, R. (2007) 'Description and validation of a rapid (1h) flow cytometry test for enumerating thermophilic bacteria in milk powders', *Journal of Applied Microbiology,* 102(4), 909-915.

Forsythe, S. J. and Hayes, P. R. (2000) 'Fondamental principle of Microbiology' in Forsythe, S. J. and Hayes, P. R., eds., *Food Hygiene, Microbiology and HACCP - Third Edition*, Gaithersburg, USA: An Aspen Publication Inc., -20.

Foschino, R., Picozzi, C., Civardi, A., Bandini, M. and Faroldi, P. (2003) 'Comparison of surface sampling methods and cleanability assessment of stainless steel surfaces subjected or not to shot peening', *Journal of Food Engineering,* 60(4), 375-381.

Fratamico, P. M., Bagi, L. K. and Pepe, T. (2000) 'A multiplex polymerase chain reaction assay for rapid detection and identification of *Escherichia coli* O157 : H7 in foods and bovine feces', *Journal of Food Protection,* 63(8), 1032-1037.

Freeman, S. E. and Thompson, B. D. (1988) 'Evaluation of densitometry data using interactive computer graphics: Application to DNA agarose gels', *International Journal of Bio-Medical Computing,* 22(2), 121-133.

Freeman, W. M., Robertson, D. J. and Vrana, K. E. (2000) 'Fundamentals of DNA hybridization arrays for gene expression analysis', *Biotechniques,* 29(5), 1042-+.

Fretz, R., Pichler, J., Sagel, U., Much, P., Ruppitsch, W., Pietzka, A. T., Stoger, A., Huhulescu, S., Heuberger, S., Appl, G., Werber, D., Stark, K., Prager, R., Flieger, A., Karpiskova, R., Pfaff, G. and Allerberger, F. (2010) 'Update: Multinational listeriosis outbreak due to 'Quargel', a sour milk curd cheese, caused by two different *L. monocytogenes* serotype 1/2a strains, 2009-2010', *Eurosurveillance,* 15(16), 2-3.

Frye, D. M., Zweig, R., Sturgeon, J., Tormey, M., LeCavalier, M., Lee, I., Lawani, L. and Mascola, L. (2002) 'An outbreak of febrile gastroenteritis associated with delicatessen meat contaminated with *Listeria monocytogenes*', *Clinical Infectious Diseases,* 35(8), 943-949.

Fung, D. Y. C. (2000) 'Rapid methods and automation in microbiology: A review', *Irish Journal of Agricultural and Food Research,* 39(2), 301-307.

Furet, J.-P., Quénée, P. and Tailliez, P. (2004) 'Molecular quantification of lactic acid bacteria in fermented milk products using real-time quantitative PCR', *International Journal of Food Microbiology,* 97(2), 197-207.

Gahan, C. G. M., Odriscoll, B. and Hill, C. (1996) 'Acid adaptation of *Listeria monocytogenes* can enhance survival in acidic foods and during milk fermentation', *Applied and Environmental Microbiology,* 62(9), 3128-3132.

Gandhi, M. and Chikindas, M. L. (2007) '*Listeria*: A foodborne pathogen that knows how to survive', *International Journal of Food Microbiology,* 113(1), 1-15.

Gannon, V. P. J., Dsouza, S., Graham, T., King, R. K., Rahn, K. and Read, S. (1997) 'Use of the flagellar H7 gene as a target in multiplex PCR assays and improved specificity in identification of enterohemorrhagic Escherichia coli strains', *Journal of Clinical Microbiology,* 35(3), 656-662.

Garcia-Cayuela, T., Tabasco, R., Pelaez, C. and Requena, T. (2009) 'Simultaneous detection and enumeration of viable lactic acid bacteria and bifidobacteria in fermented milk by using propidium monoazide and real-time PCR', *International Dairy Journal,* 19(6-7), 405-409.

Gaynor, C. (2007) 'Food safety controls in Europe - Challenges and opportunities', *Journal of Aoac International,* 90(1), 1-5.

Gerritsen, M. J., Olyhoek, T., Smits, M. A. and Bokhout, B. A. (1991) 'Sample preparation method for polymerase chain reaction-based semiquantitative detection of Leptospira interrogans serovar hardjo subtype hardjobovis in bovine urine', *J. Clin. Microbiol.,* 29(12), 2805-2808.

Giaccone, V. and Ottaviani, F. (2007) 'Current Foodborne Pathogens - *Listeria monocytogenes*' in bioMerieux, ed. *Food Safety Handbook - Microbiological Challenges*, Marcy l'Etoile, France: 108-127.

Giacomazzi, S., Leroi, F. and Joffraud, J. J. (2005) 'Comparison of three methods of DNA extraction from cold-smoked salmon and impact of physical treatments', *Journal of Applied Microbiology,* 98(5), 1230-1238.

Gibellini, D. E., Re, M. C., Furlini, G. and La Placa, M. (1997) 'Flow Cytometry Analysis of an *In Situ* PCR for the Detection of Human Immunodeficiency Virus Type-1 (HIV-1) Proviral DNA', *Methods in Molecular Biology,* 71, 113-122.

Giglio, S., Monis, P. T. and Saint, C. P. (2003) 'Demonstration of preferential binding of SYBR Green I to specific DNA fragments in real-time multiplex PCR', *Nucleic Acids Research,* 31(22).

Giovannoni, S. J., DeLong, E. F., Olsen, G. J. and Pace, N. R. (1988) 'Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells', *Journal of Bacteriology,* 170(2), 720-726.

Giraffa, G. and Neviani, E. (2001) 'DNA-based, culture-independent strategies for evaluating microbial communities in food-associated ecosystems', *International Journal of Food Microbiology,* 67(1-2), 19-34.

Gorga, A., Claisse, O. and Lonvaud-Funel, A. (2002) 'Organisation of the genes encoding glycerol dehydratase of Lactobacillus collinoides, Lactobacillus hilgardii and Lactobacillus diolivorans', *Sciences Des Aliments,* 22(1-2), 151-160.

Gottlieb, S. L., Newbern, E. C., Griffin, P. M., Graves, L. M., Hoekstra, R. M., Baker, N. L., Hunter, S. B., Holt, K. G., Ramsey, F., Head, M., Levine, P., Johnson, G., Schoonmaker-Bopp, D., Reddy, V., Kornstein, L., Gerwel, M., Nsubuga, J., Edwards, L., Stonecipher, S., Hurd, S., Austin, D., Jefferson, M. A., Young, S. D., Hise, K., Chernak, E. D., Sobel, J. and Listeriosis Outbreak Working, G. (2006) 'Multistate outbreak of listeriosis linked to Turkey deli meat and subsequent changes in US regulatory policy', *Clinical Infectious Diseases,* 42(1), 29-36.

Goulet, V. and Marchetti, P. (1996) 'Listeriosis in 225 Non-pregnant Patients in 1992: Clinical Aspects and Outcome in Relation to Predisposing Conditions', *Scandinavian Journal of Infectious Diseases,* 28(4), 367-374.

Gram, L., Ravn, L., Rasch, M., Bruhn, J. B., Christensen, A. B. and Givskov, M. (2002) 'Food spoilage - interactions between food spoilage bacteria', *International Journal of Food Microbiology,* 78(1-2), 79-97.

Gray, M. L. and Killinge.Ah (1966) '*Listeria monocytogenes* and Listeric Infections', *Bacteriological Reviews,* 30(2), 309-&.

Guilbaud, M., de Coppet, P., Bourion, F., Rachman, C., Prevost, H. and Dousset, X. (2005) 'Quantitative detection of *Listeria monocytogenes* in biofilms by real-time PCR', *Applied and Environmental Microbiology,* 71(4), 2190-2194.

Guillebault, D., Laghdass, M., Catala, P., Obernosterer, I. and Lebaron, P. (2010) 'An improved method of bacterial cell capture after flow cytometry cell sorting', *Applied and Environmental Microbiology*, AEM.00621-10.

Gunasekera, T. S., Veal, D. A. and Attfield, P. V. (2003) 'Potential for broad applications of flow cytometry and fluorescence techniques in microbiological and somatic cell analyses of milk', *International Journal of Food Microbiology,* 85(3), 269-279.

Gurtler, J. B. and Beuchat, L. R. (2005) 'Performance of media for recovering stressed cells of *Enterobacter sakazakii* as determined using spiral plating and ecometric techniques', *Applied and Environmental Microbiology,* 71(12), 7661-7669.

Hart, J. and Smith, G. (2009) 'Verocytotoxin-producing *Escherichia coli* O157 outbreak in Wrexham, North Wales, July 2009', *Euro Surveill,* 14(32).

Hassibi, B., Vikalo, H. and Hassibi, A. (2008) *Multiplex Q-PCR Arrays*,

Hein, I., Lehner, A., Rieck, P., Klein, K., Brandl, E. and Wagner, M. (2001) 'Comparison of different approaches to quantify *Staphylococcus aureus* cells by real-time quantitative PCR and application of this technique for examination of cheese', *Applied and Environmental Microbiology,* 67(7), 3122-3126.

Henke, W., Herdel, K., Jung, K., Schnorr, D. and Loening, S. A. (1997) 'Betaine improves the PCR amplification of GC-rich DNA sequences', *Nucl. Acids Res.,* 25(19), 3957-3958.

Herman, L. (1997) 'Detection of viable and dead *Listeria monocytogenes* by PCR', *Food Microbiology,* 14(2), 103-110.

Herrera-Leon, S., McQuiston, J. R., Usera, M. A., Fields, P. I., Garaizar, J. and Echeita, M. A. (2004) 'Multiplex PCR for distinguishing the most common phase-1 flagellar antigens of *Salmonella spp*', *Journal of Clinical Microbiology,* 42(6), 2581-2586.

Hibi, K., Abe, A., Ohashi, E., Mitsubayashi, K., Ushio, H., Hayashi, T., Ren, H. F. and Endo, H. (2006) 'Combination of immunomagnetic separation with flow cytometry for detection of *Listeria monocytogenes*', *Analytica Chimica Acta,* 573, 158-163.

Hocquette, J. F., Richardson, R. I., Prache, S., Medale, F., Duffy, G. and Scollan, N. D. (2005) 'The future trends for research on quality and safety of animal products', *Italian Journal of Animal Science,* 4, 49-72.

Hof, H. (1991) 'Therapeutic Activities of Antibiotics in Listeriosis', *Infection,* 19, S229-S233.

Hof, H. (2004) 'An update on the medical management of listeriosis', *Expert Opinion on Pharmacotherapy,* 5(8), 1727-1735.

Hoffmann, P. R., Gurary, A., Hoffmann, F. W., Saux, C. J. L., Teeters, K., Hashimoto, A. C., Tam, E. K. and Berry, M. J. (2007) 'A new approach for analyzing cellular infiltration during allergic airway inflammation', *Journal of Immunological Methods,* 328(1-2), 21-33.

Holm, C., Mathiasen, T. and Jespersen, L. (2004) 'A flow cytometric technique for quantification and differentiation of bacteria in bulk tank milk', *Journal of Applied Microbiology,* 97(5), 935-941.

Huang, Y.-R., Hung, Y.-C., Hsu, S.-Y., Huang, Y.-W. and Hwang, D.-F. (2008) 'Application of electrolyzed water in the food industry', *Food Control,* 19(4), 329-345.

Innis, M. A., Myambo, K. B., Gelfand, D. H. and Brow, M. A. D. (1988) 'DNA Sequencing with *Thermus aquaticus* DNA Polymerase and Direct Sequencing of Polymerase Chain Reaction-Amplified DNA', *PNAS,* 85(24), 9436-9440.

Ishii, S., Tago, K. and Senoo, K. (2010) 'Single-cell analysis and isolation for microbiology and biotechnology: methods and applications', *Applied Microbiology and Biotechnology,* 86(5), 1281-1292.

Issa, N. C., Espy, M. J., Uhl, J. R., Harmsen, W. S., Mandrekar, J. N., Gullerud, R. E., Davis, M. D. and Smith, T. F. (2005) 'Comparison of specimen processing and nucleic acid extraction by the swab extraction tube system versus the MagNA pure LC system for laboratory diagnosis of herpes simplex virus infections by LightCycler PCR', *Journal of Clinical Microbiology,* 43(3), 1059-1063.

Issa, N. C. and Thompson, R. L. (2001) 'Staphylococcal toxic shock syndrome - Suspicion and prevention are keys to control', *Postgraduate Medicine,* 110(4), 55-+.

Issack, M. I., Hendriksen, R. S., Lun, P. L. K., Lutchun, R. K. S. and Aarestrup, F. M. (2009) '*Salmonella enterica* Serovar Typhimurium in Mauritius Linked to Consumption of Marlin Mousse', *Foodborne Pathogens and Disease,* 6(6), 739-741.

Iversen, C. and Forsythe, S. (2004) 'Isolation of Enterobacter sakazakii and other Enterobacteriaceae from powdered infant formula milk and related products', *Food Microbiology,* 21(6), 771-777.

Janda, J. M. (2006a) '*Escherichia coli*' in Janda, J. M. and Abbott, S. L., eds., *The Enterobacteria - Second Edition*, Washington, USA: ASM Press.

Janda, J. M. (2006b) 'The family Enterobacteriaceae : Taxonomic considerations' in Janda, J. M. and Abbott, S. L., eds., *The Enterobacteria - Second Edition*, Washington, USA: ASM Press.

Jaroszeski, M. J. and Radcliff, G. (1999) 'Fundamentals of flow cytometry', *Molecular Biotechnology,* 11(1), 37-53.

Jasson, V., Jacxsens, L., Luning, P., Rajkovic, A. and Uyttendaele, M. (2010) 'Alternative microbial methods: An overview and selection criteria', *Food Microbiology,* 27(6), 710-730.

Jemmi, T. and Stephan, R. (2006) '*Listeria monocytogenes*: food-borne pathogen and hygiene indicator', *Revue Scientifique Et Technique-Office International Des Epizooties,* 25(2), 571-580.

Johnson-Green, P. (2002) 'Diagnostic Systems' in *Introduction to Food Biotechnology*, Courtice, Canada: CRC Press, 151-180.

Jorgensen, H. J., Mathisen, T., Lovseth, A., Omoe, K., Qvale, K. S. and Loncarevic, S. (2005) 'An outbreak of staphylococcal food poisoning caused by enterotoxin H in mashed potato made with raw milk', *Fems Microbiology Letters,* 252(2), 267-272.

Josefsen, M. H., Lofstrom, C., Hansen, T. B., Christensen, L. S., Olsen, J. E. and Hoorfar, J. (2010) 'Rapid Quantification of Viable *Campylobacter* Bacteria on Chicken Carcasses, Using Real-Time PCR and Propidium Monoazide Treatment, as a Tool for Quantitative Risk Assessment', *Applied and Environmental Microbiology,* 76(15), 5097-5104.

Julia, O., Comas, J. and Vives-Rego, J. (2000) 'Second-order functions are the simplest correlations between flow cytometric light scatter and bacterial diameter', *Journal of Microbiological Methods,* 40(1), 57-61.

Junge, B. and Berghof-Jager, K. (2006) 'Roche/BIOTECON Diagnostics LightCycler foodproof *L. monocytogenes* detection kit in combination with ShortPrep foodproof II kit - Performance-Tested Method(SM) 070401', *Journal of Aoac International,* 89(2), 374-398.

Junttila, J. R., Niemela, S. I. and Hirn, J. (1988) 'Minimum Growth Temperatures of *Listeria monocytogenes* and non-hemolytic *Listeria*', *Journal of Applied Bacteriology,* 65(4), 321-327.

Kalvatchev, Z. N., Tsekov, I. D., Slavov, S. N. and Draganov, P. I. (2010) 'Effective Light-Upon-Extension Real-Time PCR Primer Systems for Rapid Detection of Human Viruses', *Lab Medicine,* 41(3), 150-155.

Kaprelyants, A. S. and Kell, D. B. (1993) 'The Use of 5-cyano-2,3-ditolyl tetrazolium chrloride and Flow Cytometry for the Visualization of Respiratory Activity in Individual Cells of *Micrococcus luteus*', *Journal of Microbiological Methods,* 17(2), 115-122.

Katcher, H. L. and Schwartz, I. (1994) 'A Distinctive Property of Tth DNA-Polymerase - Enzymatic Amplification in the Presence of Phenol', *Biotechniques,* 16(1), 84-&.

Kennedy, J. and Wall, P. (2007) 'Food Safety Challenges' in BioMerieux, ed. *Food Safety Handbook - Microbiological Challenges*, Marcy l'Etoile, France: 8-19.

Kim, C. H., Khan, M., Morin, D. E., Hurley, W. L., Tripathy, D. N., Kehrli, M., Oluoch, A. O. and Kakoma, I. (2001) 'Optimization of the PCR for detection of *Staphylococcus aureus nuc* gene in bovine milk', *Journal of Dairy Science,* 84(1), 74-83.

Kim, H. J. and Cho, J. C. (2008) 'Rapid and Sensitive Detection of *Listeria monocytogenes* Using a PCR-Enzyme-Linked Immunosorbent Assay', *Journal of Microbiology and Biotechnology,* 18(11), 1858-1861.

Klein, P. and Juneja, V. (1997) 'Sensitive detection of viable *Listeria monocytogenes* by reverse transcription-PCR', *Applied and Environmental Microbiology,* 63(11), 4441-4448.

Knutsson, R., Blixt, Y., Grage, H., Borch, E. and Radstrom, P. (2002) 'Evaluation of selective enrichment PCR procedures for *Yersinia enterocolitica*', *International Journal of Food Microbiology,* 73(1), 35-46.

Knutsson, R. and Rådström, P. (2002) 'Detection of Pathogenic *Yersinia enterocolitica* by a Swab Enrichment PCR Procedure' in, 311-324.

Ko, W. C., Paterson, D. L., Sagnimeni, A. J., Hansen, D. S., Von Gottberg, A., Mohapatra, S., Casellas, J. M., Goossens, H., Mulazimoglu, L., Trenholme, G., Klugman, K. P., McCormack, J. G. and Yu, V. L. (2002) 'Community-acquired *Klebsiella pneumoniae* bacteremia: Global differences in clinical patterns', *Emerging Infectious Diseases,* 8(2), 160-166.

Kobayashi, H., Kubota, J., Fujihara, K., Honjoh, K., Ito, M., Fujiki, N., Nakabe, M., Oda, S., Satoyama, T., Takasu, K., Nakanishi, H. and Miyamoto, T. (2009a) 'Simultaneous Enrichment of *Salmonella spp*, *Escherichia coli* O157:H7, *Vibrio parahaemolyticus*, *Staphylococcus aureus*, *Bacillus cereus*, and *Listeria monocytogenes* by Single Broth and Screening of the Pathogens by Multiplex Real-time PCR', *Food Science and Technology Research,* 15(4), 427-438.

Kobayashi, H., Oethinger, M., Tuohy, M. J., Hall, G. S. and Bauer, T. W. (2009b) 'Unsuitable distinction between viable and dead *Staphylococcus aureus* and *Staphylococcus epidermidis* by ethidium bromide monoazide', *Letters in Applied Microbiology,* 48(5), 633-638.

Kodaka, H., Mizuochi, S., Teramura, H. and Nirazuka, T. (2005) 'Comparison of the Compact Dry TC method with the Standard Pour Plate method (AOAC Official Method 966.23) for determining aerobic colony counts in food samples - Performance-Tested Method(SM)', *Journal of Aoac International,* 88(6), 1702-1713.

Kostrzynska, M. and Bachand, A. (2006) 'Application of DNA microarray technology for detection, identification, and characterization of food-borne pathogens', *Canadian Journal of Microbiology,* 52(1), 1-8.

Kothary, M. H. and Babu, U. S. (2001) 'Infective dose of foodborne pathogens in volunteers: A review', *Journal of Food Safety,* 21(1), 49-73.

Kubista, M., Andrade, J. M., Bengtsson, M., Forootan, A., Jonak, J., Lind, K., Sindelka, R., Sjoback, R., Sjogreen, B., Strombom, L., Stahlberg, A. and ZoriC, N. (2006) 'The real-time polymerase chain reaction', *Molecular Aspects of Medicine,* 27(2-3), 95-125.

Kumar, T. D. K., Murali, H. S. and Batra, H. V. (2009) 'Simultaneous detection of pathogenic *B. cereus*, *S. aureus* and *L. monocytogenes* by multiplex PCR', *Indian Journal of Microbiology,* 49(3), 283-289.

Kunchev, M., Alexandrov, E., Kamarinchev, B., Marinov, K., Gergova, I. and Mekouchinov, K. (2007) 'Effective LUX (Light upon eXtension) primer system for early and rapid detection of *Coxiella burnetii*', *Biotechnology & Biotechnological Equipment,* 21(3), 338-340.

Kunicka, A. (2007) 'Evaluation of the TEMPO (R) system: An automated method for food microbiological quality control', *Journal of Biotechnology,* 131(2), S69-S69.

Kusunoki, H., Tsukamoto, T., Gibas, C. F. C., Dalmacio, I. F. and Uemura, T. (1996) 'Application of flow cytometry for the detection of *Escherichia coli* O157', *Journal of the Food Hygienic Society of Japan,* 37(6), 390-394.

Lambertz, S. T., Lindqvist, R., Ballagi-Pordany, A. and Danielsson-Tham, M. L. (2000) 'A combined culture and PCR method for detection of pathogenic *Yersinia enterocolitica* in food', *International Journal of Food Microbiology,* 57(1-2), 63-73.

Lampel, K. A., Jagow, J. A., Trucksess, M. and Hill, W. E. (1990) 'Polymerase Chain Reaction for Detection of Invasive *Shigella flexneri* in Food', *Applied and Environmental Microbiology,* 56(6), 1536-1540.

Lantz, P.-G., Knutsson, R., Blixt, Y., Al-Soud, W. A., Borch, E. and Radstrom, P. (1998) 'Detection of pathogenic Yersinia enterocolitica in enrichment media and pork by a multiplex PCR: a study of sample preparation and PCR-inhibitory components', *International Journal of Food Microbiology,* 45(2), 93-105.

Lantz, P.-G., Matsson, M., Wadstrom, T. and Radstrom, P. (1997) 'Removal of PCR inhibitors from human faecal samples through the use of an aqueous two-phase system for sample preparation prior to PCR', *Journal of Microbiological Methods,* 28(3), 159-167.

Le Loir, Y., Baron, F. and Gautier, M. (2003) '*Staphylococcus aureus* and food poisoning', *Genetics and Molecular Research,* 2(1), 63-76.

Levin, R. E. (2003) 'Application of the polymerase chain reaction for detection of *Listeria monocytogenes* in foods: A review of methodology', *Food Biotechnology,* 17(2), 99-116.

Liu, G., Han, Y., Li, X. and Song, S. (2006) 'Applicability of a rapid method based on immunomagnetic capture-fluorescent PCR assay for *Campylobacter jejuni*', *Food Control,* 17(7), 527-532.

Loddenkotter, B., Becker, K., Hohoff, C., Brinkmann, B. and Bajanowski, T. (2005) 'Real-time quantitative PCR assay for the detection of *Helicobacter pylori*: no association with sudden infant death syndrome', *International Journal of Legal Medicine,* 119(4), 202-206.

Loewen, P. C. and Switala, J. (1995) 'Template Secondary Structure can increase the Error Frequency of the DNA Polymerase from *Thermus aquaticus*', *Gene,* 164(1), 59-63.

Lofdahl, M., Ivarsson, S., Andersson, S., Langmark, J. and Plym-Forshell, L. (2009) 'An outbreak of *Shigella dysenteriae* in Sweden, May-June 2009, with sugar snaps as the suspected source', *Euro Surveill,* 14(28).

Lopez-Enriquez, L., Rodriguez-Lazaro, D. and Hernandez, M. (2007) 'Quantitative detection of Clostridium tyrobutyricum in milk by real-time PCR', *Applied and Environmental Microbiology,* 73(11), 3747-3751.

Lopez, C., Feltri, A., Leotta, G., Gonzalez, G., Manfredi, E., Gottardi, G., Elder, M., De las Carreras, S., Patri, C., Guajardo, F., San Martin, A. and Rivas, M. (2008) 'Foodborne disease outbreak in EI Huecu community, province of Neuquen', *Revista Argentina De Microbiologia,* 40(4), 198-203.

Low, J. C. and Donachie, W. (1997) 'A review of *Listeria monocytogenes* and Listeriosis', *Veterinary Journal,* 153(1), 9-29.

MacDonald, P. D. M., Whitwam, R. E., Boggs, J. D., MacCormack, J. N., Anderson, K. L., Reardon, J. W., Saah, J. R., Graves, L. M., Hunter, S. B. and Sobel, J. (2005) 'Outbreak of listeriosis among Mexican immigrants as a result of consumption of illicitly produced Mexican-Style cheese', *Clinical Infectious Diseases,* 40(5), 677-682.

Mackay, I. M. (2004) 'Real-time PCR in the microbiology laboratory', *Clinical Microbiology and Infection,* 10(3), 190-212.

Mackay, I. M., Bustin, S. A., Andrade, J. M., Kubista, M. and Sloots, T. P. (2007a) 'Quantification of Microorganisms: not Human, not Simple, not Quick' in Mackay, I. M., ed. *Real-time PCR in Microbiology - From Diagnosis to Characterization*, Caister Academic Press, 133-182.

Mackay, I. M., Mackay, F. M., Nissen, M. D. and Sloots, T. P. (2007b) 'Introduction to Real-time PCR' in Mackay, I. M., ed. *Real-time PCR in Microbiology - From Diagnosis to Characterization*, Norfolk, UK: Caister Academic Press, 1-39.

Mafu, A. A., Pitre, M. and Sirois, S. (2009) 'Real-Time PCR as a Tool for Detection of Pathogenic Bacteria on Contaminated Food Contact Surfaces by Using a Single Enrichment Medium', *Journal of Food Protection,* 72(6), 1310-1314.

Makino, S. I., Kawamoto, K., Takeshi, K., Okada, Y., Yamasaki, A., Yamamoto, S. and Igimi, S. (2005) 'An outbreak of food-borne listeriosis due to cheese in Japan, during 2001', *International Journal of Food Microbiology,* 104(2), 189-196.

Manafi, M. (1996) 'Fluorogenic and chromogenic enzyme substrates in culture media and identification tests', *International Journal of Food Microbiology,* 31(1-3), 45-58.

Marsh, T. L. (1999) 'Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products', *Current Opinion in Microbiology,* 2(3), 323-327.

Marsh, T. L., Saxman, P., Cole, J. and Tiedje, J. (2000) 'Terminal restriction fragment length polymorphism analysis program, a web-based research tool for microbial community analysis', *Applied and Environmental Microbiology,* 66(8), 3616-3620.

Mason, D. J., Shanmuganathan, S., Mortimer, F. C. and Gant, V. A. (1998) 'A fluorescent gram stain for flow cytometry and epifluorescence microscopy', *Applied and Environmental Microbiology,* 64(7), 2681-2685.

Maurer, J. J., Schmidt, D., Petrosko, P., Sanchez, S., Bolton, L. and Lee, M. D. (1999) 'Development of primers to O-antigen biosynthesis genes for specific detection of Escherichia coli O157 by PCR', *Applied and Environmental Microbiology,* 65(7), 2954-2960.

McClelland, R. G. and Pinder, A. C. (1994) 'Detection of Low Levels of Specific *Salmonella* Species by Fluorescent Antibodies and Flow Cytometry', *Journal of Applied Bacteriology,* 77(4), 440-447.

McClure, J. (2007) 'Current Foodborne Pathogens - Staphylococci' in BioMerieux, ed. *Food Safety Handbook - Microbiological Challenges*, Marcy l'Etoile, France.

McCrea, J. K., Liu, C. Y., Ng, L. K. and Wang, G. H. (2007) 'Detection of the *Escherichia coli* pathogenic gene *eae* with three real-time polymerase chain reaction methods', *Canadian Journal of Microbiology,* 53(3), 398-403.

McIngvale, S. C., Elhanafi, D. and Drake, M. A. (2002) 'Optimization of reverse transcriptase PCR to detect viable Shiga-toxin-producing Escherichia coli', *Applied and Environmental Microbiology,* 68(2), 799-806.

McKillip, J. L., Jaykus, L. A. and Drake, M. (1998) 'rRNA stability in heat-killed and UV-irradiated enterotoxigenic *Staphylococcus aureus* and *Escherichia coli* O157 : H7', *Applied and Environmental Microbiology,* 64(11), 4264-4268.

McLauchlin, J., Mitchell, R. T., Smerdon, W. J. and Jewell, K. (2004) '*Listeria monocytogenes* and listeriosis: a review of hazard characterisation for use in microbiological risk assessment of foods', *International Journal of Food Microbiology,* 92(1), 15-33.

Mitchell, S. L., Budhiraja, S., Thurman, K. A., Thacker, W. L. and Winchell, J. M. (2009) 'Evaluation of two real-time PCR chemistries for the detection of *Chlamydophila pneumoniae* in clinical specimens', *Molecular and Cellular Probes,* 23(6), 309-311.

Miwa, N., Kawamura, A., Masuda, T. and Akiyama, M. (2001) 'An outbreak of food poisoning due to egg yolk reaction-negative *Staphylococcus aureus*', *International Journal of Food Microbiology,* 64(3), 361-366.

Morgan, C. A., Bigeni, P., Herman, N., Gauci, M., White, P. A. and Vesey, G. (2004) 'Production of precise microbiology standards using flow cytometry and freeze drying', *Cytometry Part A,* 62A(2), 162-168.

Murray, E. G. D., Webb, R. A. and Swann, M. B. R. (1926) 'A disease of rabbits characterised by a large mononuclear leucocytosis caused by a hitherto undescribed bacillus *Bacterium monocytogenes*', *Journal of Pathology and Bacteriology,* 29, 407-39.

Muska, A., Peck, E. and Palmer, S. (2007) 'Standards and Controls: Concepts for Preparation and Use in Real-Time PCR Applications' in Mackay, ed. *Real-Time PCR in Microbiology*, Norfolk, UK: Caister Academic Press, 133-182.

Mustapha, A. and Li, Y. (2006) 'Molecular Detection of Foodborne Bacterial Pathogens' in Maurer, J., ed. *PCR Methods in Foods*, Athens, USA: Springer, 69-90.

Mylonakis, E., Paliou, M., Hohmann, E. L., Caldewood, S. B. and Wing, E. J. (2002) 'Listeriosis During Pregnancy: A Case Series and Review of 222 Cases', *Medicine,* 81(4), 260-269.

Nam, H. M., Srinivasan, V., Gillespie, B. E., Murinda, S. E. and Oliver, S. P. (2005) 'Application of SYBR Green real-time PCR assay for specific detection of *Salmonella spp.* in dairy farm environmental samples', *International Journal of Food Microbiology,* 102(2), 161-171.

Nema, V., Agrawal, R., Kamboj, D. V., Goel, A. K. and Singh, L. (2007) 'Isolation and characterization of heat resistant enterotoxigenic *Staphylococcus aureus* from a food poisoning outbreak in Indian subcontinent', *International Journal of Food Microbiology,* 117(1), 29-35.

Nicholson, F. A., Groves, S. J. and Chambers, B. J. (2005) 'Pathogen survival during livestock manure storage and following land application', *Bioresource Technology,* 96(2), 135-143.

Niederhauser, C., Candrian, U., Hofelein, C., Jermini, M., Buhler, H. P. and Luthy, J. (1992) 'Use of Polymerase Chain reaction for Detection of *Listeria monocytogenes* in Food', *Applied and Environmental Microbiology,* 58(5), 1564-1568.

Nilsson, A., Lambertz, S. T., Stalhandske, P., Norberg, P. and Danielsson-Tham, M. L. (1998) 'Detection of *Yersinia enterocolitica* in food by PCR amplification', *Letters in Applied Microbiology,* 26(2), 140-144.

Nilsson, W. B. and Strom, M. S. (2002) 'Detection and identification of bacterial pathogens of fish in kidney tissue using terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes', *Diseases of Aquatic Organisms,* 48(3), 175-185.

Niyogi, S. K. (2005) 'Shigellosis', *Journal of Microbiology,* 43(2), 133-143.

Nocker, A. and Camper, A. K. (2006) 'Selective removal of DNA from dead cells of mixed bacterial communities by use of ethidium monoazide', *Applied and Environmental Microbiology,* 72(3), 1997-2004.

Nocker, A. and Camper, A. K. (2009) 'Novel approaches toward preferential detection of viable cells using nucleic acid amplification techniques', *Fems Microbiology Letters,* 291(2), 137-142.

Nocker, A., Cheung, C. Y. and Camper, A. K. (2006) 'Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells', *Journal of Microbiological Methods,* 67(2), 310-320.

Nocker, A., Sossa-Fernandez, P., Burr, M. D. and Camper, A. K. (2007) 'Use of propidium monoazide for live/dead distinction in microbial ecology', *Applied and Environmental Microbiology,* 73(16), 5111-5117.

Noel, H., Hofhuis, A., De Jonge, R., Heuvelink, A. E., De Jong, A., Heck, M., De Jager, C. and van Pelt, W. (2010) 'Consumption of Fresh Fruit Juice: How a Healthy Food Practice Caused a National Outbreak of *Salmonella Panama* Gastroenteritis', *Foodborne Pathogens and Disease,* 7(4), 375-381.

Nogva, H. K., Rudi, K., Naterstad, K., Holck, A. and Lillehaug, D. (2000) 'Application of 5 '-nuclease PCR for quantitative detection of *Listeria monocytogenes* in pure cultures, water, skim milk, and unpasteurized whole milk', *Applied and Environmental Microbiology,* 66(10), 4266-4271.

Nolan, T., Mueller, R. and Bustin, S. (2007) 'QPCR:Target Preparation' in Mackay, ed. *Real-Time PCR in Microbiology*, Norfolk, UK: Caister Academic Press, 71-99.

Nordentoft, S., Christensen, H. and Wegener, H. C. (1997) 'Evaluation of a fluorescence-labelled oligonucleotide tide probe targeting 23S rRNA for in situ detection of *Salmonella* serovars in paraffin-embedded tissue sections and their rapid identification in bacterial smears', *Journal of Clinical Microbiology,* 35(10), 2642-2648.

Nordgren, J., Bucardo, F., Dienus, O., Svensson, L. and Lindgren, P. E. (2008) 'Novel light-upon-extension real-time PCR assays for detection and quantification of genogroup I and II noroviruses in clinical specimens', *Journal of Clinical Microbiology,* 46(1), 164-170.

Nyyssonen, M., Piskonen, R. and Itavaara, M. (2006) 'A targeted real-time PCR assay for studying naphthalene degradation in the environment', *Microbial Ecology,* 52(3), 533-543.

O'Brien, S. B., Duffy, G., Daly, D., Sheridan, J. J., Blair, I. S. and McDowell, D. A. (2005) 'Detection and recovery rates achieved using direct plate and enrichment/immunomagnetic separation methods for *Escherichia coli* O157 : H7 in minced beef and on bovine hide', *Letters in Applied Microbiology,* 41(1), 88-93.

O'Hanlon, K. A., Catarame, T. M. G., Blair, I. S., McDowell, D. A. and Duffy, G. (2005) 'Comparison of a real-time PCR and an IMS/culture method to detect Escherichia coli O26 and O111 in minced beef in the Republic of Ireland', *Food Microbiology,* 22(6), 553-560.

O'Hanlon, K. A., Catarame, T. M. G., Duffy, G., Blair, I. S. and McDowell, D. A. (2004) 'Rapid detection and quantification of *E. coli* O157/O26/O111 in minced beef by real-time PCR', *Journal of Applied Microbiology,* 96(5), 1013-1023.

Obermaier, I., Tellmann, G. and Goldstein, C. (2009) *LightCycler® Real-Time PCR Systems - Application Manual,* Mannheim: Roche Diagnostics GmbH.

Oliveira, T., Barbut, S. and Griffiths, M. W. (2005) 'A robotic DNA purification protocol and real-time PCR for the detection of *Campylobacter jejuni* in foods', *Journal of Food Protection,* 68(10), 2131-2135.

Olsen, S. J., Patrick, M., Hunter, S. B., Reddy, V., Kornstein, L., MacKenzie, W. R., Lane, K., Bidol, S., Stoltman, G. A., Frye, D. M., Lee, I., Hurd, S., Jones, T. F., LaPorte, T. N., Dewitt, W., Graves, L., Wiedmann, M., Schoonmaker-Bopp, D. J., Huang, A. J., Vincent, C., Bugenhagen, A., Corby, J., Carloni, E. R., Holcomb, M. E., Woron, R. F., Zansky, S. M., Dowdle, G., Smith, F., Ahrabi-Fard, S., Ong, A. R., Tucker, N., Hynes, N. A. and Mead, P. (2005) 'Multistate outbreak of *Listeria monocytogenes* infection linked to delicatessen turkey meat', *Clinical Infectious Diseases,* 40(7), 962-967.

Ooi, SayÂ T. and Lorber, B. (2005) 'Gastroenteritis Due to *Listeria monocytogenes*', *Clinical Infectious Diseases,* 40(9), 1327-1332.

Oravcova, K., Trncikova, T. and Kaclikova, E. (2007) 'Comparison of three real-time PCR-based methods for the detection of *Listeria monocytogenes* in food', *Journal of Food and Nutrition Research,* 46(2), 63-67.

Oravcova, K., Trncikova, T., Kuchta, T. and Kaclikova, E. (2008) 'Limitation in the detection of *Listeria monocytogenes* in food in the presence of competing *Listeria innocua*', *Journal of Applied Microbiology,* 104(2), 429-437.

Ott, S. J., Musfeldt, M., Ullmann, U., Hampe, J. and Schreiber, S. (2004) 'Quantification of Intestinal Bacterial Populations by Real-Time PCR with a Universal Primer Set and Minor Groove Binder Probes: a Global Approach to the Enteric Flora', *J. Clin. Microbiol.,* 42(6), 2566-2572.

Oyofo, B. A., Thornton, S. A., Burr, D. H., Trust, T. J., Pavlovskis, O. R. and Guerry, P. (1992) 'Specific Detection of *Campylobacter jejuni* and *Campylobacter coli* by using Polymerase Chain detection', *Journal of Clinical Microbiology,* 30(10), 2613-2619.

Park, Y. S., Lee, S. R. and Kim, Y. G. (2006) 'Detection of *Escherichia coli* O157 : H7, *Salmonella spp.*, *Staphylococcus aureus* and *Listeria monocytogenes* in kimchi by multiplex polymerase chain reaction (mPCR)', *Journal of Microbiology,* 44(1), 92-97.

Pavlov, A. R., Pavlova, N. V., Kozyavkin, S. A. and Slesarev, A. I. (2004) 'Recent developments in the optimization of thermostable DNA polymerases for efficient applications', *Trends in Biotechnology,* 22(5), 253-260.

Peel, M., Donachie, W. and Shaw, A. (1988) 'Temperature-dependant Expression of Flagella of *Listeria monocytogenes* Studied by Electron Microscopy, SDS-PAGE and Western Blotting', *Journal of General Microbiology,* 134, 2171-2178.

Peresi, J. T. M., Kaku, M., Gelli, D. S., De Lima, S. I. and Duarte, V. L. S. (1996) 'Comparison of Tryptic soy broth with 10 percent salt and Giolitti-Cantoni broth used in determination of coagulase positive staphylococci in foods', *Revista do Instituto Adolfo Lutz,* 56(1), 83-86.

Phillips, A. P. and Martin, K. L. (1988) 'Limitations of flow cytometry for the specific detection of bacteria in mixed populations', *Journal of Immunological Methods,* 106(1), 109-117.

Pichler, J., Much, P., Kasper, S., Fretz, R., Auer, B., Kathan, J., Mann, M., Huhulescu, S., Ruppitsch, W., Pietzka, A., Silberbauer, K., Neumann, C., Gschiel, E., de Martin, A., Schuetz, A., Gindl, J., Neugschwandtner, E. and Allerberger, F. (2009) 'An outbreak of febrile gastroenteritis associated with jellied pork contaminated with *Listeria monocytogenes*', *Wiener Klinische Wochenschrift,* 121(3-4), 149-156.

Pinto, B., Chenoll, E. and Aznar, R. (2005) 'Identification and typing of food-borne *Staphylococcus aureus* by PCR-based techniques', *Systematic and Applied Microbiology,* 28(4), 340-352.

Piraee, M. and Vining, L. C. (2002) 'Use of degenerate primers and touchdown PCR to amplify a halogenase gene fragment from *Streptomyces venezuelae*', *Journal of Industrial Microbiology & Biotechnology,* 29(1), 1-5.

Pirie, J. H. (1927) 'A new disease of veld rodents "Tiger river disease"', *South African Institute of Medical Research Publications,* 3, 163-86.

Powell, H. A., Gooding, C. M., Garrett, S. D., Lund, B. M. and McKee, R. A. (1994) 'Proteinase Inhibition of the Detection of *Listeria monocytogenes* in Milk Using the Polymerase Chain Reaction', *Letters in Applied Microbiology,* 18(1), 59-61.

Poyart-Salmeron, C., Carlier, C., Trieu-Cuot, P., Courvalin, P. and Courtieu, A. L. (1990) 'Transferable plasmid-mediated antibiotic resistance in *Listeria monocytogenes*', *The Lancet,* 335(8703), 1422-1426.

Pryor, R. J. and Wittwer, C. T. (2006) 'Real-time polymerase chain reaction and melting curve analysis', *Methods in Molecular Biology*, 19-32.

Quaglia, N. C., Normanno, G., Dambrosio, A., Celano, G. V., Parisi, A., Firinu, A. and Buonavoglia, C. (2005) 'Multiplex-touchdown PCR assay for the detection and genotyping of *Helicobacter pylori* from artificially contaminated sheep milk', *Journal of Food Protection,* 68(10), 2136-2139.

Radji, M., Malik, A. and Widyasmara, A. (2010) 'Rapid detection of *Salmonella* in food and beverage samples by polymerase chain reaction', *Malesian Journal of Microbiology,* 6(2), 166-170.

Rådström, P., Knutsson, R., Wolffs, P., Lövenklev, M. and Löfström, C. (2004) 'Pre-PCR processing - Strategies to Generate PCR-Compatible Samples', *Molecular Biotechnology,* 26, 133-146.

Raybourne, R. B. (2001) 'Flow cytometric detection of pathogenic *E. coli* in food', *Curr Protoc Cytom,* Chapter 11, Unit 11.6.

Rentenaar, I. M. F. (1996) 'MicroVal, a challenging Eureka project', *Food Control,* 7(1), 31-36.

Ririe, K. M., Rasmussen, R. P. and Wittwer, C. T. (1997) 'Product differentiation by analysis of DNA melting curves during the polymerase chain reaction', *Analytical Biochemistry,* 245(2), 154-160.

Rocourt, J. and Jacquet, C. (1995) 'Surveillance of Human Listeriosis in France - The Role of the National Reference Center', *Medecine Et Maladies Infectieuses,* 25, 177-183.

Rossmanith, P., Krassnig, M., Wagner, M. and Hein, I. (2006) 'Detection of *Listeria monocytogenes* in food using a combined enrichment/real-time PCR method targeting the prfA gene', *Research in Microbiology,* 157(8), 763-771.

Rothe, G. (2009) 'Technical Background and Methodological Principles of Flow Cytometry' in Sack, U., Tárnok, A. and Rothe, G., eds., *Background and Methodological Principles*, Cellular Diagnostics. Basics, Methods and Clinical Applications of Flow Cytometry ed., Basel, Switzerland: Karger, 53-88.

Rudi, K., Moen, B., Dromtorp, S. M. and Holck, A. L. (2005) 'Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples', *Applied and Environmental Microbiology,* 71(2), 1018-1024.

Rychlik, W. (1995) 'Selection of primers for Polymerase Chain Reaction', *Molecular Biotechnology,* 3(2), 129-134.

Sachidanandham, R. and Gin, K. Y.-H. (2003) *Flow cytometric detection of β-D-glucuronidase gene in wild-type bacterial cells using in-situ PCR* Wiley Subscription Services, Inc., A Wiley Company.

Sails, A. D., Fox, A. J., Bolton, F. J., Wareing, D. R. A. and Greenway, D. L. A. (2003) 'A real-time PCR assay for the detection of *Campylobacter jejuni* in foods after enrichment culture', *Applied and Environmental Microbiology,* 69(3), 1383-1390.

Sanderson, K. and Nichols, D. (2003) *Genetic techniques: PCR, NASBA, hybridisation and microaarrays - Detecting pathogens in food,* Woodhead Publishing Limited ed.

Schmid, D., Fretz, R., Winter, P., Mann, M., Hoger, G., Stoger, A., Ruppitsch, W., Ladstatter, J., Mayer, N., de Martin, A. and Allerberger, F. (2009) 'Outbreak of staphylococcal food intoxication after consumption of pasteurized milk products, June 2007, Austria', *Wiener Klinische Wochenschrift,* 121(3-4), 125-131.

Schmitt, M., Schulerschmid, U. and Schmidtlorenz, W. (1990) 'Temperature Limits of Growth, TNase and Enterotoxin Production of *Staphylococcus aureus* strains isolated from foods', *International Journal of Food Microbiology,* 11(1), 1-20.

Schoenhals, G. and Whitfield, C. (1993) 'Comparative analysis of flagellin sequences from *Escherichia coli* strains possesing serologically distinct flagellar filaments with a shared complex surface pattern', *Journal of Bacteriology,* 175(17), 5395-5402.

Seeliger, H. P. R. (1975) 'Serovariants of *Listeria monocytogenes* and other *Listeria* species', *Acta Microbiologica Academiae Scientiarum Hungaricae,* 22(2), 179-181.

Sen, K., Shay Fout, G., Haugland, R., Moulton, C., Grimm, A., Di Giovanni, G., Feige, M. A., Birkenhauer Best, J., Lott, G., Scheller, J., Reilly, E., Connell, K., and Marshall, M. (2004) Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples. In U. S. E. O. o. G. W. a. D. Water (Ed.). Cincinatti, USA.

Seo, K. H. and Brackett, R. E. (2005) 'Rapid, specific detection of *Enterobacter sakazakii* in infant formula using a real-time PCR assay', *Journal of Food Protection,* 68(1), 59-63.

Shapiro, H. M. (2003a) 'Flow sorting' in Wiley-Liss, ed. *Practical Flow Cytometry - Fourth Edition*, Hoboken, New Jersey.: John Wiley and Sons Inc., 257-272.

Shapiro, H. M. (2003b) 'Using Flow Cytometers: Applications, Extensions and Alternatives' in Wiley-Liss, ed. *Practical Flow Cytometry - Fourth Edition*, Hoboken, New Jersey.: John Wiley and Sons Inc., 443-542.

Sheridan, G. E. C., Masters, C. I., Shallcross, J. A. and Mackey, B. M. (1998) 'Detection of mRNA by reverse transcription PCR as an indicator of viability in *Escherichia coli* cells', *Applied and Environmental Microbiology,* 64(4), 1313-1318.

Shetty, A., McLauchlin, J., Grant, K., O'Brien, D., Howard, T. and Davies, E. M. (2009) 'Outbreak of *Listeria monocytogenes* in an oncology unit associated with sandwiches consumed in hospital', *Journal of Hospital Infection,* 72(4), 332-336.

Shinoda, S., Yamamoto, S., Tomochika, K. and Miyoshi, S. (1997) 'Enterohemorrhagic *Escherichia coli* O157:H7 infection', *Japanese Journal of Toxicology and Environmental Health,* 43(1), 1-14.

Siguret, V., Ribba, A. S., Cherel, G., Meyer, D. and Pietu, G. (1994) 'Effect of Plasmid Size on Transformation Efficiency by Electroporation of *Escherichia coli* DH5 Alpha', *Biotechniques,* 16(3), 422-426.

Silliker, J. H. and Gabis, D. A. (1973) 'ICMSF Methods Studies .1. Comparison of Analytical Schemes for Detection of *Salmonella* in Dried Foods', *Canadian Journal of Microbiology,* 19(4), 475-479.

Slavov, S., Kalvatchev, Z., Tsekov, I., Simeonov, P., Hristova, L., Kotsev, J., Mladenov, D. and Tsvetkov, M. (2008) 'Novel light-upon-extension (LUX) real-time PCR primer system for rapid detection of *Polyomavirus hominis* 1 (BKV) in clinical samples', *Biotechnology & Biotechnological Equipment,* 22(2), 759-761.

Smith, N. H. and Selander, R. K. (1990) 'Sequence invariance of the antigen coding central region of the phase 1 flagellar filament gene (*fliC*) amopng strains of *Salmonella typhimurium*', *Journal of Bacteriology,* 172(2), 603-609.

Španová, A., Rittich, B., Karpíšková, R., Čechová, L. and Škapová, D. (2000) 'PCR identification of *Salmonella* cells in food and stool samples after immunomagnetic separation', *Bioseparation,* 9(6), 379-384.

Sperber, W. A., Moorman, M. A. and Freier, T. A. (2001) 'Cultural Methods for the Enrichment and Isolation of Microorganisms' in Pouch Downes, F. and Ito, K., eds., *Microbiological Examination of Foods*, 4 ed., Washington, USA: American Public Health Association, 45-52.

Starbuck, M. A. B., Hill, P. J. and Stewart, G. (1992a) 'Ultra Sensitive Detection of Listeria-Monocytogenes in Milk by the Polymerase Chain-Reaction (Pcr)', *Letters in Applied Microbiology,* 15(6), 248-252.

Starbuck, M. A. B., Hill, P. J. and Stewart, G. (1992b) 'Ultra Sensitive Detection of *Listeria monocytogenes* in Milk by the Polymerase Chain Reaction (PCR)', *Letters in Applied Microbiology,* 15(6), 248-252.

Stiles, M. E. (2000) 'Less recognized and Suspected Foodborne Bacterial Pathogens' in Lund, B. M., Baird-Parker, T. C. and Gould, G. W., eds., *The Microbiological Safety and Quality of Food* Gaithersburg, USA: An Aspen Publication, 1394-1419.

Su, T.-Z., Schweizer, H. and Oxender, D. L. (1990) 'A novel phosphate-regulated expression vector in Escherichia coli', *Gene,* 90(1), 129-133.

Szabo, E. A. and Mackey, B. M. (1999) 'Detection of *Salmonella enteritidis* by reverse transcription polymerase chain reaction (PCR)', *International Journal of Food Microbiology,* 51(2-3), 113-122.

Szostkova, M. and Horakova, D. (1998) 'The effect of plasmid DNA sizes and other factors on electrotransformation of *Escherichia coli* JM109', *Bioelectrochemistry and Bioenergetics,* 47(2), 319-323.

Taboada, E. N., Acedillo, R. R., Carrillo, C. D., Findlay, W. A., Medeiros, D. T., Mkytczuk, O. L., Roberts, M. J., Valencia, C. A., Farber, J. M. and Nash, J. H. E. (2004) 'Large-scale comparative genomics meta-analysis of *Campylobacter jejuni* isolates reveals low level of genome plasticity', *Journal of Clinical Microbiology,* 42(10), 4566-4576.

Takahashi, H., Iwade, Y., Konuma, H. and Hara-Kudo, Y. (2005) 'Development of a quantitative real-time PCR method for estimation of the total number of *Vibrio parahaemolyticus* in contaminated shellfish and seawater', *Journal of Food Protection,* 68(5), 1083-1088.

Tamarapu, S., McKillip, J. L. and Drake, M. (2001) 'Development of a multiplex polymerase chain reaction assay for detection and differentiation of *Staphylococcus aureus* in dairy products', *Journal of Food Protection,* 64(5), 664-668.

Tavolaro, P., Ferrati, A. R., Destro, M. T., Landgraf, M. and Franco, B. D. G. d. M. (2005) 'Performance of two ready-to-use systems for enumeration of aerobic mesophilic microorganisms in frozen goat milk', *Brazilian Journal of Microbiology,* 36, 295-300.

Techathuvanan, C. and D'Souza, D. H. (2011) 'Optimization of Rapid *Salmonella enterica* Detection in Liquid Whole Eggs by SYBR Green I-Based Real-Time Reverse Transcriptase-Polymerase Chain Reaction', *Foodborne Pathogens and Disease,* 8(4), 527-534.

Techatruvanan, C., Draughon, F. A. and D'Souza, D. H. (2010) 'Real-Time Reverse Transcriptase PCR for the Rapid and Sensitive Detection of *Salmonella Typhimurium* from Pork', *Journal of Food Protection,* 73(3), 507-514.

Thaddeus, F. M. and Bryant, R. G. (2001) 'Sampling Plans, Sample Collection, Shipment, and Preparation for Analysis' in Pouch Downes, F. and Ito, K., eds., *Microbiological Examination of Foods*, 4 ed., Washington, USA: American Public Health Association, 13-24.

Tilden, J., Young, W., McNamara, A. M., Custer, C., Boesel, B., LambertFair, M., Majkowski, J., Vugia, D., Werner, S. B., Hollingsworth, J. and Morris, J. G. (1996) 'A new route of transmission for *Escherichia coli*: Infection from dry fermented salami', *American Journal of Public Health,* 86(8), 1142-1145.

Townsend, S., Hurrell, E. and Forsythe, S. (2008) 'Virulence studies of *Enterobacter sakazakii* isolates associated with a neonatal intensive care unit outbreak', *Bmc Microbiology,* 8, 9.

Trncikova, T., Hruskova, V., Oravcova, K., Pangallo, D. and Kaclikova, E. (2009) 'Rapid and Sensitive Detection of *Staphylococcus aureus* in Food Using Selective Enrichment and Real-Time PCR Targeting a New Gene Marker', *Food Analytical Methods,* 2(4), 241-250.

Tse, C. and Capeau, J. (2002) 'Quantification des acides nucléiques par PCR quantitative en temps réel', *Annales de Biologie Clinique,* 61(3), 279-293.

Tyagi, A., Saravanan, V. and Karunasagar, I. (2009) 'Detection of *Vibrio parahaemolyticus* in tropical shellfish by SYBR green real-time PCR and evaluation of three enrichment media', *International Journal of Food Microbiology,* 129(2), 124-130.

Uhl, J. R., Adamson, S. C., Vetter, E. A., Schleck, C. D., Harmsen, W. S., Iverson, L. K., Santrach, P. J., Henry, N. K. and Cockerill, F. R. (2003) 'Comparison of LightCycler PCR, Rapid Antigen Immunoassay, and Culture for Detection of Group A *Streptococci* from Throat Swabs', *J. Clin. Microbiol.,* 41(1), 242-249.

Vaerman, J. L., Saussoy, P. and Ingargiola, I. (2004) 'Evaluation of real-time PCR data', *Journal of Biological Regulators and Homeostatic Agents,* 18(2), 212-214.

Van Acker, J., De Smet, F., Muyldermans, G., Bougatef, A., Naessens, A. and Lauwers, S. (2001) 'Outbreak of necrotizing enterocolitis associated with *Enterobacter sakazakii* in powdered milk formula', *Journal of Clinical Microbiology,* 39(1), 293-297.

Van Doorne, H., Baird, R. M., Hendriksz, D. T., Van Der Kreek, D. M. and Pauwels, H. P. (1981) 'Liquid Modification of Baird Parker's Medium for the Selective Enrichment of *Staphylococcus aureus*', *Journal of Microbiology,* 47(3), 267-278.

Varadaraj, K. and Skinner, D. M. (1994) 'Denaturants or Cosolvents Improve the Specificity of PCR Amplification of a G+C-Rich DNA Using Genetically-Engineered DNA-Polymerases', *Gene,* 140(1), 1-5.

Varma, M., Field, R., Stinson, M., Rukovets, B., Wymer, L. and Hauglanda, R. (2009) 'Quantitative real-time PCR analysis of total and propidium monoazide-resistant fecal indicator bacteria in wastewater', *Water Research,* 43(19), 4790-4801.

Vesey, G., Deere, D., Gauci, M. R., Griffiths, K. R., Williams, K. L. and Veal, D. A. (1997) 'Evaluation of fluorochromes and excitation sources for immunofluorescence in water samples', *Cytometry,* 29(2), 147-154.

Villalobo, E. and Torres, A. (1998) 'PCR for detection of *Shigella spp* in mayonnaise', *Applied and Environmental Microbiology,* 64(4), 1242-1245.

Vulindlu, M., Charlett, A., Surman, S. and Lee, J. V. (2004) 'Comparison of agar-based methods for the isolation and enumeration of heterotrophic bacteria with the new Multidose IDEXX (TM) SimPlate method', *Water Science and Technology,* 50(1), 277-280.

Walker, R. E., Petersen, J. M., Stephens, K. W. and Dauphin, L. A. (2010) 'Optimal swab processing recovery method for detection of bioterrorism-related *Francisella tularensis* by real-time PCR', *Journal of Microbiological Methods,* 83(1), 42-47.

Wallner, G., Amann, R. and Beisker, W. (1993) *Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms* Wiley Subscription Services, Inc., A Wiley Company.

Wallner, G., Fuchs, B., Spring, S., Beisker, W. and Amann, R. (1997) 'Flow sorting of microorganisms for molecular analysis', *Applied and Environmental Microbiology,* 63(11), 4223-4231.

Walsh, D., Duffy, G., Sheridan, J. J., Blair, I. S. and McDowell, D. A. (1998) *Comparison of Selective and Non Selective Enrichment Media for the Isolation of Listeria Species from Retail Foods* Blackwell Publishing Ltd.

Wang, H., Farber, J. M., Malik, N. and Sanders, G. (1999) 'Improved PCR detection of Campylobacter jejuni from chicken rinses by a simple sample preparation procedure', *International Journal of Food Microbiology,* 52(1-2), 39-45.

Wang, L., Li, Y. and Mustapha, A. (2009) 'Detection of viable *Escherichia coli* O157:H7 by ethidium monoazide real-time PCR', *Journal of Applied Microbiology,* 107(5), 1719-1728.

Wang, X. W., Jothikumar, N. and Griffiths, M. W. (2004) 'Enrichment and DNA extraction protocols for the simultaneous detection of *Salmonella* and *Listeria monocytogenes* in raw sausage meat with multiplex real-time PCR', *Journal of Food Protection,* 67(1), 189-192.

Wesley, I. V., Wesley, R. D., Heisick, J., Harrell, F. and Wagner, D. (1990) 'Characterization of *Listeria monocytogenes* Isolates by Southern Blot Hybridization', *Veterinary Microbiology,* 24(3-4), 341-353.

Witthuhn, R. C., Kemp, F. and Britz, T. J. (2007) 'Isolation and PCR detection of *Enterobacter sakazakii* in South African food products, specifically infant formula milks', *World Journal of Microbiology & Biotechnology,* 23(2), 151-157.

Wohlsen, T., Bates, J., Vesey, G., Robinson, W. A. and Katouli, M. (2006) 'Evaluation of the methods for enumerating coliform bacteria from water samples using precise reference standards', *Letters in Applied Microbiology,* 42(4), 350-356.

Wu, C., Cai, J. and YANG, R. (2004) 'In situ PCR and Detection by Flow Cytometer of tdh-containing Bacteria *Vibrio parahaemolyticus*', *Acta Microbiologica Sinica,* (3), 399-401.

Wu, J. S., Lee, C., Wu, C. C. and Shiue, Y. L. (2004) 'Primer design using genetic algorithm', *Bioinformatics,* 20(11), 1710-1717.

Xu, R.-s., Chen, R., Lin, C.-h., Yang, Z.-j. and Chen, G.-w. (2008) 'Development of a LUX™ PCR assay for rapid detection of *Vibrio parahaemolyticus*', *Chinese Veterinary Science,* 38(2), 1075-1079.

Xu, W., Li, S.-f., Liu, J. and Hu, D. (2008) 'The development of duplex real-time PCR for detection of *Listeria monocytogenes* and *Shigella*', *Zhonghua Weishengwuxue He Mianyixue Zazhi,* 28(10), 946-950.

Yamaguchi, N., Sasada, M., Yamanaka, M. and Nasu, M. (2003) 'Rapid detection of respiring *Escherichia coli* O157 : H7 in apple juice, milk, and ground beef by flow cytometry', *Cytometry Part A,* 54A(1), 27-35.

Yan, Z. N., Vorst, K. L., Zhang, L. and Ryser, E. T. (2007) 'Use of one-ply composite tissues in an automated optical assay for recovery of Listeria from food contact surfaces and poultry-processing environments', *Journal of Food Protection,* 70(5), 1263-1266.

Yang, H., Qu, L. W., Wimbrow, A. N., Jiang, X. P. and Sun, Y. P. (2007) 'Rapid detection of *Listeria monocytogenes* by nanoparticle-based immunomagnetic separation and real-time PCR', *International Journal of Food Microbiology,* 118, 132-138.

Yoon, S. Y., Chung, G. T., Kang, D. H., Ryu, C. S., Yoo, C. K. and Seong, W. K. (2005) 'Application of real-time PCR for quantitative detection of Clostridium botulinum type A toxin gene in food', *Microbiology and Immunology,* 49(6), 505-511.

Zahraei Salehi, T., Mahzounieh, M. and Saeedzadeh, A. (2005) 'Detection of *InvA* Gene in Isolated *Salmonella* from Broilers by PCR Method', *International Journal of Poultry Science,* 4(8), 557-559.

Zarda, B., Amann, R., Wallner, G. and Schleifer, K. H. (1991) 'Identification of Single Bacterial Cells Using Digoxigenin-labeled, Ribosomal RNA Targeted Oligonucleotides', *Journal of General Microbiology,* 137, 2823-2830.

Zhang, D., Zhang, H., Yang, L., Guo, J., Li, X. and Feng, Y. (2009) 'Simultaneous Detection of *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enterica* and *Escherichia coli* O157:H7 in Food Samples using Multiplex PCR Method', *Journal of Food Safety,* 29(3), 348-363.

# Thesis objectives

This research study was undertaken to generate novel PCR and fluorescent activated cell sorting (FACS) methods for the detection of a range a foodborne pathogens with a particular focus on:

(i) The selection of highly specific PCR primers to develop simplex and duplex SYBR Green-based real-time PCR methods for the detection of *S. aureus* and *L. monocytogenes* in minimally enriched food samples.

(ii) The development and the assessment of a LUX™-based real-time PCR method for the detection of the Enterobacteriaceae and a comparison with a commercially available molecular method in infant formula milk samples.

(iii) The generation of innovative real-time PCR standards using FACS for absolute quantification of microorganisms, with *S. aureus* as a model; the evaluation of stability of these calibrants.

(iv) The assessment of the applicability of the above real-time PCR methods in the enumeration of viable bacteria swabbed from food-processed environments with the development of a suitable sample preparation using the SETS.

# CHAPTER TWO

## Selection of optimal primer sets for use in a duplex SYBR Green-based real-time PCR protocol for the detection of *L. monocytogenes* and *S. aureus* in foods[1]

[1] The work described in this chapter has been published in *Journal of Food Safety* (Martinon A. and Wilkinson M., 2011), "Selection of optimal primer sets for use in a duplex SYBR Green-based real-time PCR protocol for the detection of *L. monocytogenes* and *S. aureus* in foods", DOI 10.1111/j.1745-4565.2011.00301.x. (Appendix One)

## ABSTRACT

A low cost duplex SYBR Green-based real-time PCR for the simultaneous detection of *Listeria monocytogenes* and *Staphyloccocus aureus* in foods was developed following selection of optimal primers. For *L. monocytogenes*, the set targeting the listeriolysin O gene (*hlyA* primers) was more specific than the one annealing to the metalloprotease gene (*mpl* primers). For *S. aureus*, the *nuc* primers targeting the thermonuclease gene were highly specific. Simplex SYBR Green-based real-time PCR methods for the separate detection of *L. monocytogenes* and *S. aureus* were performed. Finally, the developed duplex real-time PCR was applied to foods spiked with these microorganisms using a simple enrichment step in buffered peptone water at 37°C for 18 h. Melting temperatures were sufficiently different for identification with intra and inter-assay coefficients of variation in melting temperature of 0.08% and 0.20%, respectively. Detection limits were 7 CFU/g in coleslaw for *L. monocytogenes* and 2 CFU/g in raw minced meat for *S. aureus*, as confirmed using the commercial kits and plate counting.

This multiplex real-time PCR method provides a potential two-in-one screening enabling simultaneous detection of positive samples of *S. aureus* and *L. monocytogenes* in minimally enriched food samples. Considering the simplicity, low cost, rapidity and acceptable reproducibility shown for the detection of *S. aureus* and *L. monocytogenes*, this duplex method may be used in food analysis prior to further potentially more expensive investigations by giving an initial indication of contamination and discarding of negative samples. This method may contribute to the validation and the verification of Hazard Analysis Critical Control Plans, Good Hygiene Practices and the acceptability of batches in food industries. In regard to EC (European Community) microbiological criteria regulation, this alternative method may be applied especially for the analysis of *L. monocytogenes* in ready-to-eat foods and *S. aureus* in dairy products.

# 1. INTRODUCTION

Most outbreaks of food poisoning are related to ingestion of contaminated foods arising from time and temperature abuse, inadequate preparation or insufficient hygiene during production. *Listeria monocytogenes* and *Staphyloccocus aureus* are of major concern in the food industry as their presence may indicate a failure of quality control systems during production. EC regulations (Anonymous, 2005a) require conventional culture methods according to ISO standards in official food control laboratories. In contrast, an increasing number of commercial real-time PCR kits for qualitative detection of such foodborne pathogens are becoming available (Choudhurry *et al.*, 2006; Jasson *et al.*, 2010; Oravcova *et al.*, 2007) in either simplex or multiplex systems. Overall, ready-to-use PCR systems while being convenient for qualitative detection are still regarded by the food industry as being too expensive to be applied for routine analysis. The uptake of real-time PCR for routine testing by the food industry would therefore be advanced by the availability of standardized protocols enabling detection of multiple pathogens. Despite the increasing commercial availability of PCR and real-time PCR kits (Glynn *et al.*, 2006) standardized and simplified real-time PCR methods are not in regular use in food quality applications.

Development of simplex real-time PCR methods applicable to food microbiology is ongoing with a range of in-house protocols reported for the detection of *L. monocytogenes* (O'Grady *et al.*, 2009) or *S. aureus* (Chiang *et al.*, 2007; Trncikova *et al.*, 2009) in enriched food samples. Such methodology may be extended to the simultaneous detection of multiple target DNA sequences via multiplex real-time PCR (Elizaquivel and Aznar, 2008; Fukushima *et al.*, 2003; Gubala, 2006; Lee *et al.*, 2009; Omiccioli *et al.*, 2009; Wang *et al.*, 2004).

The purpose of multiplex PCR is to simultaneously amplify segments of target DNA, using more than one pair of primers in the assay, where time and reaction costs are minimised (Shi *et al.*). Multiplex PCR methods have been recently reported for the simultaneous detection of *L. monocytogenes* and *S. aureus* in foods and broth cultures (Kim *et al.*, 2007; Kobayashi *et al.*, 2009a; Kumar *et al.*, 2009; Zhang *et al.*, 2009). However, limitations of these methods include time consuming with conventional multiplex PCR and additional costs in chemicals and machine features using probes for multiplex-based real-time PCR procedures. In contrast, SYBR Green I is a commonly used fluorescent dye which does not require specific and costly probe design, is easy to use (Kugelman *et al.*, 2009) and binding is not affected by potential

mutations of the target gene (Guilbaud *et al.*, 2005). Aldea *et al.* (2002) and Fernandez *et al.* (2006) commented on the simpler and cheaper use of SYBR Green I in comparison to the increased handling and the possible loss of detection found for probes. Indeed probes include many fluorescent labels, instead of just one molecule which is inserted into the amplicon. The SYBR Green I detection format is suitable for melting curve analysis where PCR products can be identified as a function of their melting temperature ($T_M$). However, as SYBR Green I binds to any double stranded DNA, notably primer dimmers, the primer sets used must be highly specific and generate a single PCR product (Ririe *et al.*, 1997). To date, multiplex real-time PCR systems or commercial kits for the simultaneous detection of *L. monocytogenes* and *S. aureus*, using a low cost detection format, have yet to be developed.

This study was therefore undertaken to develop a duplex SYBR Green based-protocol as an alternative simple lower cost method for the simultaneous detection of *L. monocytogenes* and *S. aureus*. Two commercial real-time PCR systems were evaluated for the detection of both microorganisms. Then, suitable primer sets were selected for the detection of *L. monocytogenes* and *S. aureus*, using PCR and Primer Blast Software. Subsequently, optimal primer sets were evaluated in a simplex SYBR Green-based real-time PCR, and finally a duplex SYBR Green-based real-time PCR method was developed and evaluated for the simultaneous detection of *L. monocytogenes* and *S. aureus* in minimally enriched food samples spiked with known bacterial levels and compared to the commercial PCR systems and traditional plate method.

## 2. MATERIALS AND METHODS

### 2.1. Bacterial strains

Type strains of *L. monocytogenes* (ATCC 19115, 13451), *Vibrio parahaemolyticus* (ATCC 17802), *Aeromonas hydrophila* (ATCC 7966) and *Campylobacter jejuni* (ATCC 29428) and Enterobacteriaceae: *Escherichia coli* (ATCC 11775), *Serratia marcescens* (ATCC 13880), *Enterobacter aerogenes* (ATCC 13048), *Salmonella typhimurium* (ATCC 13311), *Erwinia persicina* (ATCC 1381), *Shigella flexneri* (ATCC 9199), *Klebsiella pneumoniae* (ATCC 700603), *Yersinia*

*enterocolitica* (ATCC 9610), were obtained from Microbiologics Inc, Saint Cloud, USA. *S. aureus* (NCTC 8325) was obtained from the National Collection of Type Cultures (Health Protection Agency Culture Collections, Salisbury, UK). *S. aureus* UL (University of Limerick) isolate, *Cronobacter sakazakii*, *Enterobacter cloacae*, *Pseudomonas aeruginosa* and *Proteus mirabilis* were obtained from the culture collection of the Department of Life Sciences, University of Limerick. These strains were selected as most of them are foodborne pathogens themselves or they may be present in the background flora within food samples. Strains were stored on Protect beads 109 (LangenBach services Ltd, Dublin, Ireland) at – 20°C until cultivation.

## 2.2. Culture conditions

Pure cultures were prepared for primer optimisation. *L. monocytogenes*, *S. aureus* and the *Enterobacteriaceae* were grown overnight on nutrient agar (NA; Oxoid Ltd, Basingstoke, UK) at 37˚C, except *Erwinia persicina* which was incubated at 30˚C. *V. parahaemolyticus* was grown overnight at 35˚C on tryptic soya agar (TSA; Oxoid Ltd). *A. hydrophila* was grown overnight at 35˚C on Columbia blood agar (Oxoid Ltd). *P. aeruginosa* was incubated overnight on nutrient agar at 25˚C. *C. jejuni* was grown on Columbia blood agar in a microaerophilic environment (CampyGen 2.5 l, Oxoid Ltd) for 72 hours at 37°C. Prior to DNA extraction, a preculture of each strain was made by inoculating one loopful of each culture into a flask of 30 ml of nutrient broth (NB; Oxoid Ltd) with overnight shaking at 37˚C for *S. aureus* and the Enterobacteriaceae, except for *E. percisina* that was cultured at 30°C. Tryptic Soya Broth (TSB; Oxoid Ltd) was used for growth of *L. monocytogenes* (37˚C), the *Vibrionaceae* (35˚C), and *P. aeruginosa* (25˚C). Following this, 300 µl of preculture was transferred into 30 ml of fresh broth. A culture of each strain was obtained under the conditions outlined above and grown to exponential growth phase. *C. jejuni* colonies grown on a Columbia agar plate were suspended in 1 ml of 0.85% saline sterile water prior to DNA extraction.

For spiking of food samples, cultures were prepared by inoculating separately one loopful of *L. monocytogenes* in 30 ml of TSB and one loopful of *S. aureus* in 30 ml of NB with overnight shaking at 37°C. Expected bacterial concentrations were ~$10^9$ CFU.ml$^{-1}$. The inocula were serially diluted to obtain the required bacterial concentration for spiking.

## 2.3. Analysis of artificially inoculated food samples

A range of food samples were purchased from local supermarkets in Limerick city including: ready-to-eat meals, tagliatelle and ham, chicken supreme, vegetable soup, chicken soup, cottage pie, ice cream, coleslaw, cottage cheese and raw minced beef. The food samples were prepared in triplicate according to standard methods ISO 8261:2002 (milk and milk products), ISO 6887-2:2003 (meat and meat products), ISO 6887-4:2003 (products other than milk and milk products, meat and meat products and fish and fishery products), ISO 6887-1:1999 (preparation of test samples for microbiological examination), and were artificially inoculated according to ISO 16140:2003 Microbiology of food and animal feeding stuffs — Protocol for the validation of alternative methods) (Anonymous 1999, 2002, 2003a, 2003b, 2003d). The food samples were treated as described in Figure 2.10.

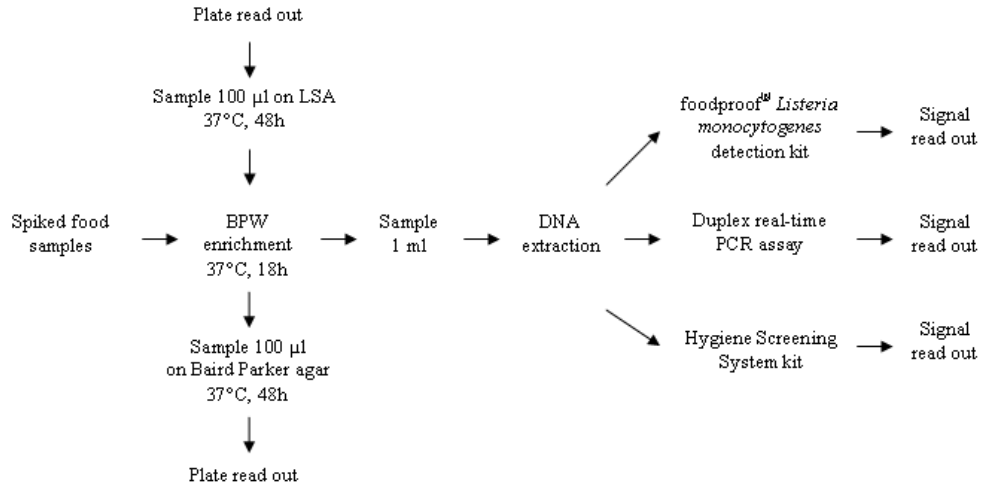


**Figure 2.10.** Analysis work flow of the artificially inoculated food samples.

25 g of each food were previously inoculated with 1 ml of overnight culture for *L. monocytogenes* or *S. aureus* to achieve final concentrations of the order of 1, 10, 100 or 1 x $10^3$ CFU per 25 g of food. Negative samples consisted of preparing a food sample without any bacterial inoculation. Each food sample was transferred into 225 ml of Buffered Peptone Water (BPW, Oxoid) and homogenized bags (Seward Medical, London, UK) using a Blender Stomacher 400 (Seward Medical). A simple enrichment consisted of incubating the contents of each stomacher bag for 18 h at 37°C.

After incubation, the presence of *L. monocytogenes* and *S. aureus* was confirmed by plating out 100 µl of each enrichment culture on *Listeria* Selective Agar (LSA, Oxoid) for *L. monocytogenes* or Baird Parker agar (Oxoid) for *S. aureus*. Plates were incubated at 37°C for 48 h. Typical *L. monocytogenes* colonies on LSA were black surrounded by a black halo. Characteristic colonies of *S. aureus* on Baird Parker agar were grey-black, shiny, convex and surrounded by a zone of clearing.

## 2.4. DNA extractions

DNA used for primer optimisation was extracted following manufacturer's instructions for Gram negative or Gram positive bacteria using a DNeasy Blood and Tissue Kit (Qiagen, Crawley, UK) based on DNA purification through chromatography columns. For each strain, DNA was extracted from pellets obtained from cultures in the exponential growth phase following centrifugation of 1 ml of a bacterial suspension at 5000 x g for 10 min in a Sigma centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). DNA quantifications were performed by spectrophotometry at 260 nm using a Nanodrop Spectrophotometer ND-1000 (Thermo Scientific, Wilmington, USA). Finally, DNA concentrations were adjusted to 1 ng per volume depending on the PCR protocol. A mix of *L. monocytogenes* and *S. aureus* DNA was also prepared at final DNA concentrations of 1 ng / 2 µl for each DNA species.

For food sample analysis, the DNA isolation using DNeasy Blood and Tissue Kit was assessed on 1 ml of BPW enrichment culture, following the manufacturer's instructions.

The sensitivity of DNA detection in pure cultures was determined by serially diluting *L. monocytogenes* or *S. aureus* DNA to 3 copies per 2 µl. Assuming that the target gene was present as a single-copy in the 2.9-mbp-sized genome (Nelson *et al.*, 2004), 1 ng of *L. monocytogenes* DNA therefore equals $3.1 \times 10^5$ copies of the entire genome. From the 2.7- to 2.8-mbp-sized genome of *S. aureus* (Mlynarczyk *et al.*, 1998), 1 ng of *S. aureus* DNA corresponds to $6.10^5$ copies of the entire genome provided the target gene is present as a single-copy.

## 2.5. Commercial real-time PCR systems

### 2.5.1. foodproof® Listeria monocytogenes Detection Kit

This commercial kit (Biotecon Diagnostics GmbH, Potsdam, Germany) uses sequence-specific primers and probes that amplify and simultaneously detect both a 149-bp fragment of a metalloprotease gene (*mpl*) located within the virulence cluster of *L. monocytogenes* and a fragment of the Internal Control (IC) (Anonymous, 2007a). This IC contained chimeric non-relevant DNA added to the master mix amplifiable by the same primer set as the target DNA. Each reaction mix or glass capillary contained 2 µl of foodproof® *Listeria monocytogenes* Detection Mix which included HybProbes probes 10X, 2 µl of foodproof® Enzyme Master Mix, 10× concentrated, and 1 µl foodproof® Uracil-DNA Glycosylase made up with PCR-grade water to 15 µl. The Enzyme Master Mix itself contains the foodproof® Enzyme Solution combined with foodproof® Reaction Mix including primer sets, dUTPs, DNA polymerase and $MgCl_2$. The foodproof® Uracil-DNA Glycosylase was included to prevent cross contaminations occurring with DNA residues containing uracil-glycosidic bonds at U-DNA sites, as dUTP instead of dNTP may have been used in previous runs for the synthesis of PCR products. 5 µl of sample DNA were added to each PCR reaction.

The positive control consisted of a PCR reaction where the template DNA is replaced by a control DNA, a stabilised plasmid solution, provided with the kit (foodproof® Control Template). In the case of the negative control, template DNA was replaced by PCR-grade water. Experiments were carried out using a LightCycler® 1.2 (Roche Diagnostics GmbH, Mannheim, Germany). Amplification conditions were as follows: one cycle of 37°C for 2 min and 95°C for 10 min, followed by 45 cycles of heating at 20°C/s to 95°C, holding at 95°C for 0 s, cooling at 20°C/s to 59°C, holding at 59°C for 30 s where fluorescence was acquired (single acquisition), heating at to 72°C, and holding at 72°C for 5 s. After amplification, a melting curve analysis was performed by heating at 20°C/s to 95°C for 1 min, cooling at 20 °C/s to 40°C for 1 min, and slowly heating at 0.1°C/s to 80°C with fluorescence collection in continuous acquisition mode. The final step involved cooling to 40°C during 30 s at 20°C/s. HybProbes probes, contained in the foodproof® Detection Mix, were designed to bind specifically to the IC, allowing detection in channel F3 or 705 nm, whereas the *L. monocytogenes* DNA was detected in channel F2 or 640 nm.

The use of this kit required the creation of a colour compensation file or colour compensation object with the LightCycler® 1.2. This was a prerequisite for the

unambiguous discrimination of *L. monocytogenes* DNA and Internal Control DNA amplification in the dual-color experiment. As crosstalk may be observed between single channels when using differently labelled HybProbe probes in multiplex dual-colour experiments the LightCycler® Color Compensation Set (Roche Diagnostics GmbH) was used to compensate for this event. The generated colour-compensation file was used in all subsequent real-time PCR experiments with the foodproof® *Listeria monocytogenes* detection kit.

### 2.5.2. Hygiene Screening System kit for *S. aureus*

The Hygiene Screening System kit (Anonymous, 2007b) is designed for the rapid identification of *Staphylococcus* spp, *Micrococcus* spp or *Corynebacterium* from environmental samples in pharmaceutical industries. A melting curve analysis is used to identify *S. aureus* from other species detected in a sample by this kit. A 20-µl standard reaction contains a ready-to-use primer and HybProbe (Biotecon Diagnostics) mix for the specific amplification and detection of DNA of *Staphylococcus* spp (Hygiene Screening System Master Mix, 16 µl) to which 1 µl of Enzyme Mix (*Taq* Polymerase / UNG; Biotecon Diagnostics) is added separately. A stabilized solution of plasmid DNA acting as an internal control is provided to check the quality of amplification (Hygiene Screening System Internal Control, 1 µl). Finally, 2.5 µl of DNA sample is added. The positive control comprised of 2.5 µl Hygiene Screening System Positive Control, a stabilised plasmid solution that replaces the DNA template. The negative control consisted of 2.5 µl of DNA grade water instead of the template. Amplification conditions for 35 cycles were as follows: 95°C for 2 s, 62 °C for 20 s (single acquisition), and 72 °C for 10 s. Melting curve analysis was performed at 95 °C for 0 s, 40 °C for 45 s, and 80 °C for 0 s with temperature transition rate of 0.1 °C/s (continuous acquisition) followed by a cooling step of 40 °C for 30 s.

The HybProbes probes are designed to specifically bind to the IC, allowing detection in channel F3 or 705 nm, whereas the *Staphylococcus* DNA is detected in channel F2 or 640 nm.

In the case of positive results, a further melting curve analysis was performed to differentiate between the closely related genera *Staphyloccocus* and *Micrococcus*, and also for a limited further characterization of detected *Staphylococcus* species. *S. aureus* was detected on channel F3 with a main peak at 57°C (+/- 1.5 °C).

Protocols have been developed and optimised by Biotecon Diagnostics on the LightCycler® 2.0 (Roche Diagnostics GmbH). However, the LightCycler® 1.2 used in the present study possesses the required channels for identification of *S. aureus*.

To our knowledge, no evaluation of these commercial kits has been reported in the literature. In consequence, it was of interest to assess these two kits and give a critical opinion on their use for food microbiology purposes.

### 2.5.3. In-house real-time PCR assays

#### *2.5.3.1. Primer selection*

All primers were purchased from MWG Eurofins Operon (Ebersberg, Germany).

For the detection of *L. monocytogenes*, two primer sets were evaluated: (1) *mpl* primers targeting the metalloprotease gene of *L. monocytogenes* and previously tested by Scheu *et al.* (1999) in a PCR-ELISA. The *mpl* primers are purported to target a 149-bp fragment of the gene. The sequences of the primers were as follows: 5'-GAAAAAGCATTTGAAGCCAT-3' (forward primer) and 5'-GCAACTTCCGGCTCAGC-3' (reverse primer), and (2) *hly A* primers targeting the listeriolysin O of *L. monocytogenes* (Nogva *et al.*, 2000). This set was designed according to the DNA sequence coding for the listeriolysin O gene. The sequence of the forward primer was 5'-TGCAAGTCCTAAGACGCCA-3' and the sequence of the reverse primer was 5'-CACTGCATCTCCGTGGTATACTAA-3'. The expected PCR product size was 112-bp.

For the detection of *S. aureus*, a primer set targeting the *nuc* gene was assayed since the detection of *S. aureus* by PCR and real-time PCR using this primer set has been previously described in a number of reports (Brakstad *et al.*, 1992; Hein *et al.*, 2001; Ikeda *et al.*, 2005; Kim *et al.*, 2001; B. Pinto *et al.*, 2005). The primers were designed to amplify a 279-bp fragment from the gene encoding for a thermonuclease. The forward primer was 5'-GCGATTGATGGTGATACGGT-3' the reverse primer 5'-AGCCAA GCCTTGACGAACTAAAGC-3'.

The specificity of the primer sets was tested using *in silico* PCR analysis against complete genome sequences of *S. aureus*, *L. monocytogenes* and other species. All sequences were provided by the National Centre for Biotechnology Information (NCBI) nucleotide database. The Primer BLAST (Basic Local Alignment Search

Tool) program, available on http://www.ncbi.nlm.nih.gov/tools/primer-blast/, was used for simulated PCR with the primer sets.

The specificity of primers for the detection of *L. monocytogenes* and *S. aureus* was tested separately as a first step. Then primer sets were combined in order to create a detection method in duplex using real-time PCR.

### *2.5.3.2. PCR conditions for detection of L. monocytogenes and S. aureus*

For conventional PCR, mixes were prepared with the ready-to-use kit from Roche Diagnostics GmbH: FastStart *Taq* DNA polymerase, dNTPack 5 U/µl. On the basis of a final 50 µl reaction volume, the master mixture contained 48 µl of 4 mM $MgCl_2$ (2 mM from 10X PCR buffer and 2 mM from $MgCl_2$ solution), 500 nM of forward primer, 500 nM of reverse primer, dNTP mixture and the FastStart *Taq* DNA Polymerase to which 2 µl of DNA sample was added to each reaction.

PCR was performed on a G-Storm GS2 Thermal Cycler (Genetic Research Instrumentation, Braintree, UK) for the detection of *L. monocytogenes* or *S. aureus*. The PCR programmes were carried out as shown in Table 2.9, using annealing temperatures of 59°C for *mpl* primers, 60°C for *hly A* primers and 62°C for *nuc* primers.

**Table 2.9.** PCR and real-time PCR conditions using the *mpl, hly A* and *nuc* primer sets.

| Program | PCR | SYBR Green-based real-time PCR |
|---|---|---|
| Pre-Incubation | 95°C, 6 min | 95°C, 10 min |
| Amplification | 28 cycles | 35 cycles |
| | 95°C, 30 s | 95°C, 5 s |
| | [a]°C, 15 s | [a]°C, 10 s |
| | 72°C, 30s | 72°C, 20 s (Single) |
| Melting | N/A[b] | 95°C, 0 s |
| | | 65°C, 10 s |
| | | 95°C, 0 s |
| | | 0.2°C/s (Continuous) |
| Cooling | N/A | 40°C, 30 s |

[a] specific to each primer set used, similar to conventional PCR annealing temperatures

[b] non applicable

Each PCR product was subsequently run on a 2% agarose electrophoresis gel and stained using SYBR Safe™ (Molecular Probes, Eugene, USA) and visualized with a transilluminator (Syngen, Frederick, USA) under UV light. The Hyperladder II, 50-2,000bp (Bioline Ltd, London, UK) was used as a molecular marker.

#### *2.5.3.3. Simplex and duplex SYBR Green-based real-time PCR*

When testing the primer sets, conventional PCR procedures were adapted to provide a simplex real-time procedure on the LightCycler® 1.2 (Roche Diagnostics GmbH), using LightCycler® FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH).

In each capillary, the 20-µl reaction mix contained 1X concentration LightCycler-FastStart DNA Master SYBR Green (Roche Diagnostics GmbH); 4 mM $MgCl_2$; 500 nM concentration of each primer; and 2 µl of the template. A duplex real-time PCR was then evaluated based on combining the two primer sets in the same reaction mix with the same concentrations of reagents and same volume of template, applying the $T_M$ average from two primer sets.

Times and temperatures applied are shown in Table 2.9 and DNA amplification was followed in real-time in channel F1 or 530 nm.

The intra and inter-assay reproducibility of the duplex PCR system was evaluated as described by Fan *et al.* (2007). The intra-assay evaluation corresponded to a unique PCR run where triplicates per sample were analysed. The inter-assay evaluation was equivalent to three separate PCR runs where for each assay with data retrieved from triplicates for each sample. The coefficients of variation (CV) for Ct and $T_M$ values were calculated by dividing the standard deviation (SD) by the mean Ct or $T_M$ for intra and inter-assays.

The duplex based real-time PCR was evaluated in the inoculated food samples and compared with the commercial real-time PCR systems and the traditional microbiological detection methods described above (Figure 2.10).

# 3. RESULTS AND DISCUSSION

## 3.1. Evaluation of the commercial real-time PCR systems

### 3.1.1. foodproof® *Listeria monocytogenes* detection kit

PCR kinetic profiles revealed that extracted *L. monocytogenes* DNAs and the positive control were amplified and detected at 640 nm. A mean Ct of 21.21 (SD equal to 0.28) for *L. monocytogenes* ATCC 19115 and *L. monocytogenes* ATCC 13451 DNA was determined (Table 2.10A). The positive control had a Ct equal to 24.61 for the assay.

**Table 2.10.** Intra-assay crossing point values and sensitivities obtained from triplicate samples analysed using various real-time PCR systems for the detection of (A) *L. monocytogenes* ATCC 19115 and 13451 and (B) *S. aureus* NCTC 8325 and UL isolate.

**A**

| Real-time PCR system | Ct[a] (SD) | Sensitivity (copies) |
|---|---|---|
| foodproof® *Listeria monocytogenes* Detection kit | 18.97 (0.17) | 3 |
| Simplex SYBR-Green with *hly A* primer set | 19.65 (0.2) | 30 |
| Duplex SYBR-Green based | 18.67 (0.01) | 30 |

**B**

| Real-time PCR system | Ct[a] (SD) | Sensitivity (copies) |
|---|---|---|
| Hygiene Screening Detection kit | 18.51 (0.17) | 60 |
| Simplex SYBR-Green with *nuc* primer set | 20.57 (0.19) | 60 |
| Duplex SYBR-Green based | 17.18 (0.01) | 6 |

[a] Mean Ct value corresponded to the amplification of 1 ng of DNA (pure culture) per PCR reaction

In contrast, the negative control was not amplified. Visualisation at 705 nm showed that the IAC was amplified in each sample, including the negative control. This commercial real-time PCR system, described by Junge and Berghof-Jager (2006) has been validated by AOAC (Association of Official Agricultural Chemists). Scheu *et al.* (1998) suggested that the increasing uptake of commercial PCR systems would eventually lead to standardized procedures which are a prerequisite to routine analysis by the food industry using these kits. Malorny *et al.* (2003) included an IAC to PCR

methods to monitor the robustness of diagnostic PCR which is a criterion for standardization. As regards the frequency of when IACs are included in PCR, various real-time PCR methods have been recently developed with the inclusion of an IAC (O'Grady *et al.*, 2009; Rip and Gouws, 2009; Rossmanith *et al.*, 2006). Some researchers include an IAC in order to overcome the possibility of false negative results (Hoorfar, 2004) and to monitor the presence of PCR inhibitors (Malorny *et al.*, 2003). However, a significant number of PCR related publications do not include an IAC in their procedures. Barkham (2004) suggested that an IAC should not be mandatory, at least in clinical diagnostics, mainly because of the risk of increasing the cost of PCR testing. Another criterion mentioned by Malorny *et al.* (2003) was the analytical and diagnostic accuracy where "a selective PCR-based method comprises inclusivity (detection of the target-pathogen from a wide range of strains) and exclusivity (lack of response from a relevant range of closely related but non target strains)". Biotecon Diagnostics claim to have evaluated the inclusivity of this kit using 102 *L. monocytogenes* isolates and the exclusivity using 60 non-*L. monocytogenes* bacteria. The company reported that a relative detection limit of 1 to 10 cells per 25 g sample could be achieved in various food samples. Biotecon Diagnostics stated that the kit can detect down to $10^3$ - $10^4$ CFU/ml in enrichment cultures, depending on the sample preparation kit used (Anonymous, 2007a). In the present study, 1 ng of *L. monocytogenes* DNA or $3.5.10^5$ copies per reaction was detected at a mean Ct equal to 18.97 (SD equal to 0.17) in the case of a pure culture. The LightCycler® instrument can detect a X-fold difference in template concentration, according to a statistical variability of Ct in multiple PCR reactions (Anonymous, 2009a). For instance, to detect a 10-fold change, the determined ΔCt must statistically confirm a 3.32 cycle change, assuming that a reaction has an efficiency of 2, which means that at each 10-fold dilution the Ct value is increased by adding 3.32. Therefore, 1 fg of *L. monocytogenes* DNA or 3 copies can theoretically be detected at a mean Ct equal to 35.57. In parallel, serial dilutions of DNA copy numbers amplified by real-time PCR confirmed that 3 copies could be detected at a mean Ct equal to 37.56 (SD equal to 0.5) with an efficiency of 1.91 (Table 2.10A). While the foodproof® *Listeria monocytogenes* detection kit appeared to have a high specificity and low detection limit, a single kit for 96 real-time PCR reactions equates to a high cost per single reaction for routine analysis or research. Therefore, in-house real-time PCR protocols were developed to obtain alternatives lower cost real-time PCR assays.

### 3.1.2. Hygiene Screening System kit – *Staphylococcus aureus*

This commercial real-time PCR system is a typical example of multiplex real-time PCR system using a combination of primer sets and hybridization probes. At 640 nm, *S. aureus* DNAs and the positive control were amplified, whereas the negative control was not. *S. aureus* NCTC and UL isolate were detected at a mean Ct equal to 18.51 (SD equal to 0.17). In this study, the sensitivity of this commercial kit was found to be 60 copies for a mean Ct equal to 35.91 (SD equal to 0.20) (Table 2.10B). The positive control was detected later at a Ct equal to 19.27. When switching detection channel to 750 nm, amplification curves were observed even for the negative control confirming the reliability of the PCR using the internal control. To confirm the amplification of *S. aureus*, a melting curve analysis was performed. At 640 nm, a single peak/shoulder > 64°C was observed for the positive sample (Figure 2.11A).

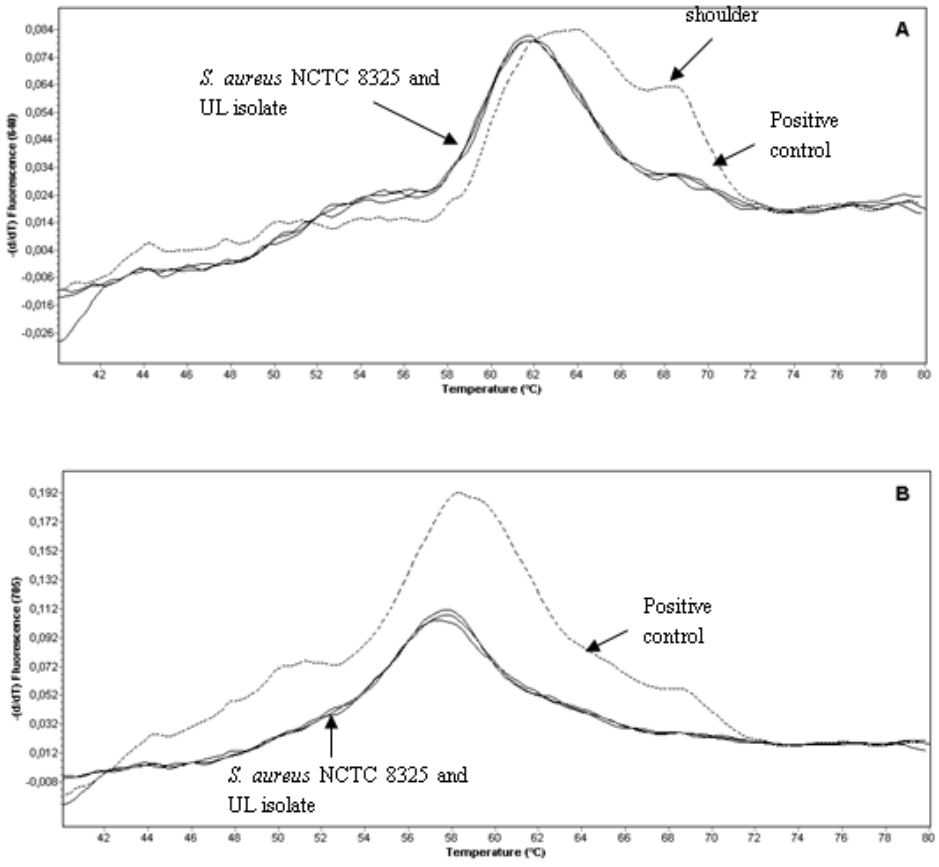


**Figure 2.11.** Melting curves of *S. aureus* NCTC 8325, UL isolate and positive control analysed using the Hygiene Screening System kit and detected in (A) channel F2 at 640 nm or (B) channel F3 at 705 nm.

The presence of a shoulder on the melting curve may arise as an internal melting domain because of long PCR products, as described by Ririe *et al.* (1997), which can be a fingerprint for species identification. However, this shoulder was quite difficult to define for *S. aureus* NCTC 8325 and UL isolate in comparison to the positive control. Therefore, a highly experienced analyst would appear to be required to discriminate such a region on the melting curve using the commercial kit.

At 705 nm, *S. aureus* NCTC 8325 and UL isolate could be identified in both test DNA samples, where the mean $T_M$ was 57.57°C (SD equal to 0.09), which confirmed the identification of *S. aureus* (Figure 2.11B).

The Hygiene Screening System kit allows rapid qualitative detection of the presence of *S. aureus* in contaminated samples while identifiying the species using melting curve analysis. The kit has also been designed for performing a fast and reliable multiplex PCR system that detects other *Staphylococcus*, *Micrococcus,* and *Corynebacterium* species.

## 3.2. *mpl* primers

The *mpl* gene was characterised by Domann *et al.* (1991) as a gene unique to *L. monocytogenes* species and physically linked to the listeriolysin gene of these strains. The *mpl* primers were purported to target a 149-bp fragment of the *mpl* gene, according to Scheu *et al.* (1999), whose work has been referenced in the manual instructions of the foodproof® *Listeria monocytogenes* detection kit. *In silico* PCR analysis using Primer Blast, as shown on Table 2.11 indicated poor specificity for *L. monocytogenes*.

**Table 2.11.** *In silico* PCR analysis using the *mpl, hlyA* and *nuc* primer sets with Primer Blast Software.

| ***Bacterial species*** | **Primer sets** | | |
|---|---|---|---|
| | ***mpl*** | ***hlyA*** | ***nuc*** |
| *Budvicia aquatica* | -[b] | - | - |
| *Cedecea davisae* | - | - | - |
| *Citrobacter braakii* | - | - | - |
| *Citrobacter farmeri* | - | - | - |
| *Citrobacter freundii* | - | - | - |
| *Citrobacter koseri* | +[a] | - | - |
| *Cronobacter sakazakii* | + | - | - |
| *Enterobacter aerogenes* | - | - | - |
| *Enterobacter cloacae* | + | - | - |
| *Erwinia persicina* | - | - | - |
| *Escherichia coli* | + | - | - |
| *Escherichia coli 0157:H7* | + | - | - |
| *Ewingella Americana* | - | - | - |
| *Hafnia alvei* | - | - | - |
| *Klebsiella pneumoniae* | + | - | - |
| *Klebsiella oxytoca* | + | - | - |
| *Kluyvera ascorbata* | - | - | - |
| *Kluyvera intermedia* | - | - | - |
| *Pantoea agglomerans* | - | - | - |
| *Plesiomonas shigelloides* | - | - | - |
| *Proteus mirabilis* | + | - | - |
| *Proteus vulgaris* | - | - | - |
| *Salmonella typhimurium* | - | - | - |
| *Salmonella enteritidis* | + | - | - |
| *Serratia liquefaciens* | - | - | - |
| *Serratia marcescens* | + | - | - |
| *Shigella flexneri* | + | - | - |
| *Shigella sonnei* | + | - | - |
| *Yersinia enterocolitica* | + | - | - |
| *Yersinia pseudotuberculosis* | + | - | - |
| *Yersinia rohdei* | - | - | - |
| *Acinetobacter baumanii* | + | - | - |
| *Aeromonas hydrophila* | + | - | - |
| *Aeromonas punctata* | - | - | - |
| *Aeromonas sobria* | - | - | - |
| *Alcaligenes faecalis* | - | - | - |
| *Bacillus cereus* | + | - | - |

| | | | |
|---|---|---|---|
| *Bacillus subtilis* | + | - | - |
| *Campylobacter coli* | - | - | - |
| *Campylobacter jejuni* | - | - | - |
| *Enterococcus faecalis* | + | - | - |
| *Listeria innocua* | + | - | - |
| *Listeria monocytogenes* | + | + | - |
| *Micrococcus spp.* | + | - | - |
| *Pseudomonas aeruginosa* | + | - | - |
| *Pseudomonas fluorescens* | + | - | - |
| *Pseudomonas putida* | + | - | - |
| *Staphylococcus aureus* | + | - | + |
| *Staphyloccoccus capitis* | - | - | - |
| *Staphyloccoccus lentus* | - | - | - |
| *Staphyloccoccus xylosus* | + | - | - |
| *Vibrio parahaemolyticus* | + | - | - |
| *Vibrio vulnificus* | + | - | - |

[a] target templates found

[b] target templates not found

Complementary PCR analysis showed non-specific detection for selected species such as *C. sakazakii*, *E. cloacae*, *S. aureus* or *V. parahaemolyticus*. The PCR system developed by Scheu *et al.* (1999) appeared to be specific for *L. monocytogenes* when combined with probes. The inclusion of probes can circumvent non-specific amplifications by providing an additional layer of specificity (Nakken *et al.*, 2009). However, based on the current data, this primer set would not appear to be applicable for the subsequent development of a SYBR Green-based method for detection of *L. monocytogenes*.

### 3.3. *hly A* primers

The *hly A* primer set anneals to the listeriolysin O gene of *L. monocytogenes*. This targeted sequence has been reported in several PCR and real-time PCR applications and uses different primer sequences targeting the same gene (Amagliani *et al.*, 2004; Bansal, 1996; Guilbaud *et al.*, 2005). Aznar and Alarcón (2002) compared primer set specificities, notably primers that target the listeriolysin O, for the detection of *L. monocytogenes*. According to these workers, only one primer set was shown to be specific among the primer sets targeting the listeriolysin O gene, which confirmed that non-specific amplification can occur. Nogva *et al.* (2000)

selected a region with 100% homology between the *L. monocytogenes* sequences and with little homology to the sequences reported from other species with a gene encoding for the same type of protein using a *TaqMan*®-based PCR assay. However, a Primer Blast evaluation over a range of species showed a high specificity of the primer set for *L. monocytogenes* (Table 2.11). Data obtained in this study following PCR indicated that *L. monocytogenes* DNA was detected as a 112-bp fragment which could be identified on the agarose electrophoresis gel under UV (Figure 2.12). Therefore, the *hly A* primer set appeared to be a better candidate for the detection of *L. monocytogenes* using real-time PCR methods on account of its specificity.

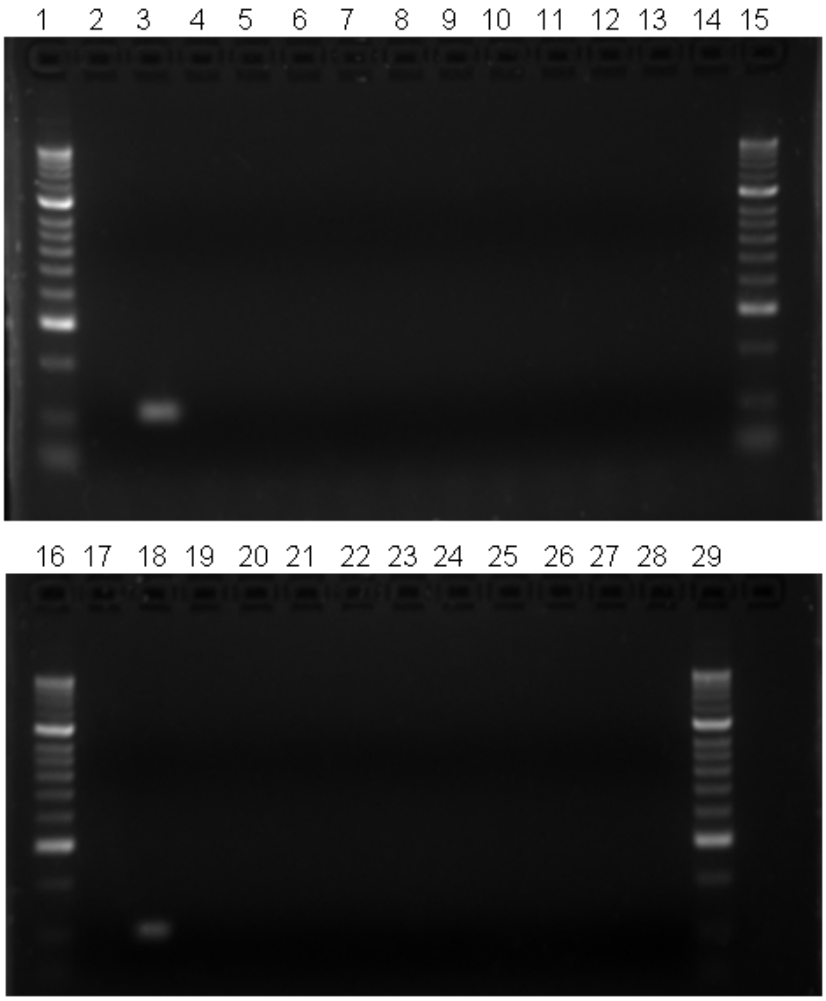


**Figure 2.12.** Amplification results obtained by PCR using the *hly A* primers.
Lanes 1, 15, 16, 29, Hyperladder II, 50-2,000bp; lane 2, 17, negative control (no DNA template in the PCR reaction); lane 3, *L. monocytogenes* ATCC 19115, lane 18 *L. monocytogenes* ATCC 13451 ; lane 4, *E. coli* ; lane 5, *E. aerogenes* ; lane 6, *E. cloacae* ; lane 7, *C. sakazakii* ; lane 8, S. *typhimurium* ; lane 9, *S. Flexneri*; lane 10 *K. pneunomiae* ; lane 11 *Y. enterocolitica* ; lane 12, *E. percisina* ; lane 13, *S. marcescens* ; lane 14, *P. mirabilis* ; lane 19, *V. parahaemolyticus* ; lane 20, *A. hydrophila* ; lane 21, *P. aeruginosa* ; lane 22, *S. aureus* ; lane 23, *S. capitis* ; lane 24, *S. lentus* ; lane 25, *S. xylosus* ; lane 26, *Micrococcus spp* ; lane 27, *B. cereus* ; lane 28, *C. jejuni*.

## 3.4. *nuc* primers

*S. aureus* DNAs were amplified using *nuc* gene primers. The DNA fragments were observed on SYBR Safe stained 2% agarose electrophoresis gel under UV which showed that a 279-bp PCR product was present (Figure 2.13).

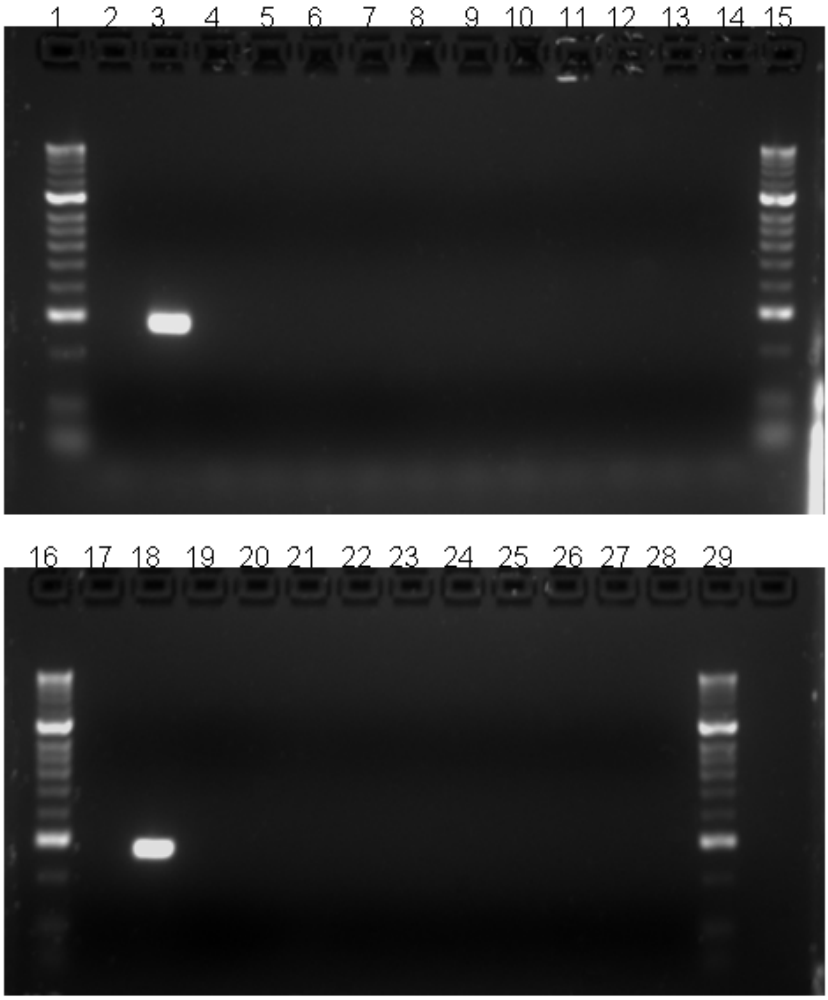


**Figure 2.13.** Amplification results obtained by PCR using the *nuc* primers.
Lanes 1, 15, 16, 29, Hyperladder II, 50-2,000bp ; lane 2, 17, negative control (no DNA template in the PCR reaction) ; lane 3, *S. aureus* NCTC 8325, lane 18, *S. aureus* UL isolate ; lane 4, *E. coli*; lane 5, *E. aerogenes* ; lane 6, *E. cloacae* ; lane 7, *C. sakazakii* ; lane 8, S. *typhimurium* ; lane 9, *S. flexneri* ; lane 10 *K. pneumoniae* ; lane 11 *Y. enterocolitica* ; lane 12, *E. percisina* ; lane 13, *S. marcescens* ; lane 14, *P. mirabilis* ; lane 19, *V. parahaemolyticus* ; lane 20, *A. hydrophila* ; lane 21, *P. aeruginosa* ; lane 22, *L. monocytogenes* ATCC 19115 ; lane 23, *S. capitis* ; lane 24, *S. lentus* ; lane 25, *S. xylosus* ; lane 26, *Micrococcus spp* ; lane 27, *B. cereus* ; lane 28, *C. jejuni*.

Among all the bacterial DNAs tested, only *S. aureus* was detected using the *nuc* primers while other *Staphylococcus* and *Micrococcus* species gave negative responses for this primer set. Data obtained confirmed findings reported by Alarcon *et al.* (2006) which support the use of PCR-based procedures using the same primer set

and combined with enrichment in different types of food. Consequently, the *nuc* primer set was considered to be a suitable candidate for the detection of *S. aureus* in a SYBR Green real-time PCR method.

## 3.5. Simplex real-time PCR

Conventional PCR was converted to real-time PCR protocols using a universal PCR mix where only the primer sets were modified in respect of the particular pathogen to be detected.

For the detection of *L. monocytogenes*, real-time PCR assays using mixes prepared with *hly A* primers were performed. The amplification curves obtained confirmed that both *L. monocytogenes* ATCC 19115 and ATCC 13451 strains were detected at a mean Ct of 19.65 (SD equal to 0.20), as shown on Table 2.10. After 35 cycles, non-specific amplifications appeared. In the case of *S. aureus*, data obtained indicated that the primer set was highly specific as *S. aureus* DNA was detected only at a mean Ct of 20.57 (SD equal to 0.19) for *S. aureus* NCTC 8325 and UL isolate. After 38 cycles, non-specific amplifications occurred as an included *L. monocytogenes* DNA sample and the negative control displayed some fluorescence.

Fernandez *et al.* (2006) outlined the main limitation of SYBR Green I as being the likelihood of false positive results. This may occur in cases where the dye binds to double-stranded DNA other than the target, such as primer-dimers which decrease the specificity. As all double-stranded DNA are detected, SYBR Green becomes less sensitive than fluorogenic probes, in the presence of primer-dimers (Ciglenecki *et al.*, 2008). The risk of formation of primer-dimers is usually related to a sub-optimal concentration of primers, a low target copy number or if the target is absent in the sample (Fernandez *et al.*, 2006). In order to differentiate specific PCR products from potential artefacts, a melting curve analysis was conducted following both real-time PCR procedures which confirmed the reliability of the methods, as shown on Figure 2.14.

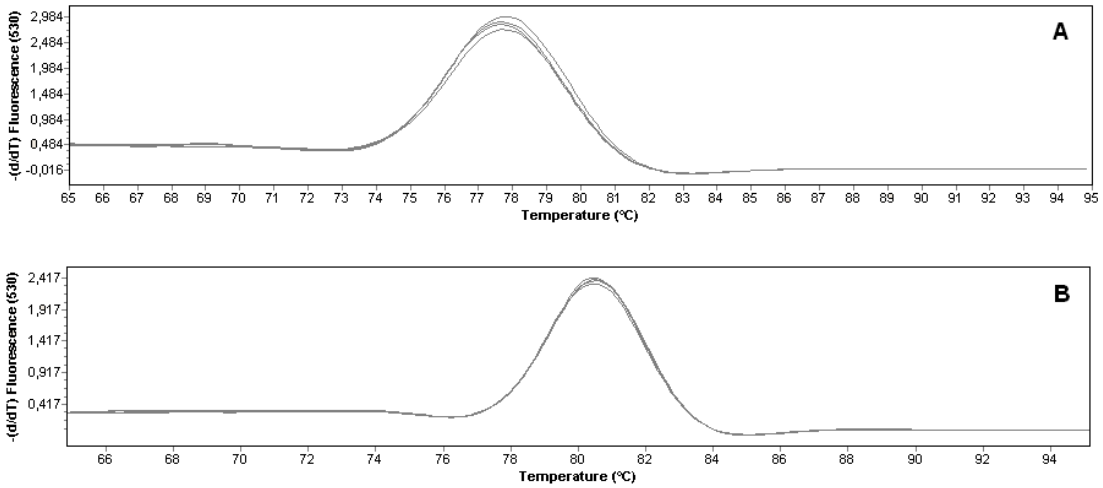


**Figure 2.14.** Melting curve profiles of simplex SYBR Green-based PCR changing the primers sequence for the identification of (A) *L. monocytogenes* and (B) *S. aureus*.

For each amplified DNA sample, generation of a single and sharply defined melting curve with a narrow peak indicated that the PCR products were pure and homogenous without the presence of primer-dimers which melt at relatively low temperatures and have broader peaks. The identity of *L. monocytogenes* was confirmed when the $T_M$ of the PCR product approached 77.99°C (SD equal to 0.07) whereas the *S. aureus* PCR product had a mean $T_M$ of 80.48°C (SD equal to 0.02) (Figure 2.14).

The sensitivity of the SYBR Green-based real-time PCR methods was assessed using a series of dilutions of genomic DNA subsequently converted into copy numbers as described previously for the assessment of the commercial real-time PCR systems. For both real-time PCR methods, using pure cultures, the detection limit was 30 copies (Table 2.10). Each simplex real-time PCR appeared to be highly specific but still required a separate run on the LightCycler® because of the difference in annealing temperatures. For this reason, a combination of both primer sets was assayed for the development of a duplex real-time PCR as a two in one solution in a single PCR run.

## 3.6. Development of a duplex SYBR Green-based real-time PCR

DNA samples extracted from pure culture of *S. aureus* and *L. monocytogenes* were amplified using the newly developed duplex system. Mean melting $T_M$ and SD for each PCR product were compared with $T_M$ data obtained using the TMUtility_1.5 Software (Idaho Technology Inc, Salt Lake City, USA), as shown in Table 2.12. Positive data were confirmed in the range $T_M$+/-3SD for each targeted gene.

**Table 2.12.** Melting temperatures (actual and calculated) of the PCR products obtained by duplex real-time PCR.

| | ***nuc* gene (*S. aureus*)** | ***hlyA* gene (*L. monocytogenes*)** |
|---|---|---|
| **Calculated $T_M$** | 81.76 | 78.95 |
| **Mean $T_M$ (SD) (°C)** | 79.90 (0.07) | 78.05 (0.05) |
| **$T_M$ range (Mean+/-3SD) (°C)** | 79.7 - 80.1 | 77.9 - 78.2 |

The specificity of the newly developed duplex real-time PCR method was assessed using a range of bacterial species, including *L. monocytogenes* and *S. aureus*. Amplification curves were observed for the samples containing *L. monocytogenes* DNA with a mean Ct of 18.67 (SD equal to 0.01) and *S. aureus* DNA with a mean Ct equal to 17.18 (SD equal to 0.01) (Table 2.10B) and the mix of *L. monocytogenes / S. aureus DNAs* with mean intra-assay Ct of 16.75 (SD equal to 0.12). The sensitivities of the duplex system were 30 copies (mean Ct of 32.15, SD equal to 0.09) for *L. monocytogenes* and 6 copies (mean Ct of 33.78, SD equal to 0.07) for *S. aureus*, in the case of samples containing either one microorganism or the other.

The melting curve profiles (Figure 2.15A) showed that no primer-dimers were formed during the PCR runs which would suggest that no excessive regions of complementarity existed between the two primers.

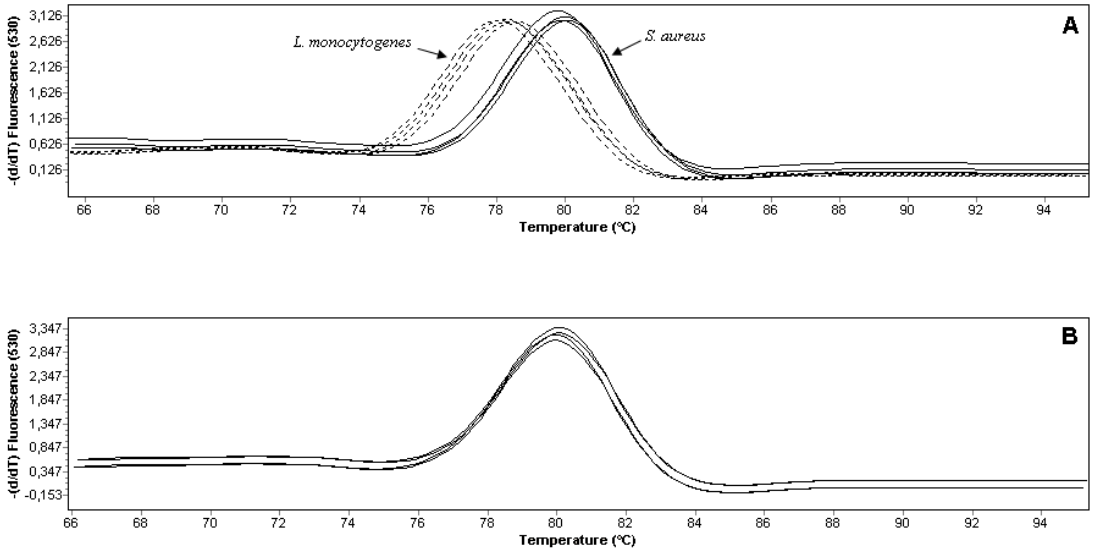


**Figure 2.15.** Melting curve profiles obtained from (A) samples containing *L. monocytogenes* DNA only or *S. aureus* DNA only and (B) samples containing both *L. monocytogenes* and *S. aureus* DNAs in a duplex real-time PCR.

In the current study, the real-time PCR assays in duplex consisted of combining the *hly A* and *nuc* primer sets for the simultaneous detection of *L. monocytogenes* and *S. aureus*. According to Wu *et al.* (2007), the performance of multiplex PCR systems depends first on the primer design and the difference in $T_M$ within one primer pair should not exceed 5°C, which is in agreement with the two primer sets used in this study ($\Delta T_M$ equal to 2°C). PCR products should ideally be between 100 and 2,000 bp-long with a difference in size of more than 50 bp. Based on the data obtained for the duplex PCR methodology developed in this study these criteria appeared met (Figure 2.15A and 2.16).

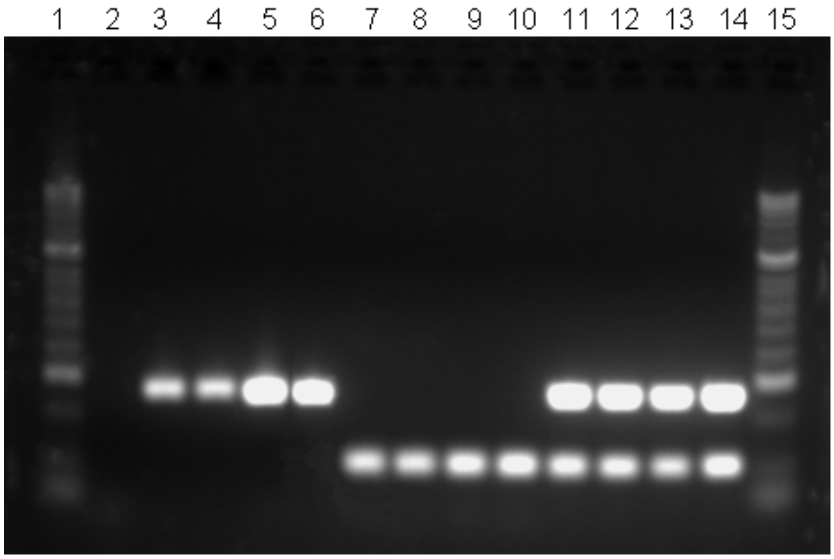


**Figure 2.16.** PCR products obtained after SYBR Green-based duplex real-time PCR using *hly A* and *nuc* primers for the simultaneous detection of *S. aureus* and *L. monocytogenes*.
Lanes 1, 15, Hyperladder II, 50-2,000bp ; lane 2, negative control (no DNA template in the PCR reaction) ; lanes 3, 4, 5, 6, *S. aureus* DNA samples only ; lanes 7, 8, 9, 10, *L. monocytogenes* DNA samples only ; lanes 11, 12, 13, 14, *S. aureus* and *L. monocytogenes* DNA in the same sample.

Distinct $T_M$ values were obtained for *L. monocytogenes* and *S. aureus strains* when analysed individually or in a mixture. When *L. monocytogenes* DNA was amplified, the $T_M$ was between 77.9 °C - 78.2 °C, whereas the observed $T_M$ for *S. aureus* was within the range 79.7°C - 80.1°C (Table 2.12). This data would satisfy the criterion of Ririe *et al.* (1997) who suggested that PCR product $T_M$s should differ by 2°C. However, in this study similar melting curves profiles were obtained for *S. aureus* DNA and *L. monocytogenes* / *S. aureus* DNA mixes, where the mean $T_M$ value was 80.10°C (SD equal to 0.01) (Figure 2.15B), which suggested the presence of a single PCR product instead of two. In contrast, analysis of multiplex PCR by agarose gel electrophoresis (Figure 2.16) showed amplification of both gene targets.

However these data were in agreement with Giglio *et al.* (2003a) regarding the preferential binding of SYBR Green I to amplicons with the highest Guanine + Cytosine percentage (G+C %) and hence the preferential binding to the largest of the two PCR products. The G+C content calculated for each PCR product was determined using the TMUtility_1.5 Software. For *L. monocytogenes* PCR product, G+C% was equal to 38.4% whereas in the case of *S. aureus*, the G+C% was 36.2%. Because G+C% are close to each other, it is reasonable to speculate that SYBR Green I may preferentially bind to *S. aureus* amplicon because of its larger size. Therefore, the

main technical issue for this duplex real-time PCR method would arise if food samples were simultaneously contaminated with *L. monocytogenes* and *S. aureus*, which may cause a misinterpretation of data. Kumar *et al.* (2009) developed a multiplex PCR system for the simultaneous detection of *B. cereus*, *S. aureus* and *L. monocytogenes* in case of coexistence of these pathogens in spiked food/milk samples and their similarities in terms of symptoms and incubation period. It was claimed that this assay could identify all the three species individually or in combination using conventional multiplex PCR on an agarose electrophoresis gel. While data analysis was simpler than for multiplex real-time PCR procedure, it was more time consuming and less sensitive. Wang *et al.* (2004) reported detection by duplex SYBR Green real-time PCR of *S. enteritidis* DNA and *L. monocytogenes* independently or combined in the same raw sausage sample. In their study, two distinct melting peaks were observed for samples containing both *S. enteritidis* and *L. monocytogenes*, one peak corresponded to *S. enteritidis* and the other was attributed to *L. monocytogenes*.

Overall, the identification by melting curve analysis of microorganisms in combination in a sample depends on the assay. The causes of variation in melting curve profiles have not been fully explained. However, Giglio *et al.* (2003a) and Gundry *et al.* (2003) attributed this to the redistribution of SYBR Green from PCR products with a lower $T_M$ to a higher $T_M$ during melting curve analysis. While it is not credible to exclude the possibility of samples being contaminated simultaneously by *S. aureus* and *L. monocytogenes* no such food poisoning outbreaks caused by the ingestion of foods contaminated by both microorganisms have been reported to date. In regard to technical challenges in simultaneously detecting of *S. aureus* and *L. monocytogenes* in a sample, it may be therefore more advantageous to run the real-time PCR assay as two tests rather than in duplex.

## 3.7. Analysis of food samples and assay reproducibility

Sensitivities were first determined for each type of spiked food using the newly developed duplex SYBR Green-based real-time PCR procedure. Secondly, in-house real-time PCR data were confirmed by a comparison with the commercial real-time PCR kits on the sample indicating the detection limit and the samples that were 10 times more or 10 times less contaminated. The foodproof® *Listeria monocytogenes* detection kit and the Hygiene Screening Detection kit provided the same sensitivity data as the duplex real-time PCR and by plating on selective media, as shown on

Table 2.13. Sensitivities were defined by plate count data obtained after initial spiking with suspensions of *L. monocytogenes* or *S. aureus*, and computed by factoring in the dilution factor applied to each food sample.

**Table 2.13.** Sensitivities[a] of the duplex SYBR Green-based real-time PCR in CFU / mass of food obtained in the analysis of 9 types of food samples after spiking of *L. monocytogenes* or *S. aureus* and 18 h enrichment with BPW.

| Food Samples | *L. monocytogenes* | *S. aureus* |
|---|---|---|
| Tagliatelle and Ham | 5 CFU / 25 g | 6 CFU / 25 g |
| Chicken Supreme | 5 CFU / 25 g | 6 CFU / 25 g |
| Vegetable Soup | 3 CFU / 25 g | 5 CFU / 25 g |
| Chicken Soup | 3 CFU / 25 g | 5 CFU / 25 g |
| Cottage pie | 3 CFU / 25 g | 5 CFU / 25 g |
| Ice Cream | 18 CFU / 25 g | 6 CFU / 25g |
| Coleslaw | 7 CFU / g | 6 CFU / 25g |
| Cottage Cheese | 6 CFU / 25 g | 5 CFU / 25g |
| Minced Meat | 6 CFU / 25 g | 2 CFU / g |

[a] Sensitivities were defined according to qualitative duplex real-time PCR data (presence/absence) on enriched samples analysed in triplicate and previously spiked with serial dilutions of an inoculum where the initial concentration was defined by plate counting on NA.

In microbiological food analysis, an enrichment step of 18 to 24 h is generally required prior to PCR (Candrian, 1995). This step appears to be essential for the detection of bacteria in foods at minimum concentration levels from 1 to 10 Colony Forming Units (CFU) per g of food. Nevertheless, such enrichment media, notably a selective one, require hazardous supplements and additional costs in consumables (Duffy *et al.*, 2001). Therefore, the possibility of eliminating or minimizing the enrichment step is of potential interest in order to simplify and even shorten the time of food analysis. In this study, the use of BPW allowed the recovery of low bacterial levels, depending on the food sample analysed, the sensitivity of the method varied (Table 2.13). In this respect, the recovery of *L. monocytogenes* normally depends on enrichment to amplify low bacterial populations or injured cells in food products. However, the repair and detection of sub-lethally injured *Listeria* may be inhibited by the use of selective agents in such enrichment media (Donnelly, 2002; Duffy *et al.*,

2001). The ISO 11290-1 reference method (Anonymous, 2004b), uses half Fraser broth and Fraser broth for the detection of *L. monocytogenes* and is time consuming. Besse *et al.* (2005) and Oravcova *et al.* (2008) suggested shortening this two-step enrichment. Indeed, similar or slightly higher recoveries of *L. monocytogenes* were obtained in the presence of *L. innocua* after the first enrichment in half Fraser compared to the second enrichment with Fraser broth. Many publications report the limitations of such media in relation to the coexistence of other *Listeria* species (Beumer *et al.*, 1996; Curiale and Lewus, 1994; Duh, 1993; Macdonald and Sutherland, 1994). This may have an impact on the detection of *L. monocytogenes* based on the fact that *L. innocua* has a faster growth rate. In contrast, Besse *et al.* (2005) discussed the effect of enrichment, using the ISO 11290-1 reference method, on samples contaminated with *Listeria* species, where *L. innocua* was able to grow more easily in various foods than *L. monocytogenes*. The predominance of *L. innocua* over *L. monocytogenes* could also be explained by the higher initial contamination level of *L. innocua* (Besse *et al.*, 2005). As an alternative to half Fraser and Fraser broths, Oravcova *et al.* (2008) obtained similar *L. monocytogenes* recoveries using a non-selective enrichment broth such as Brain Heart Infusion. In the current study, it is reasonable to speculate that BPW, used for enrichment, may be sufficiently reliable for the recovery of *L. monocytogenes* or *S. aureus*. Studies comparing BPW with selective enrichment broth showed that similar bacterial recoveries were obtained for the detection of *L. monocytogenes* and other *Listeria* species in food samples as described by Walsh *et al.* (1998a) where the selectivity for *L. monocytogenes* was increased. However, the recovery depends on the inherent buffering capacity. Dairy products are an example of foods with a low inherent buffering capacity and Walsh *et al.* (1998a) advised instead the use of a highly buffered non selective medium. In contrast, duplex real-time PCR results indicated positive samples for *L. monocytogenes* in cottage cheese and ice cream samples enriched with BPW with a detection limit of 6 and 18 CFU / 25 g of food. The lowest sensitivity was obtained in coleslaw with *L. monocytogenes* (7 CFU / g). However, this detection limit is still satisfactory for the analysis of such pathogens in foods. Duffy *et al.* (2001) compared the performances of BPW and University of Vermont Media (UVM) for the recovery of *L. monocytogenes* in minced meat samples and obtained similar results after 24 h of enrichment. In the current study, enriched food samples could be analysed after 18 h, which represents a gain of 6 h.

For the detection of *S. aureus*, the EN ISO 6888-3 (Anonymous, 2003i) is the reference standard currently available and describes a selective enrichment step with Giolitti and Cantoni broth followed by isolation of colonies with characteristic morphology and subsequent identification tests. The use of BPW for enrichment of samples contaminated with *S. aureus* has not been reported in the literature. However, *S. aureus* is recognized as not having specific nutrient requirements and *S. aureus* appeared to grow well in BPW. Data for duplex real-time PCR in the tested food samples found the lowest detection limit of 2 CFU / g of minced meat (Table 2.13).

As regards reproducibility of the data obtained using the LightCycler® 1.2 (Table 2.14), the crossing point for both targeted gene was 0.20% for intra-assay CV and 0.63% for inter-assay CV in the case of DNA extracted from pure cultures. In comparison, for spiked foods an intra-assay CV of 0.32% and inter assay of 0.70% CV were obtained.

**Table 2.14.** Reproducibility data of the duplex SYBR Green-based real-time PCR.

| | | DNA Samples[d] | |
|---|---|---|---|
| | | **Pure cultures** | **Spiked foods** |
| **CV (%)[a] Intra-assay[b]** | *Ct* | 0.20 | 0.32 |
| | $T_M$ | 0.04 | 0.08 |
| **CV (%)[a] Inter-assay[c]** | *Ct* | 0.63 | 0.70 |
| | $T_M$ | 0.19 | 0.20 |

[a] The CV was calculated by dividing SD by the mean Ct and $T_M$ values obtained by measurement

[b] Mean CVs calculated from the replicates measured for both targeted genes

[c] Mean CVs calculated from data obtained in three separate PCR runs. In each run, the mean of the triplicates corresponding to both targeted genes was used as the value for the Ct and $T_M$ from each run.

[d] For comparison purposes according to the nature of the sample, CVs were calculated in the case of DNA extracted directly from a pure culture (1 ng / 2 µl) and DNA extracted from 1 ml of enriched food sample (cottage cheese).

Similar CV% for intra-assay and inter-assay crossing points were observed for both targeted genes. In contrast, the intra-assay CV obtained for $T_M$ was lowest using pure cultures, which confirmed a satisfactory degree of precision within the same assay when defining $T_M$ data. Moreover, the CV obtained for $T_M$ was much lower than that obtained for inter-assay Ct for both types of samples. Because of the relatively

small error between assays in the determination of $T_M$ values the duplex real-time PCR system appeared to be a reproducible analytical method.

In the current study, an evaluation of two commercial kits from Biotecon Diagnostics's range showed advantages in using these systems for the detection of *L. monocytogenes* and *S. aureus*. While the kits are easy to use, rapid, reliable and highly specific the high cost per reaction may preclude their widespread use in the food industry. In this study combining optimal primer sets in a duplex real-time PCR platform was shown to allow simultaneous detection of *L. monocytogenes* and *S. aureus* with low sensitivities found in minimally enriched food products. SYBR Green I identified two different amplification products by melting curve analysis in spiked samples with detection limits of 2 CFU / g in minced beef spiked with *S. aureus* and 7 CFU / g in coleslaw spiked with *L. monocytogenes*. BPW recovered both pathogens in foods in 18h and food analysis including DNA extraction and duplex real-time PCR took ~1 h 30 min to 2 h depending on the number of samples for analysis. This multiplex real-time PCR method provides a two in one screening solution to detect positive samples of *S. aureus* or *L. monocytogenes* in minimally enriched food samples. The main limitation is that it can exclude simultaneously native samples of *S. aureus* and *L. monocytogenes*. In the presence of both microorganisms in the same sample, only *S. aureus* will be detected using SYBR Green in duplex real-time PCR. However while double contaminations of this type cannot be excluded, they remain rare in foods.

## 4. CONCLUSION

In conclusion, the newly developed method may be validated as an alternative analytical method to be widely accepted. On this aspect, inclusivity and exclusivity of the method may be tested over a wider range of strains among foodborne and non-foodborne pathogens, including *L. monocytogenes* and *S. aureus* subspecies. An appropriate internal amplification control may be required as a quality control feature for the uptake of the assay by industry. Interlaboratory studies may be conducted in the analysis of defined food samples comparing ISO methods versus alternative method and using different real-time PCR devices in order to confirm the application of this duplex method.

# 5. REFERENCES

Alarcon, B., Vicedo, B. and Aznar, R. (2006) 'PCR-based procedures for detection and quantification of *Staphylococcus aureus* and their application in food', *Journal of Applied Microbiology,* 100(2), 352-364.

Aldea, C., Alvarez, C. P., Folgueira, L., Delgado, R. and Otero, J. R. (2002) 'Rapid detection of herpes simplex virus DNA in genital ulcers by real-time PCR using SYBR green I dye as the detection signal', *Journal of Clinical Microbiology,* 40(3), 1060-1062.

Amagliani, G., Brandi, G., Omiccioli, E., Casiere, A., Bruce, I. J. and Magnani, M. (2004) 'Direct detection of *Listeria monocytogenes* from milk by magnetic based DNA isolation and PCR', *Food Microbiology,* 21(5), 597-603.

Anonymous (1999) 'ISO 6887-1:1999 Microbiology of food and animal feeding stuffs -- Preparation of test samples, initial suspension and decimal dilutions for microbiological examination -- Part 1: General rules for the preparation of the initial suspension and decimal dilutions'.

Anonymous (2002) 'ISO 8261:2002 Milk and milk products -- General guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiological examination, Geneva, Switzerland'.

Anonymous (2003a) 'ISO 6887-2:2003 Microbiology of food and animal feeding stuffs -- Preparation of test samples, initial suspension and decimal dilutions for microbiological examination -- Part 2: Specific rules for the preparation of meat and meat products'.

Anonymous (2003b) 'ISO 6887-4:2003 Microbiology of food and animal feeding stuffs -- Preparation of test samples, initial suspension and decimal dilutions for microbiological examination -- Part 4: Specific rules for the preparation of products other than milk and milk products, meat and meat products, and fish and fishery products'.

Anonymous (2003c) 'ISO 6888-3:2003 Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) -- Part 3: Detection and MPN technique for low numbers, Geneva, Switzerland'.

Anonymous (2003d) 'ISO 16140:2003 Microbiology of food and animal feeding stuffs -- Protocol for the validation of alternative methods, Geneva, Switzerland'.

Anonymous (2004) 'ISO 11290-1:2004 Microbiology of food and animal feeding stuffs -- Horizontal method for the detection and enumeration of *Listeria monocytogenes*, Geneva, Switzerland'.

Anonymous (2005) 'Commission Regulation (EC) No 2073/2005 of 15 November, 2005 on microbiological criteria for foodstuffs',

Anonymous (2007a) 'foodproof® *Listeria monocytogenes* detection kit - Hybridization Probes (LC 1.x, 2.0) - Instruction manual - Biotecon Diagnostics GmbH, Potsdam, Germany'.

Anonymous (2007b) 'Hygiene Screening System (*Staphylococcus, Micrococcus, Corynebacterium*) - Hybridization Probes (LC 2.0) - Biotecon Diagnostics GmbH, Potsdam, Germany'.

Anonymous (2009) 'Detecting x-fold differences in template concentration - Roche Diagnostics', [online], available: https://www.roche-applied-science.com/techresources/techpage.jsp?page=/techresources/tr_content/tr_010100.htm [accessed 24th December 2009].

Anonymous. (2010) Microval Certificate of Compliance for the foodproof® Enterobacteriaceae plus *E. sakazakii* (*Cronobacter sakazakii*) Detection Kit, 5'-nuclease (Art No R302 15) for detection of Cronobacter spp. (*Enterobacter sakazakii*) in combination with the foodproof StarPrep One kit (Art no S 400 07) and Reagent D (Art No 500 02). In. Rotterdam, The Netherlands: Lloyd's Register Quality Assurance.

Aznar, R. and Alarcón, B. (2002) 'On the Specificity of PCR Detection of Listeria monocytogenes in Food: a Comparison of Published Primers', *Systematic and Applied Microbiology,* 25(1), 109-119.

Bansal, N. S. (1996) 'Development of a polymerase chain reaction assay for the detection of Listeria monocytogenes in foods', *Letters in Applied Microbiology,* 22(5), 353-356.

Barkham, T. (2004) 'Internal amplification control for PCR should not be mandatory in the clinical medical environment', *Journal of Clinical Microbiology,* 42(7), 3379-3379.

Besse, N. G., Audinet, N., Kerouanton, A., Colin, P. and Kalmokoff, M. (2005) 'Evolution of *Listeria* populations in food samples undergoing enrichment culturing', *International Journal of Food Microbiology,* 104(2), 123-134.

Beumer, R. R., teGiffel, M. C., Anthonie, S. V. R. and Cox, L. J. (1996) 'The effect of acriflavine and nalidixic acid on the growth of *Listeria spp* in enrichment media', *Food Microbiology,* 13(2), 137-148.

Biotecon (2007) 'foodproof® *Listeria monocytogenes* detection kit - Hybridization Probes (LC 1.x, 2.0) - Instruction manual',

Brakstad, O. G., Aasbakk, K. and Maeland, J. A. (1992) 'Detection of *Staphylococcus aureus* by Polymerase Chain Reaction, Amplification of the *nuc* gene', *Journal of Clinical Microbiology,* 30(7), 1654-1660.

Candrian, U. (1995) 'Polymerase Chain Reaction in Food Microbiology', *Journal of Microbiological Methods,* 23(1), 89-103.

Chiang, Y. C., Fan, C. M., Liao, W. W., Lin, C. K. and Tsen, H. Y. (2007) 'Real-time PCR detection of *Staphylococcus aureus* in milk and meat using new primers designed from the heat shock protein gene *htrA* sequence', *Journal of Food Protection,* 70(12), 2855-2859.

Choudhurry, R. S. R., Melles, D. C., Eadie, K., Vos, M., Wertheim, H. F. L., Verbrugh, H. A., van Belkum, A. and van Leeuwen, W. B. (2006) 'Direct detection of human *Staphylococcus aureus* carriage in the nose using the Lightcycler *Staphylococcus* kit', *Journal of Microbiological Methods,* 65(2), 354-356.

Ciglenecki, U. J., Grom, J., Toplak, I., Jemersic, L. and Barlic-Maganja, D. (2008) 'Real-time RT-PCR assay for rapid and specific detection of classical swine fever virus: Comparison of SYBR Green and TaqMan MGB detection methods using novel MGB probes', *Journal of Virological Methods,* 147(2), 257-264.

Curiale, M. S. and Lewus, C. (1994) 'Detection of *Listeria monocytogenes* in samples containing *Listeria innocua*', *Journal of Food Protection,* 57(12), 1048-1051.

Domann, E., Leimeisterwachter, M., Goebel, W. and Chakraborty, T. (1991) 'Molecular Cloning, Sequencing, and Identification of a Metalloprotease Gene from *Listeria monocytogenes* that is species specific and physically linked to the Listeriolysin gene', *Infection and Immunity,* 59(1), 65-72.

Donnelly, C. W. (2002) 'Detection and isolation of *Listeria monocytogenes* from food samples: Implications of sublethal injury', *Journal of Aoac International,* 85(2), 495-500.

Duffy, G., Walsh, D., Sheridan, J. J., Logue, C. M., Harrington, D., Blair, I. S. and McDowell, D. A. (2001) 'Comparison of selective and non-selective enrichment media in the detection of *Listeria monocytogenes* from meat containing *Listeria innocua*', *Journal of Applied Microbiology,* 90(6), 994-999.

Duh, Y. H. (1993) 'Modelling the Effect of Temperature on the Growth rate and Lag Time of *Listeria innocua* and *Listeria monocytogenes*', *Journal of Food Protection,* 56(8), 734-734.

Elizaquivel, P. and Aznar, R. (2008) 'A multiplex RTi-PCR reaction for simultaneous detection of *Escherichia coli* O157 : H7, *Salmonella spp.* and *Staphylococcus aureus* on fresh, minimally processed vegetables', *Food Microbiology,* 25(5), 705-713.

Fan, W., Hamilton, T., Webster-Sesay, S., Nikolich, M. P. and Lindler, L. E. (2007) 'Multiplex real-time SYBR Green I PCR assay for detection of tetracycline efflux genes of Gram-negative bacteria', *Molecular and Cellular Probes,* 21(4), 245-256.

Fernandez, F., Gutierrez, J., Sorlozano, A., Romero, J. M., Soto, M. J. and Ruiz-Cabello, F. (2006) 'Comparison of the SYBR Green and the hybridization probe format for real-time PCR detection of HHV-6', *Microbiological Research,* 161(2), 158-163.

Fukushima, H., Tsunomori, Y. and Seki, R. (2003) 'Duplex real-time SYBR green PCR assays for detection of 17 species of food- or waterborne pathogens in stools', *Journal of Clinical Microbiology,* 41(11), 5134-5146.

Giglio, S., Monis, P. T. and Saint, C. P. (2003) 'Demonstration of preferential binding of SYBR Green I to specific DNA fragments in real-time multiplex PCR', *Nucleic Acids Research,* 31(22), 5.

Glynn, B., Lahiff, S., Wernecke, M., Barry, T., Smith, T. J. and Maher, M. (2006) 'Current and emerging molecular diagnostic technologies applicable to bacterial food safety', *International Journal of Dairy Technology,* 59(2), 126-139.

Gubala, A. J. (2006) 'Multiplex real-time PCR detection of *Vibrio cholerae*', *Journal of Microbiological Methods,* 65(2), 278-293.

Guilbaud, M., de Coppet, P., Bourion, F., Rachman, C., Prevost, H. and Dousset, X. (2005) 'Quantitative detection of Listeria monocytogenes in biofilms by real-time PCR', *Applied and Environmental Microbiology,* 71(4), 2190-2194.

Gundry, C. N., Vandersteen, J. G., Reed, G. H., Pryor, R. J., Chen, J. and Wittwer, C. T. (2003) 'Amplicon melting analysis with labelled primers: A closed-tube method for differentiating homozygotes and heterozygotes', *Clinical Chemistry,* 49(3), 396-406.

Hein, I., Lehner, A., Rieck, P., Klein, K., Brandl, E. and Wagner, M. (2001) 'Comparison of different approaches to quantify *Staphylococcus aureus* cells by real-time quantitative PCR and application of this technique for examination of cheese', *Applied and Environmental Microbiology,* 67(7), 3122-3126.

Hoorfar, J. (2004) 'Internal amplification control for PCR should not be mandatory in the clinical medical environment - Reply', *Journal of Clinical Microbiology,* 42(7), 3379-3380.

Ikeda, T., Tamate, N., Yamaguchi, K. and Makino, S. I. (2005) 'Quantitative analysis of *Staphylococcus aureus* in skimmed milk powder by real-time PCR', *Journal of Veterinary Medical Science,* 67(10), 1037-1041.

Jasson, V., Jacxsens, L., Luning, P., Rajkovic, A. and Uyttendaele, M. (2010) 'Alternative microbial methods: An overview and selection criteria', *Food Microbiology,* 27(6), 710-730.

Junge, B. and Berghof-Jager, K. (2006) 'Roche/BIOTECON Diagnostics LightCycler foodproof *L. monocytogenes* detection kit in combination with ShortPrep foodproof II kit - Performance-Tested Method(SM) 070401', *Journal of Aoac International,* 89(2), 374-398.

Kim, C. H., Khan, M., Morin, D. E., Hurley, W. L., Tripathy, D. N., Kehrli, M., Oluoch, A. O. and Kakoma, I. (2001) 'Optimization of the PCR for detection of *Staphylococcus aureus nuc* gene in bovine milk', *Journal of Dairy Science,* 84(1), 74-83.

Kim, J. S., Lee, G. G., Park, J. S., Jung, Y. H., Kwak, H. S., Kim, S. B., Nam, Y. S. and Kwon, S. T. (2007) 'A novel multiplex PCR assay for rapid and simultaneous detection of five pathogenic bacteria: *Escherichia coli* O157 : H7, *Salmonella*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Vibrio parahaemolyticus*', *Journal of Food Protection,* 70(7), 1656-1662.

Kobayashi, H., Kubota, J., Fujihara, K., Honjoh, K., Ito, M., Fujiki, N., Nakabe, M., Oda, S., Satoyama, T., Takasu, K., Nakanishi, H. and Miyamoto, T. (2009) 'Simultaneous Enrichment of *Salmonella spp*, *Escherichia coli* O157:H7, *Vibrio parahaemolyticus*, *Staphylococcus aureus*, *Bacillus cereus*, and *Listeria monocytogenes* by Single Broth and Screening of the Pathogens by Multiplex Real-time PCR', *Food Science and Technology Research,* 15(4), 427-438.

Kugelman, G., Tapsall, J. W., Goire, N., Syrmis, M. W., Limnios, A., Lambert, S. B., Nissen, M. D., Sloots, T. P. and Whiley, D. M. (2009) 'Simple, Rapid, and Inexpensive Detection of *Neisseria gonorrhoeae* Resistance Mechanisms Using Heat-Denatured Isolates and SYBR Green-Based Real-Time PCR', *Antimicrobial Agents and Chemotherapy,* 53(10), 4211-4216.

Kumar, T. D. K., Murali, H. S. and Batra, H. V. (2009) 'Simultaneous detection of pathogenic *B. cereus*, *S. aureus* and *L. monocytogenes* by multiplex PCR', *Indian Journal of Microbiology,* 49(3), 283-289.

Lee, S. H., Jung, B. Y., Rayamahji, N., Lee, H. S., Jeon, W. J., Choi, K. S., Kweon, C. H. and Yoo, H. S. (2009) 'A multiplex real-time PCR for differential detection and quantification of *Salmonella spp.*, *Salmonella enterica* serovar *Typhimurium* and *Enteritidis* in meats', *Journal of Veterinary Science,* 10(1), 43-51.

Macdonald, F. and Sutherland, A. D. (1994) 'Important Differences between the Generation Times of *Listeria monocytogenes* and *Listeria innocua* in two *Listeria* Enrichment Broths', *Journal of Dairy Research,* 61(3), 433-436.

Malorny, B., Tassios, P. T., Radstrom, P., Cook, N., Wagner, M. and Hoorfar, J. (2003) 'Standardization of diagnostic PCR for the detection of foodborne pathogens', *International Journal of Food Microbiology,* 83(1), 39-48.

Mlynarczyk, A., Mlynarczyk, G. and Jeljaszewicz, J. (1998) 'The genome of *Staphylococcus aureus*: A review', *Zentralblatt Fur Bakteriologie-International Journal of Medical Microbiology Virology Parasitology and Infectious Diseases,* 287(4), 277-314.

Nakken, S., Aussedat, O., Kristoffersen, A. B., Holst-Jensen, A. and Tengs, T. (2009) 'UniquePrimer - a web utility for design of specific PCR primers and probes', *Annals of Microbiology,* 59(2), 391-393.

Nelson, K. E., Fouts, D. E., Mongodin, E. F., Ravel, J., DeBoy, R. T., Kolonay, J. F., Rasko, D. A., Angiuoli, S. V., Gill, S. R., Paulsen, I. T., Peterson, J., White, O., Nelson, W. C., Nierman, W., Beanan, M. J., Brinkac, L. M., Daugherty, S. C., Dodson, R. J., Durkin, A. S., Madupu, R., Haft, D. H., Selengut, J., Van Aken, S., Khouri, H., Fedorova, N., Forberger, H., Tran, B., Kathariou, S., Wonderling, L. D., Uhlich, G. A., Bayles, D. O., Luchansky, J. B. and Fraser, C. M. (2004) 'Whole genome comparisons of serotype 4b and 1/2a strains of the food-borne pathogen *Listeria monocytogenes* reveal new insights into the core genome components of this species', *Nucleic Acids Research,* 32(8), 2386-2395.

Nogva, H. K., Rudi, K., Naterstad, K., Holck, A. and Lillehaug, D. (2000) 'Application of 5 '-nuclease PCR for quantitative detection of *Listeria monocytogenes* in pure cultures, water, skim milk, and unpasteurized whole milk', *Applied and Environmental Microbiology,* 66(10), 4266-4271.

O'Grady, J., Ruttledge, M., Sedano-Balbas, S., Smith, T. J., Barry, T. and Maher, M. (2009) 'Rapid detection of *Listeria monocytogenes* in food using culture enrichment combined with real-time PCR', *Food Microbiology,* 26(1), 4-7.

Omiccioli, E., Amagliani, G., Brandi, G. and Magnani, M. (2009) 'A new platform for Real-Time PCR detection of *Salmonella spp.*, *Listeria monocytogenes* and *Escherichia coli* O157 in milk', *Food Microbiology,* 26(6), 615-622.

Oravcova, K., Trncikova, T. and Kaclikova, E. (2007) 'Comparison of three real-time PCR-based methods for the detection of *Listeria monocytogenes* in food', *Journal of Food and Nutrition Research,* 46(2), 63-67.

Oravcova, K., Trncikova, T., Kuchta, T. and Kaclikova, E. (2008) 'Limitation in the detection of *Listeria monocytogenes* in food in the presence of competing *Listeria innocua*', *Journal of Applied Microbiology,* 104(2), 429-437.

Pinto, B., Chenoll, E. and Aznar, R. (2005) 'Identification and typing of food-borne *Staphylococcus aureus* by PCR-based techniqnes', *Systematic and Applied Microbiology,* 28(4), 340-352.

Rip, D. and Gouws, P. A. (2009) 'Development of an Internal Amplification Control Using Multiplex PCR for the Detection of *Listeria monocytogenes* in Food Products', *Food Analytical Methods,* 2(3), 190-196.

Ririe, K. M., Rasmussen, R. P. and Wittwer, C. T. (1997) 'Product differentiation by analysis of DNA melting curves during the polymerase chain reaction', *Analytical Biochemistry,* 245(2), 154-160.

Rossmanith, P., Krassnig, M., Wagner, M. and Hein, I. (2006) 'Detection of Listeria monocytogenes in food using a combined enrichment/real-time PCR method targeting the prfA gene', *Research in Microbiology,* 157(8), 763-771.

Scheu, P., Gasch, A. and Berghof, K. (1999) 'Rapid detection of Listeria monocytogenes by PCR-ELISA', *Letters in Applied Microbiology,* 29(6), 416-420.

Scheu, P. M., Berghof, K. and Stahl, U. (1998) 'Detection of pathogenic and spoilage micro-organisms in food with the polymerase chain reaction', *Food Microbiology,* 15(1), 13-31.

Shi, X. M., Long, F. and Suo, B. 'Molecular methods for the detection and characterization of foodborne pathogens', *Pure and Applied Chemistry,* 82(1), 69-79.

Trncikova, T., Hruskova, V., Oravcova, K., Pangallo, D. and Kaclikova, E. (2009) 'Rapid and Sensitive Detection of *Staphylococcus aureus* in Food Using Selective Enrichment and Real-Time PCR Targeting a New Gene Marker', *Food Analytical Methods,* 2(4), 241-250.

Walsh, D., Duffy, G. and Sheridan, J. J. (1998) 'Comparison of selective and nonselective enrichment media for the isolation of *Listeria* species from retail foods', *Journal of Food Safety,* 18(2), 85-99.

Wang, X. W., Jothikumar, N. and Griffiths, M. W. (2004) 'Enrichment and DNA extraction protocols for the simultaneous detection of *Salmonella* and *Listeria monocytogenes* in raw sausage meat with multiplex real-time PCR', *Journal of Food Protection,* 67(1), 189-192.

Wu, L. C., Horng, J. T., Huang, H. Y., Lin, F. M., Huang, H. D. and Tsai, M. F. (2007) 'Primer design for multiplex PCR using a genetic algorithm', *Soft Computing,* 11(9), 855-863.

Zhang, D., Zhang, H., Yang, L., Guo, J., Li, X. and Feng, Y. (2009) 'Simultaneous Detection of *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enterica* and *Escherichia coli* O157:H7 in Food Samples using Multiplex PCR Method', *Journal of Food Safety,* 29(3), 348-363.

# CHAPTER THREE

## Comparison of in-house and commercial real-time PCR systems for the detection of Enterobacteriaceae and their evaluation within an interlaboratory study using infant formula samples[2]

[2] The work described in this chapter has been published in *Food Analytical Methods* (Martinon A., Cronin U. and Wilkinson M., 2011), "Comparison of in-house and commercial real-time PCR systems for the detection of Enterobacteriaceae and their evaluation within an interlaboratory study using infant formula samples", DOI 10.1007/s12161-010-9188-7 (Appendix Two).

## ABSTRACT

Traditional detection methods for *Enterobacteriaceae* in foods are time-consuming and laborious. The current study assessed the specificity of three real-time PCR primer sets. Set A (*IEC* primers) targeted the conserved flanking regions of the 16S rRNA, the 16S-ITS-23S gene region. Set B (*ENT* primers) annealed to *Escherichia coli* 16S ribosomal RNA gene. The third set (C) used a D-LUX™ (Light Upon eXtension) single FAM-labelled forward primer and a corresponding unlabeled primer. Set A was specific for *E. coli* and for some non-Enterobacteriaceae. SYBR Green-based real-time PCR confirmed the specificity of set B for the Enterobacteriaceae but also detected Vibrionaceae. In contrast, set C was poorly specific. However, set D including the forward LUX™ primer from set C and the reverse primer from set B had a specificity comparable to that of set B, but with higher sensitivity. This combined set was successfully applied to detect Enterobacteriaceae in infant milk formula and compared favourably with a commercial real-time PCR kit.

# 1. INTRODUCTION

The Enterobacteriaceae family is composed of widely studied microorganisms and includes several species such as *Escherichia coli, Klebsiella pneumoniae* or *Salmonella* Typhimurium which are responsible for food intoxications (Blood and Curtis, 1995). Routine monitoring of Enterobacteriaceae serves as a hygiene indicator within food processing plants and their presence typically signifies poor cleaning procedures for process surfaces or post-processing contamination of heat-processed foods. To date, most quality assurance laboratories use agar-based ISO procedures (de Boer, 1998) in order to detect and quantify Enterobacteriaceae in food products or swab samples. Combined with enrichment steps, Violet Red Bile Glucose Agar (VRBGA) has been among the most popular media for detecting Enterobacteriacaeae in foods. However, this medium is recognised as having some shortcomings (Baylis, 2006) as other strains like *Aeromonas* spp. can also grow. Consequently, the colonies that appear on VRBGA are qualified as presumptive with further confirmatory tests required. Overall, this method can take five to seven days for a definitive result which is not satisfactory for allowing rapid product release. Therefore, it is of commercial interest to accelerate this procedure by investigating alternative rapid DNA-based methods.

Detection of the Enterobacteriaceae by conventional PCR has been previously reported. Bayardelle and Zafarullah (2002) developed PCR protocols for detection of the most frequent species of the Enterobacteriaceae in blood, urine and water samples using primer sets targeting the wec gene cluster involved in the synthesis of the enterobacterial common antigen. Real-time PCR has been widely accepted because of rapidity, sensitivity, reproducibility and reduced carry-over contamination (Mackay *et al.*, 2007e). Real-time PCR protocols have also been developed and applied in food samples for the detection of Enterobacteriaceae (Nakano *et al.*, 2003; Qiu *et al.*, 2009). Nakano *et al.* (2003) used specific primers and SYBR Green I as a detection format. Currently, the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit – 5'Nuclease (Biotecon, Potsdam, Germany) uses TaqMan® probes for the qualitative detection of the Enterobacteriaceae and *Cronobacter sakazakii* particularly in infant formula.

The LUX™ (Light Upon eXtension) primer system is another commercially available tool for detection of pathogens by real-time PCR. Such primers are labelled with a single fluorophore near the 3'-end in a hairpin structure that intrinsically

quenches the fluorescence. When incorporated into double-stranded DNA, the fluorophore is de-quenched, resulting in a significant increase of fluorescence signal. The LUX™ primers are software-designed by entering the targeted DNA sequence and subsequently a range of primers are proposed for use.

The LUX™-based real-time PCR has been recognised as a cost-effective alternative to other fluorescence-based techniques (Nazarenko *et al.*, 2002). Such fluorogenic PCR has the potential to be routinely used in food industries because of rapidity, simplicity and lower cost compared with real-time PCR systems using probes for instance. LUX™ primers have been designed for use in a number of studies mainly in virology (Aitichou *et al.*, 2005; Antal *et al.*, 2007; Chen *et al.*, 2004; Nordgren *et al.*, 2008; Slavov *et al.*, 2008). However, some applications in bacteriology have been reported (Balcazar *et al.*, 2007; Kunchev *et al.*, 2007; McCrea *et al.*, 2007; S. L. Mitchell *et al.*, 2009; R.-s. Xu *et al.*, 2008).

The use of LUX™ primers for the detection of Enterobacteriaceae in food samples has not been assessed in detail and it was of interest to evaluate their potential as a new molecular tool of analysis.

In this study, the specificity of three separate PCR primer sets was evaluated for the detection of the Enterobacteriaceae. Set A (*IEC* primers) consisted of classic primers, previously tested by Maheux *et al.* (2009) and Khan *et al*. (2007), and evaluated in this study using conventional PCR. Set B (*ENT* primers) developed by Nakano *et al.* (2003) was evaluated in this study by PCR and real-time PCR, using SYBR Green I as a detection format. The LUX™ primers (set C) were designed online using the 16S ribosomal RNA gene of *E. coli*, a sequence previously used by Nakano *et al.* (2003) for the design of *ENT* primers. Set C was tested by real-time PCR. Finally, the forward primer from set C and the reverse primer from set B were combined to obtain set D and evaluated for their specificity for detection of the Enterobacteriaceae. All primer sets were tested in parallel using Primer Blast available on the National Centre for Biotechnology Information website. Subsequently, the combined primer set (primer set D) was evaluated on infant formula samples and compared with a commercial kit as part of an inter-laboratory study.

# 2. MATERIALS AND METHODS

## 2.1. Assessment of in-house and commercial real-time PCR methods using pure cultures

### 2.1.1. Bacterial strains

Type strains of Enterobacteriaceae, *E. coli* (ATCC 11775), *Enterobacter aerogenes* (ATCC 13048), *Erwinia persicina* (ATCC 1381), *K. pneumoniae* (ATCC 700603), *S.* Typhimurium (ATCC 13311), *Serratia marcescens* (ATCC 13880), *Shigella flexneri* (ATCC 9199), *Y. enterocolitica* (ATCC 9610); *A. hydrophila* (ATCC 7966), *Campylobacter jejuni* (ATCC 29428), *Listeria monocytogenes* (ATCC 19115) and *Vibrio parahaemolyticus* (ATCC 17802) were obtained from MicroBioLogics Inc, Saint Cloud, USA. *C. sakazakii*, *Enterobacter cloacae*, *Pseudomonas aeruginosa* and *Proteus mirabilis* isolates were obtained from the culture collection at the Department of Life Sciences, University of Limerick. *Staphylococcus aureus* (NCTC 8325) was obtained from the National Collection of Type Cultures (Health Protection Agency Culture Collections, Salisbury, UK). All strains were stored on Protect™ beads 109 (LangenBach Services Ltd, Dublin, Ireland) at – 20°C until cultivation.

### 2.1.2. Culture conditions

All Enterobacteriaceae were grown overnight on nutrient agar (NA; Oxoid Ltd, Basingstoke, UK) at 37˚C, except *E. persicina* which was incubated at 30˚C. *L. monocytogenes* and *S. aureus* were incubated at 37˚C overnight on NA. *V. parahaemolyticus* was grown overnight at 35˚C on tryptic soya agar (TSA; Oxoid Ltd). *A. hydrophila* was grown overnight at 35˚C on Columbia blood agar (Oxoid Ltd). *P. aeruginosa* was incubated overnight on NA at 25˚C. *C. jejuni* was grown on Columbia blood agar in a microaerophilic environment (CampyGen 2.5 l, Oxoid Ltd) for 72 h at 37°C. Prior to DNA extraction, a preculture of each strain was made by inoculating one loopful of each culture into a flask of 30 ml of nutrient broth (NB; Oxoid Ltd) with overnight shaking at 37˚C for the Enterobacteriaceae and *S. aureus*. Tryptic Soya Broth (TSB; Oxoid Ltd) was used for growth of *L. monocytogenes* (37˚C), the *Vibrionaceae* (35˚C), and *P. aeruginosa* (25˚C). Following growth, 300 µl of the preculture was transferred into 30 ml of fresh broth. A culture of each strain was obtained under the conditions outlined above and grown to exponential growth

phase. *C. jejuni* colonies grown on a Columbia agar plate were suspended in 1 ml of 0.85% saline sterile water prior to DNA extraction.

### 2.1.3. DNA extractions

DNA used for PCR and real-time PCR experiments was extracted following manufacturer's instructions for Gram negative or Gram positive bacteria using the DNeasy Tissue Kit (Qiagen, Crawley, UK) based on DNA purification through chromatography columns. For each strain, DNA was extracted from pellets obtained from cultures in the exponential growth phase following centrifugation of 1 ml of a bacterial suspension at 5000 x *g* for 10 min. DNA quantifications were performed by spectrophotometry at 260 nm using a Nanodrop Spectrophotometer ND-1000 (Thermo Scientific, Wilmington, USA). Finally, DNA concentrations were adjusted to 1 ng per 2 µl. The sensitivity of DNA detection was determined by diluting *E. coli* DNA, assuming that the targeted gene is present in 7 copies in the *E. coli* genome according to the Ribosomal RNA Operon Copy Number Database (Klappenbach *et al.*, 2001), and using 4990 kb as the size of the *E. coli* genome (Bergthorsson and Ochman, 1995). Therefore 1 ng of DNA was calculated to equal 2.6 x $10^4$ genomes.

### 2.1.4. Commercial real-time PCR detection

Enterobacteriaceae were detected using the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit – 5'Nuclease which allows rapid detection of Enterobacteriaceae and simultaneous identification of *C. sakazakii.* A 25 µl-reaction included 18 µl of Master mix containing the primers and the Hydrolysis Probes, 1 µl of foodproof® Enzyme solution and 1 µl of foodproof® IC (Internal Control) and 5 µl of sample DNA. A positive control was used for each experiment where the DNA template was replaced by the DNA from the foodproof® Control Template. The reaction purity and cross-contamination were checked by adding a negative control consisting of PCR grade water, in place of the sample. Experiments were carried out using an AB 7900 HT (Applied BioSystems Inc, Foster City, USA) or a LightCycler® 480 II (Roche Diagnostics GmbH, Mannheim, Germany). The program settings included: a single pre-incubation step: 37°C for 4 min and 95°C for 5 min, an amplification step of 40 cycles: 95°C for 10 s, 65°C for 70 s with a step down at each cycle by 0.1°C. A Hydrolysis Probe binds specifically to the IC, allowing detection in

the ROX/Texas Red channel with the AB 7900 HT. *C. sakazakii* DNA is detected in the FAM channel and the *Enterobacteriaceae* DNA is detected in the VIC/HEX.

### 2.1.5. In house methods of PCR and real-time PCR detection

#### *2.1.5.1. Primer selection*

Different primer sets were compared for their specificity for the detection of Enterobacteriaceae. All primers were purchased from MWG Eurofins Operon (Ebersberg, Germany). The specificity of the primer sets was tested using *in silico* PCR analysis against complete genome sequences of Enterobacteriaceae and non-Enterobacteriaceae. All sequences were provided by the National Centre for Biotechnology Information (NCBI) nucleotide database. The Primer BLAST (Basic Local Alignment Search Tool) program, available on http://www.ncbi.nlm.nih.gov/tools/primer-blast/, was used for simulated PCR with the primer sets for the detection the Enterobacteriaceae. This software allows searches for primer annealing sites on DNA sequences using the BLAST algorithm which is a segment-to-segment alignment principle.

Primer sets were named sets A, B, C and D (Table 3.15).

**Table 3.15.** Primers used in PCR and real-time PCR.

| Genetic target | Set | Primer | Primer Sequence | a | b | Reference |
|---|---|---|---|---|---|---|
| 16S rRNA - ITS - 23S rRNA | A | IEC – F[c] | 5′-CAATTTTCGTGTCCCCTTCG-3′ | 57 | 450 | Khan *et al*., 2007 Maheux *et al*., 2009 |
| | | IEC – R[d] | 5′-GTTAATGATAGTGTGTCGAAAC-3′ | | | |
| 16S rRNA | B | ENT – F | 5'-GTTGTAAAGCACTTTCAGTGGTGAGGAAGG-3' | 59 | 424 | Nakano *et al*., 2003 |
| | | ENT – R | 5'-GCCTCAAGGGCACAACCTCCAAG-3' | | | |
| | C | LUX – F | 5'-CGGTGTACCCGCAGAAGAAGCAC[FAM]G-3' | 55 | 69 | This study, generated by D-LUX™ Software |
| | | LUX – R | 5'-GCTTGCACCCTCCGTATTACC-3' | | | |
| | D | LUX – F | 5'-CGGTGTACCCGCAGAAGAAGCAC[FAM]G-3' | 55 | 368 | This study, primer combinations |
| | | ENT – R | 5'-GCCTCAAGGGCACAACCTCCAAG-3' | | | |

[a] Annealing temperature (°C)

[b] Amplicon size (bp)

[c] Forward

[d] Reverse

Set A or *IEC* primers were purported to target the conserved flanking regions of the 16S ribosomal RNA gene, the internal transcribed spacer region (ITS) and the 23 ribosomal RNA gene. Set B corresponded to *ENT* primers targeting the DNA sequence coding for *E. coli* 16S ribosomal RNA (accession number J01859). For set C, the *LUX*™ primers were provided by Invitrogen, Paisley, UK. These included a 6-carboxyfluorescein 3'-labelled forward primer. The online D-LUX Designer software was used for their design by specifying the *E. coli* 16S ribosomal RNA gene (accession number J01859) as the target sequence. For set D, the forward primer from set C and the reverse primer from set B were used in a new real-time PCR assay.

*2.1.5.2. PCR conditions*

For conventional PCR, mixes were prepared with the ready-to-use kit FastStart *Taq* DNA polymerase, dNTPack 5 U/µl purchased from Roche Diagnostics GmbH. On the basis of a final 50 µl reaction volume, the master mixture contained 48 µl of 4 mM $MgCl_2$ (2 mM from 10X PCR buffer and 2mM from $MgCl_2$ solution), 500 nM of forward primer, 500 nM of reverse primer, dNTP mixture and the FastStart *Taq* DNA Polymerase. 2 µl of DNA sample was added to each reaction. The PCR programmes were carried out as shown in Table 3.16. The specificity of the primer sets was tested against the bacterial strains described above.

**Table 3.16.** PCR and real-time PCR conditions using primer sets A, B, C and D.

| Program | PCR | SYBR Green-based real-time PCR | LUX™-based real-time PCR |
|---|---|---|---|
| Pre-Incubation | 95°C, 6 min | 95°C, 10 min | 50°C, 2 min |
| | | | 95°C, 2 min |
| Amplification | 28 cycles | 35 cycles | 35 cycles |
| | 95°C, 30 s | 95°C, 5 s | 94°C, 5 s |
| | [a]°C, 15 s | [a]°C, 10 s | 55°C, 10 s (Single) |
| | 72°C, 30s | 72°C, 20 s (Single) | 72°C, 10s |
| Melting | N/A[b] | 95°C, 0 s | 95°C, 0 s |
| | | 65°C, 10 s | 55°C, 15 s |
| | | 95°C, 0 s | 95°C, 0s |
| | | 0.2°C/s (Continuous) | 0.1°C/s (Continuous) |
| Cooling | N/A | 40°C, 30 s | 30°C, 30 s |

[a] specific to each primer set used, similar to conventional PCR annealing temperatures

[b] non applicable

PCR was performed on a G-Storm GS2 Thermal Cycler (Genetic Research Instrumentation Ltd, Braintree, UK). Each PCR product was subsequently run on a 2% agarose electrophoresis gel, stained using SYBR Safe™ (Molecular Probes, Eugene, USA) and visualized with a transilluminator G-BOX (Syngen, Frederick, USA) under UV light. The Perfect DNA™ 100 bp ladder (Novagen, Madison, USA) was used as a molecular marker.

### *2.1.5.3. Real-time PCR conditions*

When testing the *ENT* primers, conventional PCR procedures were adapted to a real-time procedure on a LightCycler® 1.2 (Roche Diagnostics GmbH), using LightCycler® FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH). In each capillary, a 20 µl reaction mix contained 1X concentration LightCycler® FastStart Reaction Mix SYBR Green; 4 mM $MgCl_2$; 500 nM concentration of each primer; and 2 µl of the template and carried out under conditions outlined in Table 3.16. Real-time DNA amplifications were observed in Channel 1 at 530 nm of the

LightCycler® 1.2. The *ENT* primers were also tested using a LightCycler® 480 with the same PCR mix in channel FAM/SYBR Green, except that the volume per reaction was 10 µl, using the same program settings.

The detection of Enterobacteriaceae using LUX™ primers (sets C and D) was first assayed with a LightCycler® 1.2, using glass capillaries. In each capillary, the 20 µl reaction mix contained 10 µl of Platinum® qPCR SuperMix-UDG (Invitrogen), 5 mM $MgCl_2$ (final concentration); 500 nM concentration of each primer; 1 µl of Bovine Serum Albumin; 0.12 µl of FastStart *Taq* DNA Polymerase (Roche Diagnostics GmbH); and 2 µl of the template. Real-time PCR conditions are shown in Table 3.16.

The LUX™ primers (sets C and D) were also evaluated on the LightCycler® 480 in channel FAM/SYBR Green with 10 µl reaction of the same PCR mix.

## 2.2. Analysis of Enterobacteriaceae in infant formula milk samples

An inter-laboratory study was performed across Europe as part of the MicroVal EN ISO 16140:2003 procedure for validation of an alternative method (Anonymous, 2003a). This study evaluated specifically the foodproof® kit for the detection of *Enterobacteriaceae* and *C. sakazakii* in powdered infant formula samples. The 40 samples of 100 g powdered infant formula in sterile stomacher bags were provided by RIKILT - Institute of Food Safety, Wageningen, The Netherlands. A blind study was undertaken using whereby all samples labeled EBES 1 to 40 with no further information provided to the participants.

Each sample was enriched with 900 ml pre-warmed Buffered Peptone Water (BPW; Bio Trading, Mijdrecht, The Netherlands) at 37°C and incubated for 18 h at 37°C. One milliliter of BPW culture was added to a separate tube containing 10 ml Enterobacteriaceae Enrichment broth (EE; Bio Trading) and incubated for 24 h at 37°C. Then, 50 µl of BPW culture were pipetted into 450 µl of fresh BPW and incubated for 3 h at 37°C. DNA from dead cells (100 µl of each 3 h culture) was eliminated by adding 300 µl of Reagent D (Biotecon Diagnostics, Potsdam, Germany) to remove DNA from dead bacterial cells and avoid false-positive PCR results. DNA was prepared following the manufacturer's instructions using a Start Prep One kit (Biotecon Diagnostics) by heating a cell pellet resuspended in 200 µl of the provided lysis buffer at 95°C.

In parallel, reference methods were used for comparison purposes with the suitable commercial and in-house real-time PCR systems.

For the detection of Enterobacteriaceae, the ISO 21528-1:2004 method was used (Anonymous, 2004f). A loopful of each EE broth tube was plated out on the selective isolation medium, VRBGA (Bio Trading). Characteristic colonies on VRBGA plates were pink to red or purple, with or without precipitation haloes. At least one representative colony from each VRBGA plate was subcultured onto NA plates incubated for 24 h at 37°C. An oxidase test was performed on each subculture and a colony from each plate was stabbed into a glucose agar tube for incubation 24 h at 37°C.

*C. sakazakii* was detected using the ISO/TS 22964:2006 (Anonymous, 2006a). After pre-enrichment of samples with BPW for 18 h at 37°C, 100 µl of each BPW pre-culture was transferred into a 10 ml tube of modified Lauryl Sulphate Tryptose broth, (mLST/v; Bio Trading) containing 0.1% of vancomycin and incubated for 24 h at 44°C. Each of the mLST/v broth tubes was plated out on a *C. sakazakii* Isolation Medium (CSIM; Bio Trading) and incubated for 24 h at 44°C. Typical colonies on CSIM plates appeared small to medium in size (1 – 3 mm) and were green to blue-green colonies. Atypical colonies were slightly transparent and violet colored. A presumptive colony from each plate was then subcultured on TSA (Bio Trading) and incubated for 48 h at 25°C. A single yellow pigmented colony from each TSA plate was tested on an ID 32 E System (Biomerieux, Craponne, France) for the identification of Enterobacteriaceae and other non-fastidious Gram-negative rods according to the manufacturer's instructions.

The Total Viable Count (TVC) was obtained following the ISO 4833:2003 method (Anonymous, 2003b). A 10 g reference powdered infant formula sample was dissolved into 90 ml of Peptone Physiological Salt (PFZ) (Bio Trading) and diluted to 1/100 dilution. 1 milliliter of each dilution (1/10, 1/100) was plated in duplicate with pre-melted Plate Count Agar (PCA; Bio Trading). The plates were incubated at 30°C for 72 h.

# 3. RESULTS AND DISCUSSION

## 3.1. Evaluation of the commercial detection kit

The occurrence of Enterobacteriaceae including *Salmonella spp*. (Cahill *et al.*, 2008), *Pantoea spp*, *E. hermanii*, *E. cloacae* (Estuningsih *et al.*, 2006) or *Klebsiella spp* (Gao *et al.*, 2010) has been previously demonstrated in infant formula milks. The occurrence of *C. sakazakii* is of major concern to infant formula manufacturers (Chap *et al.*, 2009; Drudy *et al.*, 2006; Giovannini *et al.*, 2008). In-house Real-time PCR systems have recently been developed for detection of *C. sakazakii* (Krascsenicsova *et al.*, 2008; Lehmacher *et al.*, 2007) or *K. pneumoniae* (Sun *et al.*, 2010) in infant powdered milks. In addition, the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit – 5'Nuclease on the LightCycler® 480 is also commercially available for these applications. As manufacturers of the commercial kit, Biotecon purport to have tested a range of 121 *C. sakazakii* strains and 120 non-*C. sakazakii* strains including various species. All *C. sakazakii* gave a signal in the FAM and VIC/HEX channels while all the non-*C. sakazakii* Enterobacteriaceae produced a signal in VIC/HEX channel only. For the detection of Enterobacteriaceae, this exclusivity was confirmed by Biotecon with more than 60 non-Enterobacteriaceae including most of the closely related genera of *Aeromonas* and *Vibrio*. None of the non-Enterobacteriaceae was detected in any channel. After an enrichment step, 1 to 10 cells per 25 to 100 g of relevant type of food sample could be detected.

In the present study, the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit – 5'Nuclease was assayed using DNA samples from Enterobacteriaceae and non-Enterobacteriaceae species. All the tested Enterobacteriaceae, including *P. mirabilis*, were detected in the VIC channel. *C. sakazakii* DNA had a Ct value equal to 18.77 whereas *K. pneumoniae* DNA was detected at a Ct equal to 20.73. In the FAM channel, the identification of *C. sakazakii* DNA was confirmed with a Ct equal to 19.90. Data obtained from real-time PCR on serial dilutions of copy numbers of *E. coli* DNA indicated a detection limit of 4 copies in a pure culture at a Ct equal to 30.08. This real-time PCR system was easy to use and appeared highly specific for the Enterobacteriaceae. To date, it is the only commercial kit available for the detection of Enterobacteriaceae by real-time PCR. In a single kit, 96 real-time PCR reactions can be performed; however the cost per reaction remains high. Therefore, if real-time PCR determinations are required to be performed in duplicate or triplicate to obtain

statistical data it may not be economically feasible to use this kit but this obviously depends on the particular end user application. For this reason, modifications to these actual protocols and in-house methods were investigated and some general cost comparisons per real-time PCR reaction were subsequently made.

## 3.2. Comparison of primer sets

Figure 3.17 shows the PCR products run on a SYBR Safe™ pre-stained 2% agarose electrophoresis gel obtained after PCR using set A. Among the range of species tested, these primers amplified only DNA from *E. coli,* a finding not in agreement with that of Maheux *et al.* (2009). These workers reported that the *IEC* primers, tested by Khan *et al.* (2007), amplified DNA belonging to species from the Enterobacteriaceae family that are phylogenetically relatively close to *E. coli* and *Shigella* spp. According to Khan *et al.* (2007), the ITS region allows discrimination of bacterial species, and the flanking highly conserved 16S and 23S ribosomal RNA genes can be targeted by primers for specific amplification of *E. coli* strains.

Data in the present study would support the findings of Khan *et al.* (2007) and would suggest that set A may not be suitable for detection of the entire Enterobacteriaceae family.

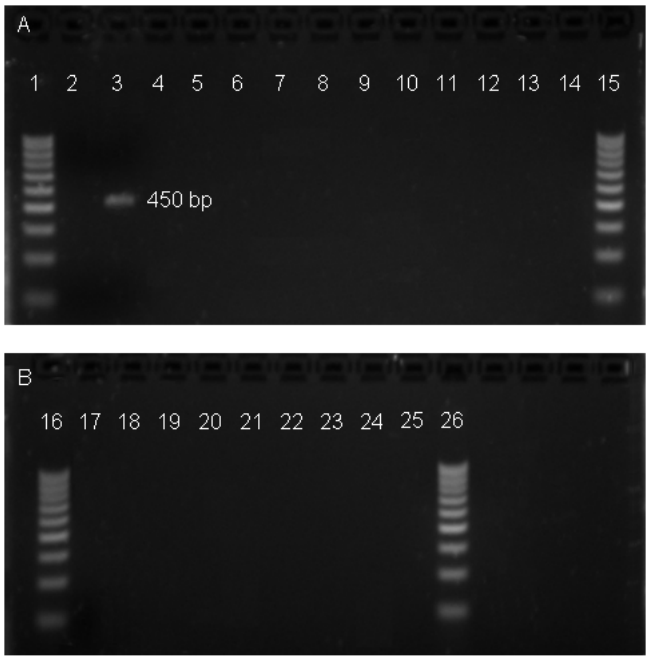


**Figure 3.17.** Amplification results obtained by PCR using the *IEC* primers (set A).
**A.** Lanes 1, 15, Perfect DNA™ 100 bp ladder ; lane 2, 17, negative control (no DNA template in the PCR reaction); lane 3, *E. coli* ; lane 4, *E. aerogenes* ; lane 5, *E. cloacae* ; lane 6, *C. sakazakii* ; lane 7, S. Typhimurium ; lane 8, *S. flexneri* ; lane 9, *K. pneumoniae* ; lane 10, *Y. enterocolitica* ; lane 11, *E. percisina* ; lane 12, *S. marcescens* ; lane 13, *P. mirabilis* ; lane 14, *V. parahaemolyticus*.
**B.** Lanes 16, 26, Perfect DNA™ 100 bp ladder lane 18, *A. hydrophila* ; lane 19, *P. aeruginosa* ; lane 20, *L. monocytogenes*; lane 21, *S. aureus* ; lane 22, *S. xylosus* ; lane 23, *Micrococcus spp* ; lane 24, *B. cereus* ; lane 25, *C. jejuni*.

*In silico* PCR analysis using Primer Blast confirmed this statement (Table 3.17). PCR procedures can be developed for the specific detection of *E. coli*, as described by Kahn *et al.* (2007). However, other species like *S. aureus* or *P. fluorescens* may also be detected according to Primer Blast data. In the present study, when specificity was tested over a range of Enterobacteriaceae and non-Enterobacteriaceae, *S. aureus* was not detected using this primer set. Therefore, the specificity of the *IEC* primers for *E. coli* requires further analysis by testing of DNA extracted from other non-Enterobacteriaceae.

**Table 3.17.** *In silico* PCR analysis using primer sets A, B, C and D with Primer Blast Software.

| Bacterial species | Set | | | |
|---|---|---|---|---|
| | A | B | C | D |
| *Budvicia aquatica* | - | + | + | + |
| *Cedecea davisae* | - | + | + | + |
| *Citrobacter braakii* | - | + | + | + |
| *Citrobacter farmeri* | - | + | + | + |
| *Citrobacter freundii* | - | + | + | + |
| *Citrobacter koseri* | - | + | + | + |
| *Cronobacter sakazakii* | - | + | + | + |
| *Enterobacter aerogenes* | - | + | + | + |
| *Enterobacter cloacae* | - | + | + | + |
| *Erwinia persicina* | - | + | + | + |
| *Escherichia coli* | + | + | + | + |
| *Escherichia coli 0157:H7* | - | + | + | + |
| *Ewingella americana* | - | + | + | + |
| *Hafnia alvei* | - | + | + | + |
| *Klebsiella pneumoniae* | - | + | + | + |
| *Klebsiella oxytoca* | - | + | + | + |
| *Kluyvera ascorbata* | - | + | + | + |
| *Kluyvera intermedia* | - | + | + | + |
| *Pantoea agglomerans* | - | - | - | - |
| *Plesiomonas shigelloides* | - | + | + | + |
| *Proteus mirabilis* | - | - | - | - |
| *Proteus vulgaris* | - | + | + | + |
| *Salmonella* Typhimurium | - | + | + | + |
| *Salmonella enteritidis* | - | + | + | + |
| *Serratia liquefaciens* | - | + | + | + |
| *Serratia marcescens* | - | + | + | + |
| *Shigella flexneri* | - | + | + | + |
| *Shigella sonnei* | - | + | + | + |
| *Yersinia enterocolitica* | - | + | + | + |
| *Yersinia pseudotuberculosis* | - | + | + | + |

| | | | | |
|---|---|---|---|---|
| *Yersinia rohdei* | - | + | + | + |
| *Acinetobacter baumanii* | - | - | + | - |
| *Aeromonas hydrophila* | - | + | + | - |
| *Aeromonas punctata* | - | + | + | + |
| *Aeromonas sobria* | - | + | + | + |
| *Alcaligenes faecalis* | - | - | + | - |
| *Bacillus cereus* | + | - | - | - |
| *Bacillus subtilis* | + | - | - | - |
| *Campylobacter coli* | - | - | + | - |
| *Campylobacter jejuni* | - | - | + | - |
| *Enterococcus faecalis* | + | - | - | - |
| *Listeria innocua* | - | - | - | - |
| *Listeria monocytogenes* | - | - | - | - |
| *Micrococcus spp.* | - | - | + | - |
| *Pseudomonas aeruginosa* | - | - | + | - |
| *Pseudomonas fluorescens* | + | - | + | - |
| *Pseudomonas putida* | - | - | + | - |
| *Staphylococcus aureus* | + | - | + | - |
| *Staphyloccoccus capitis* | - | - | - | - |
| *Staphyloccoccus lentus* | - | - | - | - |
| *Staphyloccoccus xylosus* | - | - | - | - |
| *Vibrio parahaemolyticus* | - | + | + | + |
| *Vibrio vulnificus* | - | + | + | + |

[a] target templates found
[b] target templates not found

Set B (*ENT* primers) amplified DNA from all the Enterobacteriaceae tested except for *P. mirabilis*. However, DNA from *V. parahaemolyticus* (424-bp product) was also detected using this primer set by agarose gel electrophoresis (Figure 3.18).

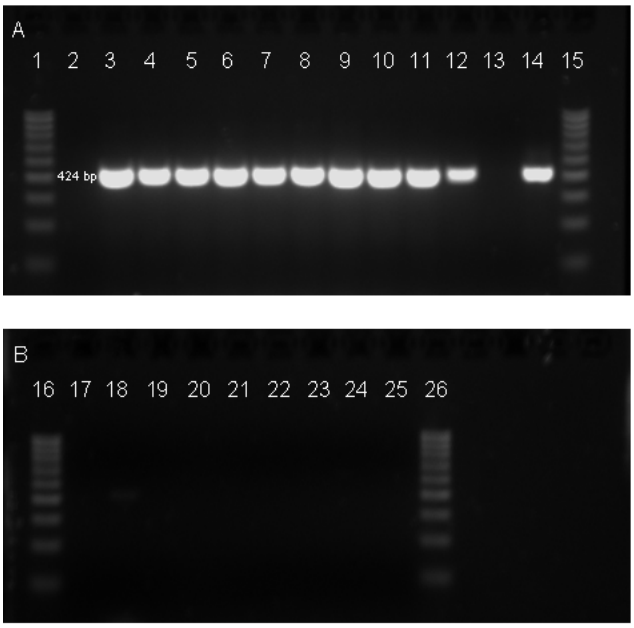


**Figure 3.18.** PCR products obtained with the *ENT* primers (set B).
**A.** Lanes 1, 15, 16, 26, Perfect DNA™ 100 bp ladder ; lane 2, 17, negative control (no DNA template in the PCR reaction); lane 3, *E. coli* ; lane 4, *E. aerogenes* ; lane 5, *E. cloacae* ; lane 6, *C. sakazakii* ; lane 7, *S.* Typhimurium ; lane 8, *S. flexneri* ; lane 9, *K. pneumoniae* ; lane 10, *Y. enterocolitica* ; lane 11, *E. percisina* ; lane 12, *S. marcescens* ; lane 13, *P. mirabilis*.
**B.** Lanes 16, 26, Perfect DNA™ 100 bp ladder ; lane 14, *V. parahaemolyticus* ; lane 18, *A. hydrophila* ; lane 19, *P. aeruginosa* ; lane 20, *L. monocytogenes*; lane 21, *S. aureus* ; lane 22, *S. xylosus* ; lane 23, *Micrococcus spp* ; lane 24, *B. cereus* ; lane 25, *C. jejuni*.

Nakano *et al.* (2003) evaluated the specificity of *ENT* primers with over 72 different bacterial species and noted amplifications for every tested strain belonging to the Vibrionaceae or Enterobacteriaceae family except *P. mirabilis*. For all other bacterial strains tested, results were negative. Data obtained in this study following PCR with the same set indicated similar results by conventional PCR, where the Enterobacteriaceae, *V. parahaemolyticus* and *A. hydrophila* were detected. Real-time PCR assays using mixes prepared with *ENT* primers were performed on both LightCycler® 1.2 and 480 instruments. The amplification curves obtained with the LightCycler® 480 confirmed that all the Enterobacteriaceae tested were detected as well as *P. mirabilis*, and *V. parahaemolyticus* and *A. hydrophila* were positive using the LightCycler® 1.2. (Table 3.18). Ct data were retrieved from real-time PCR using the LightCycler® 1.2.

**Table 3.18.** Sensitivity of primer sets B, C and D against test strains as determined by mean Ct (Cycle threshold) values determined using the automated method provided by the LightCycler® 4.1. Software.

| Tested strains | Primer set used | | |
|---|---|---|---|
| | B | C | D |
| *Escherichia coli* | 23.14 | 25.51 | 24.30 |
| *Enterobacter aerogenes* | 27.47 | 25.64 | 23.43 |
| *Enterobacter cloacae* | 28.67 | 25.79 | 26.08 |
| *Serratia marcescens* | 28.26 | 25.00 | 23.50 |
| *Erwinia percisina* | 26.85 | 26.46 | 25.68 |
| *Klebsiella pneumoniae* | 27.27 | 25.63 | 22.80 |
| *Shigella flexneri* | 22.74 | 24.83 | 24.29 |
| *Yersinia enterocolitica* | 24.71 | 25.01 | 22.95 |
| *Salmonella* Typhimurium | 29.00 | 25.32 | 25.19 |
| *Cronobacter sakazakii* | 23.52 | 25.17 | 24.81 |
| *Listeria monocytogenes* | 34.49 | 31.48 | 37.01 |
| *Staphylococcus aureus* | 31.34 | 32.28 | 37.84 |
| *Staphylococcus lentus* | 36.82 | 31.39 | >41.00 |
| *Staphylococcus xylosus* | 35.70 | 31.80 | >41.00 |
| *Staphylococcus capitis* | 33.71 | 31.89 | 37.92 |
| *Micrococcus spp* | - | 29.86 | 38.04 |
| *Campylobacter jejuni* | 38.09 | 31.96 | 36.96 |
| *Bacillus cereus* | 33.02 | 32.68 | >41.00 |
| *Pseudomonas aeruginosa* | 34.14 | 27.21 | 36.79 |
| *Vibrio parahaemolyticus* | 22.73 | 22.79 | 21.18 |
| *Aeromonas hydrophila* | 29.44 | 25.33 | 34.99 |

Primer set B appeared to be highly specific, as all the Enterobacteriaceae DNA samples tested would generate a PCR product based on Primer Blast analysis, except *Pantoea agglomerans* and *P. mirabilis*. However, the NCBI software showed detection of species from the *Vibrio* and *Aeromonas* genera, as confirmed by PCR and real-time PCR. Overall, this primer set would appear to be a good option for the detection of the Enterobacteriaceae, compared to the designed LUX™ primers (set C) which lacked specificity, as described below.

Initial assays using LUX™ primers targeting the 16S rRNA gene designed using the D-LUX™ Software (set C) were performed using the LightCycler® 1.2 with Enterobacteriaceae and non-Enterobacteriaceae. All the non-Enterobacteriaceae were detected before 35 cycles (Table 3.18). As a consequence, the primers appeared to lack a satisfactory degree of specificity over the range of strains tested. For example, *P. aeruginosa* DNA could be detected at a Ct equal to 27.22, which was quite close to the value for *E. percisina* DNA (Ct = 26.47). Moreover, fluorescence gains were observed for the negative control and the other non-Enterobacteriaceae, suggesting that forward and reverse primers were possibly forming primer-dimers beyond 30 cycles. It is reasonable to speculate that the design aspects of the LUX™ primers can impact on the specificity even if a particular DNA sequence recognised to be highly conserved among species is entered into the software.

Set D appeared to give higher specificity for the Enterobacteriaceae, as all the Enterobacteriaceae tested were detected except *P. mirabilis*. All tested non-Enterobacteriaceae, including *A. hydrophila*, were detected at or beyond 35 cycles. Therefore, cycle number should not exceed 35 in order to avoid generation of false positive data. However, Enterobacteriaceae and *V. parahaemolyticus* were detected within similar Ct values, between 21.18 and 26.08. Therefore, the specificity of primer set D appeared limited by the detection of *V. parahaemolyticus*, as confirmed by Primer Blast (Table 3.17). Using the LightCycler® 1.2, this combined primer set showed greater sensitivity as Ct values for the Enterobacteriaceae were lower than those obtained using the *ENT* primer set (Table 3.18). The detection limit was 4 cells per PCR reaction at a mean Ct equal to 32.32 for a pure culture. Non-Enterobacteriaceae species had Ct values greater than 30, except for *V. parahaemolyticus* (Ct = 21.18), as shown in Table 3.18.

Comparative studies between LUX™ primers and other detection formats have been reported recently. Xu *et al.* (2008) developed a LUX™-based real-time PCR for the detection of *V. parahaemolyticus* in seafood and obtained comparable results for rapidity, specificity and sensitivity to a *TaqMan*® probe-based real-time PCR procedure. However, Mitchell *et al.* (2009) found that the application of LUX™ primers used in the detection of *Chlamydophila pneumoniae* in clinical specimens displayed a log less sensitivity than their designed *TaqMan*-based assay. In their comparative analysis using SYBR Green I, *TaqMan*® probe and LUX™ primers as the detection format, McCrea *et al.* (2007) confirmed that the hairpin structure of the

LUX™ primers may improve the specificity of PCR by reducing mispriming and primer-dimer formation. The primers used by Castillo *et al.* (2006) were reported as being suitable for real-time PCR in the detection of Enterobacteriaceae using primers targeting the 16S ribosomal RNA gene. However, these workers used SYBR Green I technology which is purported to be less specific than LUX™ primers (Anonymous, 2010). Moreover, the LUX™ detection format could not be adapted on pre-designed sequences such as those of Castillo *et al.* (2006).

## 3.3. Limitations of the assays using primer sets B and D

Generally, with a LUX™-based real-time PCR, PCR products can be identified based to their melting temperature, $T_M$, on condition that the amplified region of the targeted gene is sufficiently variable from one species to another. In their study, Mitchell *et al.* (2009) confirmed their positive samples using melting curve analysis to ensure the specificity of the LUX™ primers. However, when using primer set D in a real-time PCR run, DNA from a range of Enterobacteriaceae, *V. parahaemolyticus* and *A. hydrophila* were amplified and melting curve analysis showed a very low variability of Tm from one species to another. The Tm values obtained were between 89°C and 90°C and indicated that the amplified region of the 16S ribosomal RNA gene may be highly conserved among the Enterobacteriaceae and the Vibrionaceae. Therefore, a melting curve analysis would not allow species identification and discrimination using this primer set. The main issue in the use of primer sets B and D is that the Vibrionaceae family was also detected. The 16S ribosomal RNA gene has been used for phylogenetic analyses (Olivier *et al.*, 2005; Wertz *et al.*, 2003). Some sequences of the gene include hypervariable regions and conserved regions. Sequence alignment using Blastn (Basic Local Alignment Search Tool nucleotide) of 16S ribosomal RNA genes of Enterobacteriaceae and Vibrionaceae showed sequences with homologies, which explains the detection of these families by the primer sets B and D.

According to Drake *et al.* (2007), all *Vibrio* species are ubiquitous in the marine environment and all species except *V. cholerae* and *V. mimicus* require sodium chloride supplementation of the media for growth. Therefore, PCR assays using primer sets B and D may not be applied for the analysis of certain samples including seafood. However, no outbreaks related to contamination with Vibrionaceae have, to

date, been reported in infant formula milks. The growth of the Vibrionaceae may be prevented using Brain Heart Infusion (BHI), as noted by Nakano *et al.* (2003). However, Wong *et al.* (2004) reported that another low salt medium such as Morita Mineral Salt (MMS)-0.5% NaCl allowed resuscitation of *V. parahaemolyticus* which was present in a viable but non-culturable state. Hence, this may provide false positive data with real-time PCR using primer sets B and D. Therefore, the use of BHI should be further tested in order to confirm the statement by Nakano *et al.* (2003).

The ISO method 21528-1:2004 for the detection of Enterobacteriaceae in foods requires an enrichment step in EE broth, which was used in the interlaboratory study. Gurtler and Beuchat (2005) and Iversen and Forsythe (2007) have reported that some *Cronobacter* strains do not grow in EE broth, which can lead to false negative results. Joosten *et al.* (2008a) advised an enrichment of the food samples with BPW only. However, Gram-positive flora may interfere with the recovery of Enterobacteriaceae. Weber *et al.* (2009) supplemented the BPW with 40 μM 8-hydroxyquinoline, 0.5 g/L ammonium iron (III) citrate, 0.1 g/L sodium deoxycholate and 0.1 g/L sodium pyruvate in order to optimise the enrichment and the selection of the Enterobacteriaceae.

## 3.4. Comparative analysis of milk samples

The results of the analysis of milk samples are shown in Table 3.19, and provide comparative data between the LUX™-based method (set D), the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit 5'-nuclease and ISO methods.

**Table 3.19.** Comparison of percentage (%) of positive results obtained in the analysis of 40 samples of 100 g powdered infant formula after spiking and detected using ISO and alternative real-time PCR methods.

| Spiking mode | Detection Enterobacteriaceae | | | Detection *C. sakazakii* | |
|---|---|---|---|---|---|
| | Set D | foodproof® kit | ISO 21528-1:2004 | foodproof® kit | ISO/TS 22964:2006 |
| EBES0[a] | 0 | 0 | 12.5 | 0 | 0 |
| EB1[b] | 100 | 100 | 100 | 0 | 37.5 |
| EB2[c] | 100 | 100 | 100 | 0 | 0 |
| CS1[d] | 37.5 | 37.5 | 37.5 | 37.5 | 37.5 |
| CS2[e] | 100 | 100 | 100 | 100 | 100 |

[a] non spiked blank samples

[b] samples spiked with low level of Enterobacteriaceae

[c] samples spiked with higher level of Enterobacteriaceae

[d] samples spiked with very low levels of *C. sakazakii*

[e] samples spiked with higher levels of *C. sakazakii*

In the interlaboratory study, five spiking modes were decided by RIKILT who gave the identification of the samples after the pooling of results: blank samples or no spiking (EBES0), samples spiked with low levels (EB1) and higher level of Enterobacteriaceae (EB2), and samples spiked with low levels (CS1) and higher levels of *C. sakazakii* (CS2). For each spiking mode, 8 samples were tested. The results of the interlaboratory study are included in the Microval certificate of compliance delivered by Lloyd's Register Quality Assurance (Anonymous, 2010). In the present study, 27 out of 40 samples tested positive for the Enterobacteriaceae and 11 out of these 27 samples tested positive for *C. sakazakii* using the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit 5'-nuclease. The samples were analysed with the LightCycler® 480 (Figure 3.19). Overall, the data obtained was in good agreement among all participants in the interlaboratory study.

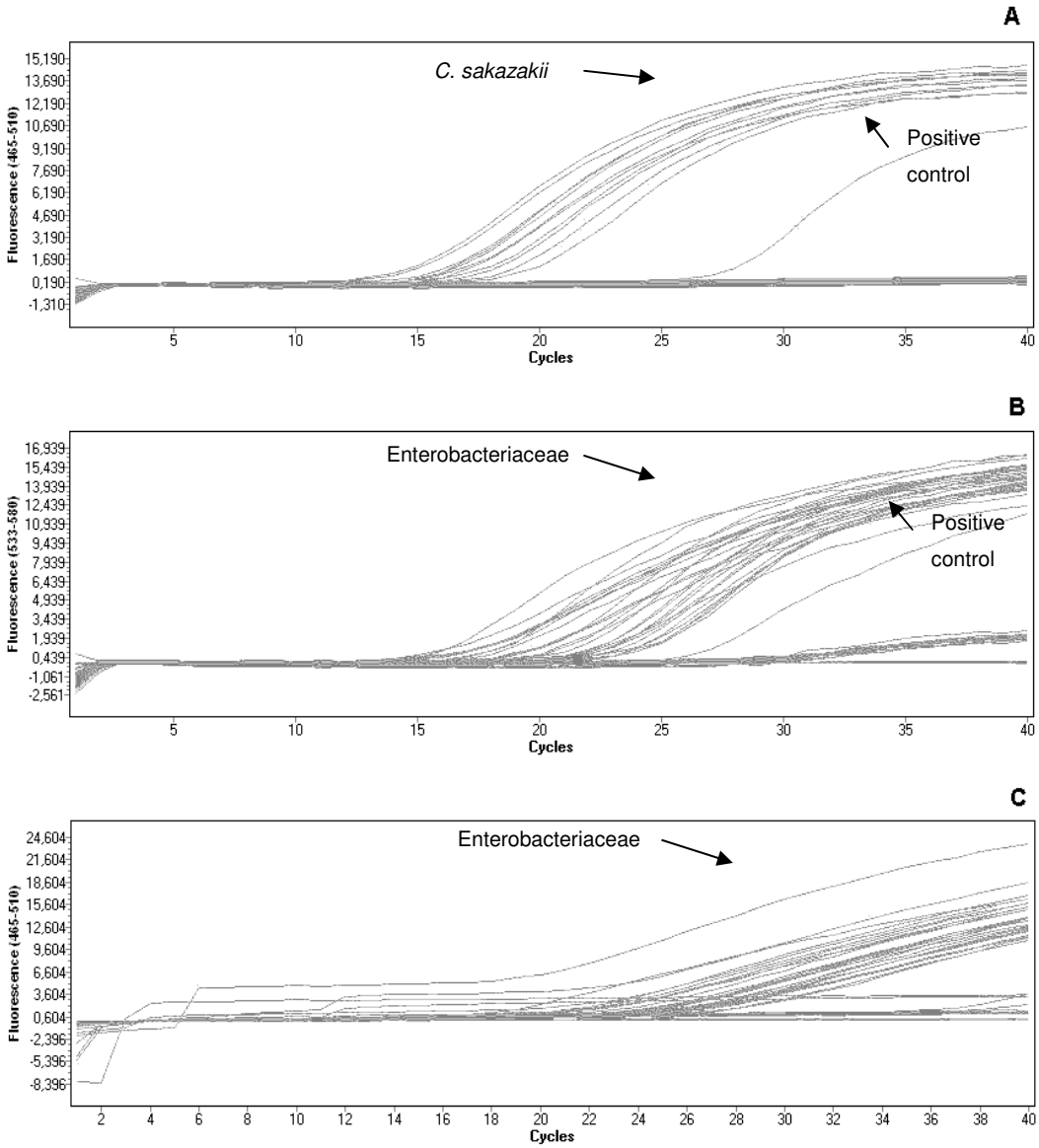


**Figure 3.19.** Amplification curves for the detection of Enterobacteriaceae using the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit 5'-nuclease on FAM channel for the detection of *C. sakazakii* on FAM channel (A) and for the Enterobacteriaceae on VIC channel (B), and using primer set D on FAM channel (C).

However, 3 false negative results were obtained for some CS1 samples among the three methods tested (also found by other participating laboratories). The occurrence of false negative results could be explained by a failure to detect *C. sakazakii*. As described by Edson *et al.* (2009) the bacteria may have failed to grow because of issues with media or incubation conditions.

One false positive result for Enterobacteriaceae was obtained among the EBES 0 samples using the ISO 21528-1:2004 method only, while both alternative methods provided the expected results. Similarly, one false positive result for *C. sakazakii* was

retrieved among the EB1 samples using the ISO/TS 22964:2006. As a hypothesis, both false positive results may have arisen from cross-contamination in the laboratory while performing the ISO procedures, more than likely after the pre-enrichment step with BPW. This indicates that the real-time PCR methods used may reduce the occurrence of such false-positive results.

## 4. CONCLUSION

In summary, Enterobacteriaceae could be detected simultaneously using primer set D or the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit. The real-time PCR with set D appeared to be as rapid and sensitive as the commercial detection kit. However, the specificity of set D was limited by the detection of the Vibrionaceae family and the non-detection of *P. mirabilis*. As shown with real-time PCR and *in silico* PCR data, primer set D can not be used for the detection of *P. mirabilis*. However, the presence of this species in food samples remains very rare and *P. mirabilis* has not been reported to date in infant formula. The identification of *C. sakazakii* was not possible using primer set D by melting curve analysis. The main commercial interest in the use of this primer set arises from its' potential lower cost based on current retail prices for the commercial kit and the costs of the individual components of the method developed in this study. Therefore, the choice is given to the user: either a lower specificity at low cost or higher specificity at higher cost with the guarantee to identify *C. sakazakii* from other Enterobacteriaceae. It should be stated that no other in-house methods or other commercial real-time PCR systems are available with the specificity of the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit for safety control in milk samples. Despite the lower specificity compared with the commercial real-time PCR kit, the LUX™-based real-time PCR system with set D may be considered as a low-cost screening method for the detection of Enterobacteriaceae. However, an internal amplification control should be synthesized and included in the amplification mixture to eliminate false-positive results and validate the assay for the food industry. This primer set could directly discriminate negative samples, while presumptive positive samples could be further analysed using the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit for Enterobacteriaceae confirmation, or even identification of *C. sakazakii*.

# 5. REFERENCES

Aitichou, M., Javorschi, S. and Ibrahim, M. S. (2005) 'Two-color multiplex assay for the identification of orthopox viruses with real-time LUX-PCR', *Molecular and Cellular Probes,* 19(5), 323-328.

Anonymous (2003a) *ISO 16140:2003: Microbiology of food and animal feeding stuffs -- Protocol for the validation of alternative methods* Geneva, Switzerland: International Organization for Standardization.

Anonymous (2003b) *ISO 4833:2003: Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of microorganisms -- Colony-count technique at 30 degrees C. ISO, Geneva, Switzerland*,

Anonymous (2004) *ISO 21528-1:2004: Microbiology of food and animal feeding stuffs - Horizontal methods for the detection and enumeration of Enterobacteriaceae - Part 1: Detection and enumeration by MPN technique with pre-enrichment. ISO, Geneva, Switzerland*, Geneva: International Organization for Standardization.

Anonymous (2006) *ISO/TS 22964:2006: Milk and milk products - Detection of Enterobacter sakazakii. ISO, Geneva, Switzerland*, Geneva: International Organization for Standardization.

Anonymous (2010) 'D-LUX™ Gene Expression Assays', [online], available: http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Nucleic-Acid-Amplification-and-Expression-Profiling/qRT-PCR/qPCR/D-LUX-Assays.html?CID=fl-lux [accessed

Anonymous (2010) Microval Certificate of Compliance for the foodproof® Enterobacteriaceae plus *E. sakazakii* (*Cronobacter sakazakii*) Detection Kit, 5'-nuclease (Art No R302 15) for detection of Cronobacter spp. (*Enterobacter sakazakii*) in combination with the foodproof StarPrep One kit (Art no S 400 07) and Reagent D (Art No 500 02). In. Rotterdam, The Netherlands: Lloyd's Register Quality Assurance

Antal, M., Farkas, T., German, P., Belak, S. and Kiss, I. (2007) 'Real-time reverse transcription-polymerase chain reaction detection of Newcastle disease virus using light upon extension fluorogenic primers', *Journal of Veterinary Diagnostic Investigation,* 19(4), 400-404.

Balcazar, J. L., Vendrell, D., de Blas, I., Ruiz-Zarzuela, I., Girones, O. and Muzquiz, J. L. (2007) 'Quantitative detection of *Aeromonas salmonicida* in fish tissue by real-time PCR using self-quenched, fluorogenic primers', *Journal of Medical Microbiology,* 56(3), 323-328.

Bayardelle, P. and Zafarullah, M. (2002) 'Development of oligonucleotide primers for the specific PCR-based detection of the most frequent Enterobacteriaceae species DNA using wec gene templates', *Canadian Journal of Microbiology,* 48(2), 113-122.

Baylis, C. L. (2006) 'Enterobacteriaceae' in Blackburn, C. d. W., ed. *Food Spoilage Microorganisms*, Campden and Chlorleywood Food Research Association, UK: Woodhead Publishing in Food Science, Technology and Nutrition, 624-667.

Bergthorsson, U. and Ochman, H. (1995) 'Heterogeneity of genome sizes among natural isolates of *Escherichia coli*', *Journal of Bacteriology,* 177(20), 5784-5789.

Blood, R. M. and Curtis, G. D. W. (1995) 'Media for total Enterobacteriaceae, coliforms and *Escherichia coli*', *International Journal of Food Microbiology,* 26(1), 93-115.

Cahill, S. M., Wachsmuth, I. K., Costarrica, M. D. and Ben Embarek, P. K. (2008) 'Powdered infant formula as a source of *Salmonella* infection in infants', *Clinical Infectious Diseases,* 46(2), 268-273.

Castillo, M., Martin-Orue, S. M., Manzanilla, E. G., Badiola, I., Martin, M. and Gasa, J. (2006) 'Quantification of total bacteria, enterobacteria and lactobacilli populations in pig digesta by real-time PCR', *Veterinary Microbiology,* 114(1-2), 165-170.

Chap, J., Jackson, P., Siqueira, R., Gaspar, N., Quintas, C., Park, J., Osaili, T., Shaker, R., Jaradat, Z., Hartantyo, S. H. P., Sani, N. A., Estuningsih, S. and Forsythe, S. J. (2009) 'International survey of *Cronobacter sakazakii* and other *Cronobacter spp.* in follow up formulas and infant foods', *International Journal of Food Microbiology,* 136(2), 185-188.

Chen, R., Huang, W. M., Lin, Z. X., Zhou, Z. F., Yu, H. Q. and Zhu, D. Z. (2004) 'Development of a novel real-time RT-PCR assay with LUX primer for the detection of swine transmissible gastroenteritis virus', *Journal of Virological Methods,* 122(1), 57-61.

de Boer, E. (1998) 'Update on media for isolation of Enterobacteriaceae from foods', *International Journal of Food Microbiology,* 45(1), 43-53.

Drake, S. L., DePaola, A. and Jaykus, L. A. (2007) 'An overview of *Vibrio vulnificus* and *Vibrio parahaemolyticus*', *Comprehensive Reviews in Food Science and Food Safety,* 6(4), 120-144.

Drudy, D., Mullane, N. R., Quinn, T., Wall, P. G. and Fanning, S. (2006) '*Enterobacter sakazakii*: An emerging pathogen in powdered infant formula', *Clinical Infectious Diseases,* 42(7), 996-1002.

Edson, D. C., Empson, S. and Massey, L. D. (2009) 'Pathogen Detection in Food Microbiology Laboratories: an Analysis of Qualitative Proficiency Test Data, 199-2007', *Journal of Food Safety,* 29(4), 521-530.

Estuningsih, S., Kress, C., Hassan, A. A., Akineden, O., Schneider, E. and Usleber, E. (2006) 'Enterobacteriaceae in dehydrated powdered infant formula manufactured in Indonesia and Malaysia', *Journal of Food Protection,* 69(12), 3013-3017.

Gao, H., Gao, Q. L., Zhang, X., Guan, C., Luo, M. H., Zhang, H. B., Liu, P., Zhang, H. Y. and Li, J. (2010) 'Improved medium for detection of *Klebsiella* in powdered milk', *Journal of Food Safety,* 30(1), 12-23.

Giovannini, M., Verduci, E., Ghisleni, D., Salvatici, E., Riva, E. and Agostoni, C. (2008) '*Enterobacter sakazakii*: An emerging problem in paediatric nutrition', *Journal of International Medical Research,* 36(3), 394-399.

Gurtler, J. B. and Beuchat, L. R. (2005) 'Performance of media for recovering stressed cells of Enterobacter sakazakii as determined using spiral plating and ecometric techniques', *Applied and Environmental Microbiology,* 71(12), 7661-7669.

Iversen, C. and Forsythe, S. J. (2007) 'Comparison of media for the isolation of Enterobacter sakazakii', *Applied and Environmental Microbiology,* 73(1), 48-52.

Joosten, H., Marugg, J., Stephan, R., Klijn, A., Jackson, T. and Iversen, C. (2008) 'A rapid and reliable alternative to ISO 21528-1 : 2004 for detection of Enterobacteriaceae', *International Journal of Food Microbiology,* 125(3), 344-346.

Khan, I. U. H., Gannon, V., Kent, R., Koning, W., Lapen, D. R., Miller, J., Neumann, N., Phillips, R., Robertson, W., Topp, E., Bochove, E. v. and Edge, T. A. (2007) 'Development of a rapid quantitative PCR assay for direct detection and quantification of culturable and non-culturable *Escherichia coli* from agriculture watersheds', *Journal of Microbiological Methods,* 69(3), 480-488.

Klappenbach, J. A., Saxman, P. R., Cole, J. R. and Schmidt, T. M. (2001) 'rrndb: the Ribosomal RNA Operon Copy Number Database', *Nucleic Acids Research,* 29(1), 181-184.

Krascsenicsova, K., Trncikova, T. and Kaclikova, E. (2008) 'Detection and Quantification of *Enterobacter sakazakii* by Real-time 5'-Nuclease Polymerase Chain Reaction Targeting the *palE* Gene', *Food Analytical Methods,* 1(2), 85-94.

Kunchev, M., Alexandrov, E., Kamarinchev, B., Marinov, K., Gergova, I. and Mekouchinov, K. (2007) 'Effective LUX (Light upon eXtension) primer system for early and rapid detection of *Coxiella burnetii*', *Biotechnology & Biotechnological Equipment,* 21(3), 338-340.

Lehmacher, A., Fiegen, M. and Hansen, B. (2007) 'Real-time PCR of *Enterobacter sakazakii* in infant formula', *Journal Fur Verbraucherschutz Und Lebensmittelsicherheit-Journal of Consumer Protection and Food Safety,* 2(2), 218-221.

Mackay, I. M., Mackay, J. F., Nissen, M. D. and Sloots, T. P. (2007) 'Real-Time PCR: History and Fluorogenic chemistries' in *Real-Time PCR in Microbiology, From Diagnosis to Characterization*, Norfolk, UK: Caister Academic Press, 1-39.

Maheux, A. F., Picard, F. J., Boissinot, M., Bissonnette, L., Paradis, S. and Bergeron, M. G. (2009) 'Analytical comparison of nine PCR primer sets designed to detect the presence of *Escherichia coli/Shigella* in water samples', *Water Research (Oxford),* 43(12), 3019-3028.

McCrea, J. K., Liu, C. Y., Ng, L. K. and Wang, G. H. (2007) 'Detection of the *Escherichia coli* pathogenic gene *eae* with three real-time polymerase chain reaction methods', *Canadian Journal of Microbiology,* 53(3), 398-403.

Mitchell, S. L., Budhiraja, S., Thurman, K. A., Thacker, W. L. and Winchell, J. M. (2009) 'Evaluation of two real-time PCR chemistries for the detection of *Chlamydophila pneumoniae* in clinical specimens', *Molecular and Cellular Probes,* 23(6), 309-311.

Nakano, S., Kobayashi, T., Funabiki, K., Matsumura, A., Nagao, Y. and Yamada, T. (2003) 'Development of a PCR assay for detection of Enterobacteriaceae in foods', *Journal of Food Protection,* 66(10), 1798-1804.

Nazarenko, I., Lowe, B., Darfler, M., Ikonomi, P., Schuster, D. and Rashtchian, A. (2002) 'Multiplex quantitative PCR using self-quenched primers labeled with a single fluorophore', *Nucleic Acids Research,* 30(9).

Nordgren, J., Bucardo, F., Dienus, O., Svensson, L. and Lindgren, P. E. (2008) 'Novel light-upon-extension real-time PCR assays for detection and quantification of genogroup I and II noroviruses in clinical specimens', *Journal of Clinical Microbiology,* 46(1), 164-170.

Olivier, A., Lee, H. Y. and Cote, J. C. (2005) 'Study of the heterogeneity of 16S rRNA genes in gamma-proteobacteria: Implications for phylogenetic analysis', *Journal of General and Applied Microbiology,* 51(6), 395-405.

Qiu, Y., Hu, Q. and Wang, W. (2009) 'Evaluation of real-time PCR in detecting enteric pathogens', *China Tropical Medicine,* 9(1), 12-13.

Slavov, S., Kalvatchev, Z., Tsekov, I., Simeonov, P., Hristova, L., Kotsev, J., Mladenov, D. and Tsvetkov, M. (2008) 'Novel light-upon-extension (LUX) real-time PCR primer system for rapid detection of *Polyomavirus hominis* 1 (BKV) in clinical samples', *Biotechnology & Biotechnological Equipment,* 22(2), 759-761.

Sun, F. L., Wu, D. C., Qiu, Z. G., Jin, M., Wang, X. W. and Li, J. W. (2010) 'Development of real-time PCR systems based on SYBR Green for the specific detection and quantification of *Klebsiella pneumoniae* in infant formula', *Food Control,* 21(4), 487-491.

Weber, C., Stephan, R., Druggan, P., Joosten, H. and Iversen, C. (2009) 'Improving the enrichment procedure for Enterobacteriaceae detection', *Food Microbiology,* 26(6), 565-572.

Wertz, J. E., Goldstone, C., Gordon, D. M. and Riley, M. A. (2003) 'A molecular phylogeny of enteric bacteria and implications for a bacterial species concept', *Journal of Evolutionary Biology,* 16(6), 1236-1248.

Wong, H. C., Wang, P. L., Chen, S. Y. and Chiu, S. W. (2004) 'Resuscitation of viable but non-culturable *Vibrio parahaemolyticus* in a minimum salt medium', *Fems Microbiology Letters,* 233(2), 269-275.

Xu, R.-s., Chen, R., Lin, C.-h., Yang, Z.-j. and Chen, G.-w. (2008) 'Development of a LUX™ PCR assay for rapid detection of *Vibrio parahaemolyticus*', *Chinese Veterinary Science,* 38(2), 1075-1079.

# CHAPTER FOUR

## Development of defined microbial population standards using Fluorescence Activated Cell Sorting for the absolute quantification of *S. aureus* using real-time PCR[3]

[3] The work described in this chapter has been published in *Molecular Biotechnology* (Martinon A., Cronin U. and Wilkinson M., 2011), "Comparison of in-house and commercial real-time PCR systems for the detection of Enterobacteriaceae and their evaluation within an interlaboratory study using infant formula samples", DOI 10.1007/s12033-011-9417-3 (Appendix Three).

## ABSTRACT

Four types of standards were assessed in a SYBR Green-based real-time PCR procedure for the quantification of *Staphylococcus aureus* in DNA samples. The standards were purified *S. aureus* genomic DNA (type A), circular plasmid DNA containing a thermonuclease (*nuc)* gene fragment (type B), DNA extracted from defined populations of *S. aureus* cells generated by Fluorescence Activated Cell Sorting (FACS) technology with (type C) or without purification of DNA by boiling (type D). The optimal efficiency of 2.016 was obtained on Roche LightCycler® 4.1. Software for type C standards, while the lowest efficiency (1.682) corresponded to type D standards. Type C standards appeared to be more suitable for quantitative real-time PCR because of the use of defined populations for construction of standard curves. Overall, Fieller Confidence Interval algorithm may be improved for replicates having a low standard deviation in Cycle Threshold values such as found for type B and C standards. Stabilities of diluted PCR standards stored at -20°C were compared after 0, 7, 14 and 30 days and were lower for type A or C standards compared with type B standards. However, FACS generated standards may be useful for bacterial quantification in real-time PCR assays once optimal storage and temperature conditions are defined.

# 1. INTRODUCTION

Quantitative real-time PCR is extensively used for the simultaneous amplification, detection and quantification of nucleic acids in many research and diagnostic applications. Most of the real-time PCR procedures in microbiology generate qualitative data by giving a simple presence or absence result. Absolute quantification of target DNA by real-time PCR can be used if it is assumed a direct relationship exists between the concentration of nucleic acid and the number of microorganisms in a sample. The Cycle Threshold (Ct) corresponding to the analysed sample is compared with other Ct data by the amplification of a dilution series of standards to generate a standard curve, which subsequently allows determination of the initial concentration of target DNA in a sample. Mackay *et al.* (2007a) noted the lack of commercial applications of quantitative PCR (qPCR) in routine microbial analysis due to difficulties in the optimization, standardization and normalization of qPCR procedures. In parallel, research laboratories have developed their own methods in real-time PCR for the absolute quantification of microorganisms in samples. However, the reliability of a real-time PCR quantification method depends on providing accurate and precise titres of microbial standards.

To date, standards for absolute quantification of microorganisms using real-time PCR are expressed as “copy number” or Colony Forming Unit (CFU). Copy number unit corresponds to the quantity of target molecules initially present in the PCR reaction. Standards in “copy number” are typically prepared using genomic DNA (Alarcon *et al.*, 2006; Guilbaud *et al.*, 2005; Hein *et al.*, 2001; Rossmanith *et al.*, 2006), plasmids containing the target sequence (Chen *et al.*, 2008; Loddenkotter *et al.*, 2005; Sails *et al.*, 2003; W. Xu *et al.*, 2008), or PCR products (Furet *et al.*, 2004).

For the quantification of bacteria as CFU per unit volume of sample, DNA standards are prepared by serial dilution of a pure bacterial culture followed by DNA extraction (Ott *et al.*, 2004; Yang *et al.*, 2007). Alternatively, standard curves can be achieved from several dilutions of a spiked food sample which generates crossing points (Cp) which are plotted against the logarithmic concentration of the serial dilutions (Berrada *et al.*, 2006a; Berrada *et al.*, 2006b; Takahashi *et al.*, 2005).

An alternative novel possibility for bacterial quantification by real-time PCR is to prepare standards with exact known quantities of microorganisms generated using single-cell isolation by FACS. In flow cytometry, physical and / or chemical characteristics of single cells or particles are measured in a fluid stream where

pressure and orifice size are controlled such that a single cell passes through once into an illumination zone (Ishii *et al.*, 2010). Cell sorting then uses electrical and/or mechanical means to separate, isolate and collect the cells of interest according to the parameters entered by the user (Shapiro, 2003b). Among its advantages, FACS allows the rapid concentration and enrichment of cell populations present in low abundance (Wallner *et al.*, 1997) or sorting of defined cell numbers into tubes or microplates (Guillebault *et al.*, 2010). However, very few cell sorting applications have been reported in the literature where cell sorting has been combined with molecular analysis techniques such as PCR. Gillebault *et al.* (2010) combined cell sorting with PCR to identify sorted marine bacterial sub-populations. Bowers *et al.* (2010) used real-time PCR combined with cell sorting to confirm the presence of a specific algal prey inside the vacuoles of sorted algal bloom species. Hoffemann *et al.* (2007) studied gene expression levels in sorted eukaryote cell populations which provided a relative quantification methodology. To date, the use of FACS has not been assessed as a potential methodology for the generation of bacterial standards for use in real-time PCR assays to improve the quantification of microrganisms of interest to the food industry such as *Staphylococcus aureus*.

*S. aureus* is an important indicator of food quality and of the efficiency of industrial cleaning procedures. This microorganism is routinely monitored by the food industry using selective agar-based methods which can take up to 48 hours for a definitive result. Therefore the development of a rapid and quantitative real-time PCR detection system for this pathogen is of both industrial and public health interest. A number of studies have described real-time PCR assays for quantification of *S. aureus* in various foods using genomic or plasmid DNA standards (Hein *et al.*, 2005; Ikeda *et al.*, 2005; Kim *et al.*, 2001; Martinon and Wilkinson, 2011a; B. Pinto *et al.*, 2005). Moreover, real-time PCR systems for the quantitative detection of *S. aureus* can be applied to clinical diagnosis, as described by Peters *et al.* (2007), for the determination of bacteremia. In this study, the thermonuclease (*nuc*) gene of *S. aureus* was selected as the target DNA for real-time PCR assays as Brakstad *et al.* (1992) demonstrated that this gene had sequences common to all tested *S. aureus* isolates and was unique to the species. Mackay *et al.* (2007a) stated that quantitative real-time PCR technology may be improved by the introduction of additional reference materials and calibrators and the release of more commercial real-time quantitative PCR kits. This lack of commercially available kits for quantification of microorganisms is itself problematic

but is further compounded by the absence of standardization among existing in-house PCR assays that enable valid comparison of methodologies and data (Kao *et al.*, 2005). Some commercial real-time PCR systems currently available on the market do not include quantitative features such as a quantification calibrator or a protocol to allow absolute quantification, whereas others use calibrated plasmid solutions to create a standard curve. Therefore, the novel strategy of using standards generated by FACS in real-time PCR was assessed to address some of the issues outlined above using *S. aureus* as the target microorganism for development of a quantification model.

In this study, the potential of DNA standards generated by cell sorting was investigated for use in real-time PCR quantification. Four DNA standards were compared for the absolute quantification of the *nuc* gene of *S. aureus* using a SYBR Green-based real-time PCR: (a) Pure genomic DNA, (b) circular plasmid DNA, (c) and (d) FACS-generated DNA standards. The resulting standard curves were compared on the basis of PCR efficiency using real-time PCR software and the stability of the diluted standards was tested over a range of storage times.

## 2. MATERIALS AND METHODS

### 2.1. Bacterial strain

The type strain of *S. aureus* (NCTC 8325) was obtained from the National Collection of Type Cultures (Health Protection Agency Culture Collections, Salisbury, UK) and was stored on Protect beads 109 (LangenBach Services Ltd, Dublin, Ireland) at – 20°C until cultivation. *S. aureus* was revived by smearing of an inoculated bead onto nutrient agar (NA; Oxoid Ltd, Basingstoke, UK). After overnight incubation at 37˚C, isolated colonies were obtained and maintained at 4°C on NA plates.

## 2.2. Type A standards (genomic DNA)

Before DNA extraction, a preculture of *S. aureus* was made by inoculating one loopful of each culture into a flask of 30 ml of nutrient broth (NB; Oxoid Ltd) with overnight shaking at 37˚C. Following this, 300 µl of preculture was transferred into 30 ml of fresh broth. A culture of *S. aureus* was obtained under the conditions outlined above and grown to exponential growth phase. DNA used for specificity evaluation was extracted following manufacturer's instructions for Gram negative or Gram positive bacteria using a DNeasy Blood and Tissue Kit (Qiagen, Crawley, UK) based on DNA purification through chromatography columns. DNA was extracted from pellets obtained from cultures in the exponential growth phase following centrifugation of 1 ml of a bacterial suspension at 5000 x *g* for 10 min in a Sigma 1-15 centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) and eluted in 200 µl of AE buffer (Qiagen). DNA quantifications were automatically determined by spectrophotometry at 260 nm using a Nanodrop Spectrophotometer ND-1000 (Thermo Scientific, Wilmington, USA) which systematically computes the 260/280nm absorbance ratio.

Standard curves were prepared by amplifying DNA samples containing 300,000 copies or 9.27 x $10^{-10}$ g of *S. aureus* DNA to 3 copies or 9.27 x $10^{-15}$ g of *S. aureus* DNA per 2 µl or PCR reaction over a 10-fold dilution series in 6 dilution points in duplicate in DNA grade water (Fisher Scientific, Loughborough, UK). A standard curve was generated from genomic DNA following Applied Biosystems´ instructions (Anonymous, 2003b) based on a 2,821,361 bp sized genome of *S. aureus* NCTC 8325 (Mlynarczyk *et al.*, 1998) assuming that the *nuc* gene was present as a single copy. The copy numbers of the *nuc* gene were calculated based on the following formula, in which *n* is the number of base pairs, *m* is the DNA mass, $N_A$ is Avogadro's number (6.02 x $10^{23}$ bp/mol) and *M* is the average molecular weight of a base pair (610 g/mol).

$$n = (m \times N_A) / M$$

$$m = n \times 1.096 \times 10^{-21} \text{ g / bp}$$

## 2.3. Type B standards (plasmid DNA)

First, a 279-bp PCR product corresponding to the insert was produced by amplifying a *S. aureus*-specific fragment of the *nuc* gene. The Genbank number of *S. aureus* NCTC 8325 is NC_007795 with SAOUHSC_00818 as the locus tag for the thermonuclease. The primers were purchased from MWG Eurofins Operon (Ebersberg, Germany). The forward primer was 5'-GCGATTGATGGTGATACGGT-3' and the reverse primer was 5'-AGCCAAGCCTTGACGAACTAAAGC-3' (Brakstad *et al.*, 1992).

The PCR mix was prepared with the FastStart *Taq* DNA Polymerase, dNTPack 5 U/µl kit (Roche Diagnostics GmbH, Mannheim, Germany). On the basis of a final 50 µl reaction volume, the master mixture contained 48 µl of 4 mM $MgCl_2$ (2 mM from 10X PCR buffer and 2mM from $MgCl_2$ solution), 500 nM of forward primer, 500 nM of reverse primer, dNTP mixture and the FastStart *Taq* DNA Polymerase to which 2 µl of *S. aureus* DNA sample (1 ng) was added to each reaction. PCR was performed on a G-Storm GS2 Thermal Cycler (Genetic Research Instrumentation, Braintree, UK). PCR programmes were carried out as shown in Table 4.20.

**Table 4.20.** PCR and real-time PCR conditions using the *nuc* primer sets.

| Program | PCR | SYBR Green-based real-time PCR |
|---|---|---|
| Pre-Incubation | 95°C, 6 min | 95°C, 10 min |
| Amplification | 35 cycles | 35 cycles |
| | 95°C, 30 s | 95°C, 5 s |
| | 62°C, 15 s | 62°C, 10 s |
| | 72°C, 30s | 72°C, 20 s (Single) |
| Melting | N/A[a] | 95°C, 0 s |
| | | 65°C, 10 s |
| | | 95°C, 0 s |
| | | 0.2°C/s (Continuous) |
| Cooling | N/A[a] | 40°C, 30 s |

[a] not applicable

Plasmid DNA standards were constructed by purifying the PCR product using a Qiaquick PCR Purification Kit (Qiagen) following the manual's instructions. DNA was quantified using a Nanodrop Spectrophotometer ND-1000. Purified PCR products were cloned using the TOPO TA pCR® 4 Cloning® Kit for Sequencing (Invitrogen Corporation, Carlsbad, USA) as per manufacturer's instructions. The plasmid was transformed into Mach1™-T1® chemically competent *Escherichia coli* cells (Invitrogen Corporation). 50 µl of transformed inoculum were spread onto Luria Bertani (LB; Sigma-Aldrich, Saint Louis, USA) agar supplemented with 50 µg/ml of ampicillin (Sigma-Aldrich). Only positive recombinants obtained after transformation of the chemically competent cells grow in this medium. Characteristic white colonies were picked and cultured overnight in LB broth (Sigma-Aldrich) containing 50 µg/ml of kanamycin (Sigma-Aldrich). Plasmids were prepared using the HiSpeed Plasmid Midi kit (Qiagen), following manufacturer's instructions. The presence of the insert was confirmed by a restriction digestion using Eco RI (Sigma-Aldrich). The circular plasmid was quantified using a Nanodrop Spectrophotometer ND-1000.

Standard curves were prepared by amplifying plasmid samples containing 300,000 copies or 1.39 x $10^{-12}$ g of plasmid DNA to 3 copies or 1.39 x $10^{-7}$ g of plasmid DNA per 2 µl or per PCR reaction over a 10-fold dilution series in 6 dilution points in duplicate in DNA grade water, according to Applied Biosystems instructions for creating a standard curve using plasmid DNA (Anonymous, 2003b), based on a 4235-bp sized of plasmid including the insert.

## 2.4. Type C and D standards (FACS generated)

A single colony of *S. aureus* NCTC 8325 was inoculated onto 5 ml of Nutrient Broth (NB; Oxoid Ltd, Basingstoke, UK) and grown overnight at 37°C with shaking at 125 rpm. The next day, 1 ml of the preculture was transferred to 49 ml of fresh NB and the culture was left shaking at 125 rpm at 37°C for 2 h. From the culture, 1 ml aliquots were pipetted into microfuge tubes and centrifuged for 10 min at 4000 x g using a Sigma centrifuge. The supernatant was discarded and the pellet was resuspended in 5 ml of sterile Phosphate Buffer Saline (PBS; Oxoid). This suspension was subsequently analysed and sorted using a standard MoFlo cell sorter (Beckman Coulter Inc, Fullerton, USA) with a 488 nm Ar laser and a 635 nm laser diode and the only modification of which was the inclusion of a photomultiplier tube for detecting

forward scattered (FSC) light. For bacterial analysis, the instrument´s 488 nm laser was set at 100 mW, with triggering on side scatter (SSC). Bacteria were separated from noise and cellular debris using combinations of FSC pulse height versus SSC pulse height and FSC pulse area versus SSC pulse area. Thereafter, single cells were discriminated from doublets and chains using FSC pulse width. Using Summit v4.0. Software, a gate was drawn around single cells and 1.0 x $10^6$ single cells were sorted into Eppendorf tubes, using the instrument´s single cell/3 drop sort mode. Sorting was performed with a 70 µm nozzle at typical drop drive frequencies of ~95,000 Hz and drop drive amplitudes of ~15 V. Charge phase and defanning were adjusted to maintain side stream integrity. If necessary, drop drive amplitude was adjusted to maintain the physical position of the last attached drop (using the stream camera) and drop delay was calculated immediately before and after each sort. Acquisition rates of 3,000–9,000 events $s^{-1}$ (eps), sort rates of 2,500–8,000 eps and abort rates of 180–200 eps were typical. Plate counts were carried out in triplicate on the day of sorting to confirm that 1.0 x $10^6$ cells per tube were sorted. Post sorting, the tubes were centrifuged at 4000 x g using an Allegra X22R centrifuge (Beckman Coulter, Brea, USA). Pellets were stored at -20°C until DNA extraction.

Type C standards were prepared by isolating DNA from sorted cells resuspended in 180 µl of lysing buffer using DNeasy Blood and Tissue Kit according to manual instructions. After DNA purification, tubes contained 1.0 x $10^6$ equivalent cells or 1.0 x $10^6$ Signal Generating Unit (SGU)/200 µl of elution buffer. Wang and Spadoro (1998) introduced the SGU to measure the concentration of template available for PCR amplification. An SGU corresponds to the smallest unit that generates a positive signal by PCR amplification or one particle containing at least one amplifiable molecule. This unit was employed in the present study when dealing with FACS generated standards.

Then serial dilutions were performed in DNA grade water over 6 points: $10^4$, 5 x $10^3$, $10^3$, 300, 100, and 10 SGU per 2 µl or per PCR reaction.

Type D standards were prepared by resuspending pellets obtained after centrifugation of sorted cells in 200 µl of DNA grade water and the inclusion of a lysing step by boiling of the cells, according to Queipo-Ortuno *et al.* (2008). Serial dilutions were performed as for preparation of type C FACS standards.

## 2.5. SYBR Green I real-time PCR assay for absolute quantification

Real-time PCR procedures were performed on a LightCycler® 1.2 (Roche Diagnostics GmbH). Primers used were the same as those used in the production of PCR products for the cloning procedure. In each capillary, the 20-µl reaction mix contained 1X concentration LightCycler-FastStart DNA Master SYBR Green (Roche Diagnostics GmbH); 4 mM $MgCl_2$; 500 nM concentration of each primer and 2 µl of the template. SYBR Green I was included as detection format for its simplicity, low cost, and as binding is not affected by mutation of the target gene (Guilbaud *et al.*, 2005), to avoid increased handling and possible loss of detection found for probes such as *TaqMan*® or HybProbes (Aldea *et al.*, 2002; Fernandez *et al.*, 2006). Indeed data obtained in the study of Martinon and Wilkinson (2011a) confirmed that the primer set targeting the *nuc* gene was highly specific for *S. aureus* and that SYBR Green I was a suitable detection format. Times and temperatures applied are displayed on Table 4.20 and DNA amplification was followed in real-time in channel F1 or at 530 nm. A no template control was included in each PCR assay.

The Roche Diagnostics LightCycler® 4.1 Software was used to measure the increase in fluorescence emitted by SYBR Green I bound to double-stranded DNA. Cp values were calculated to prepare the standard curves. Data were shown as amplification plots with fluorescence values in abscissa and the cycle number in intercept. Standard curves were displayed on a graph displaying the Cp versus the logarithm of defined copy numbers or SGU, according to the standard type. Cp values were measured in triplicate within the same PCR run.

## 2.6. Absolute quantification and statistical analysis

Standard curves were created under Excel 2003 Software from Cp data retrieved from the LightCycler® 4.1. Software. Each standard curve was assessed by the calculation of a correlation coefficient ($R^2$) that monitors the reproducibility of pipetting (Tse and Capeau, 2002) and the slope. Amplification efficiencies (E) were automatically generated by the LightCycler® 4.1. Software to give a quantitative expression of the quality of PCR. Absolute quantification analysis was performed using the automated method provided by the software, where the crossing point was identified as that where the fluorescence curve turns sharply upwards which was the

first maximum of the second derivative of the curve. An efficiency of 2 indicated that the number of target molecules was doubling at each PCR cycle (Anonymous, 2007c); E is theoretically calculated from the calibration curve slope, according to the equation:

$$E = 10^{[-1 / slope]}.$$

To obtain the efficiency value in percentage, the following formula was used:

$$E = (10^{[-1 / slope]} - 1) \times 100$$ (Yun *et al.*, 2006).

The quality of each quantification process was evaluated using Fieller's Confidence Interval (FCI) Software, an R-based algorithm software (Verderio *et al.*, 2008). Cp data collected from LightCycler® 4.1 Software were imported into FCI using Microsoft Excel as Comma Separated Values (.csv). Data were fitted to the linear regression and ANOVA (ANalysis Of Variance) models detailed by Verderio *et al.* (2008). A lack of fit test was assayed with a 95% confidence interval for regression coefficients in order to assess the efficiency of each standard curve. A plot was generated to allow the assessment of the PCR assay quality and a visual representation of the FCI graphical derivation (Verderio *et al.*, 2008).

Finally, to assess the stability of the real-time PCR assays over time using different standards, the experiments were performed using standards stored after 0, 7, 14 and 30 days at -20°C, repeated 3 times for each of the conditions. Viviskis *et al.* (1998) studied DNA conservation at 4°C and did not detect any modification in DNA quantity, quality or suitability for PCR over one month of storage, confirmed also by Farkas *et al.* (1996). However, they did not exclude the likelihood of a constant DNA degradation in samples stored at this temperature. Consequently, in the present study it was decided not to store the DNA standards at 4°C and to evaluate the uniformity of Cp values in a sample according to duration of storage at -20°C. Cp variations were estimated by calculating the intra-assay coefficient of variation (CV) per dilution point tested in triplicates. The CV was calculated by dividing the standard deviation by the mean of the measured Cp values.

# 3. RESULTS AND DISCUSSION

## 3.1. Generation of accurate populations by FACS

The present study has introduced a new concept in the preparation of quantification standards using FACS, the analytical aspect of which allows the identification and selection of cell populations and the sorting aspect of which allows rapid and precise deposition of known numbers of cells into tubes or onto plates. Scatter signals emitted by bacteria were used for discriminating the bacterial population from background noise and debris. Scatter pulse width was employed to separate single cells from doublets and chains on the principle that particles with larger diameters or cross sections produce wider pulses of scattered light (Shapiro, 2003c). In order to reduce the numbers of doublets and aggregates, mild ultrasonic treatments may be used before FACS; however energy levels applied must be controllable and reproducible (Braga *et al.*, 2003; Nebe-von-Caron *et al.*, 2000). An inherent background error of 1.9-2.5% was associated with FACS. The sorting process did not affect the viability or cellular integrity of the vast majority of sorted cells; microbial viability as assessed by plate counting was ~95% with typical yields of at least 98-99%. Furthermore, given that the sort mode used to deflect single cells into tubes would have eliminated the co-sorting of any cellular debris, including chromosomes that produced a FSC signal above threshold it seems unlikely that significant free DNA was sorted alongside intact cells. Several concerns have been raised regarding the use of standards expressed as “copy number” units which are seen as theoretical rather than experimentally derived. The accuracy of external standard quantification depends on the correct determination of standard DNA concentration that is usually measured by UV spectrophotometry giving an approximate concentration value (Tse and Capeau, 2002; Vaerman *et al.*, 2004). However, the determination of the DNA concentration is sometimes overestimated and the major drawback is that the target gene cannot be quantified in the final dilution of the standard (Wang and Spadoro, 1998). In microbiology, copy number of genomic DNA standards is calculated according to the molecular weight of the target genome. However, such data are not systematically available for a given species or strain of microorganism. In the preparation of plasmid standards, the insert is required to be purified and cloned into a vector. These operations appear to be expensive and laborious and the efficiencies of cloning and transformation vary depending on: the

nature of the insert (size, toxicity, vector-to-insert ratio, freshness of the PCR products), the plasmid size and the competent state of the cells (Anonymous, 2006b; Siguret *et al.*, 1994; Szostkova and Horakova, 1998). Also the issue of whether to linearise the plasmid or not continues to be a matter for discussion (Beld *et al.*, 2007). The use of PCR products as standards (Dhanasekaran *et al.*, 2010) may be not advised since errors may be generated during PCR when PCR products are used as templates for amplification (Loewen and Switala, 1995). Secondary structures such as stem-loops may be involved in the generation of mistakes and bias in template detection when using probes for instance. The quantification by real-time PCR in CFU per mass or volume unit (Berrada *et al.*, 2006a; Berrada *et al.*, 2006b; Ott *et al.*, 2004; Takahashi *et al.*, 2005; Yang *et al.*, 2007) is also disadvantageous as the serial dilution method employed may refer to absorbance values or enumeration in a counting chamber, and standard values must be confirmed by plate counting. Moreover, these standards can only be said to contain an approximate amount of cells, as one CFU may correspond to one cell or one cluster of cells. Furthermore, the bacterial cell may have a variety of replicons – chromosomes (Krishnapillai, 1995). Therefore, FACS-generated standards may be a potential alternative to allow accurate quantitative real-time PCR assays.

## 3.2. Comparison of amplification efficiencies using LightCycler® 4.1 Software

Standard curves were constructed using type A, B, C and D standards. The melting curve analysis of the PCR products obtained after the SYBR Green-based real-time PCR showed the primers to be specific for all of the extracted DNAs irrespective of the type of standard (Table 4.21A). Slight differences in melting temperatures ($T_M$) may be explained by the variation of salt concentrations from one reaction vessel to another which can influence the stability of the DNA helix (Tan and Chen, 2006). In this study, the melting curve profiles were considered to be similar to each other.

**Table 4.21.** Melting temperatures ($T_M$) obtained in relation to the specificity of the primers according to the calibration DNA standard amplified (A) and comparison of PCR efficiencies calculated using LightCycler® 4.1. Software and FCI Software (B).

**A.**

| Standards types | Mean value | Standard deviation |
|---|---|---|
| A | 80.61 | 0.08 |
| B | 80.72 | 0.09 |
| C | 80.14 | 0.11 |
| D | 80.51 | 0.12 |

**B.**

| Standard type | Roche LightCycler® 4.1. Software | FCI Software |
|---|---|---|
| A | 1.992 | 1.992 |
| B | 2.013 | 2.011 |
| C | 2.016 | 2.007 |
| D | 1.682 | 1.857 |

The accuracy of real-time PCR is highly dependant on the PCR efficiency. The Roche LightCycler® 4.1. and FCI Software were also utilized to define the efficiency of the real-time PCRs. As shown on Table 4.21B, efficiency values were relatively similar between both software packages.

The reaction efficiency and linearity data provided by the LightCycler® 4.1. Software were of relatively good quality, as shown in (Table 4.21B and Figure 4.20), except in the case of the standard curve type D, where outliers were observed.

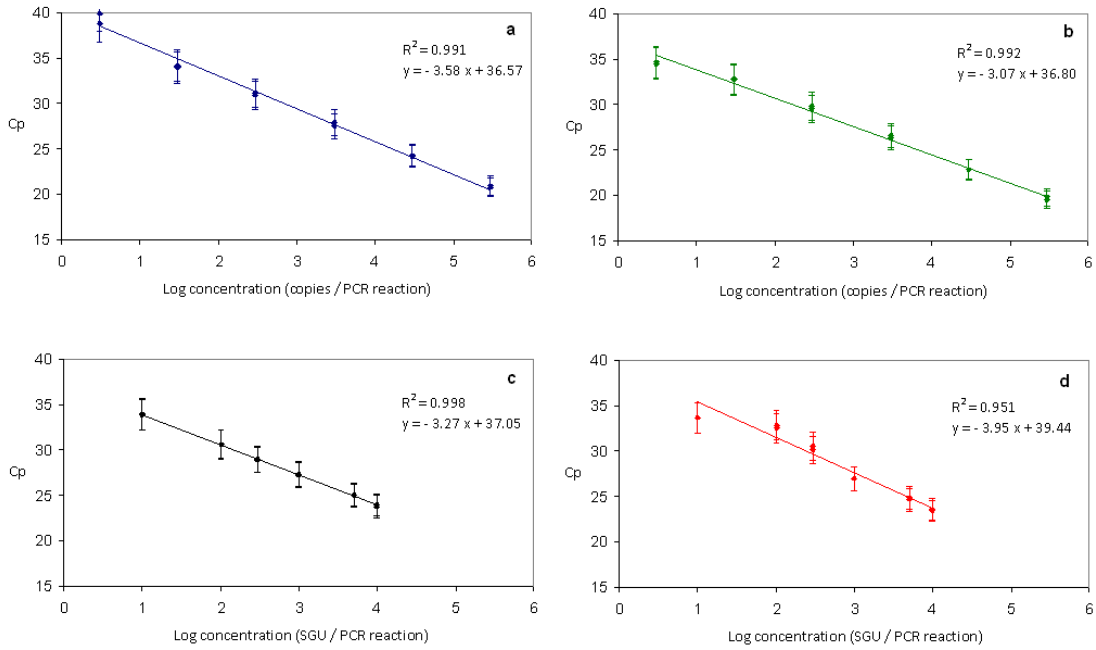


**Figure 4.20.** Standard curves showing Log of concentration (300000 to 3 copies or 10000 to 10 SGU per PCR reaction) versus crossing point (Cp) constructed using (a) type A, (b) type B, (c) type C and (d) type D standards.

Type B standard had an efficiency of 2.013 with the LightCycler® 4.1. Software. Using the type A-generated standards, the resultant standard curve may possibly result in an overestimation of the DNA content especially when DNA quantities are low, such as in the range 3 to 10 copies. To date, microbiological research and analysis by real-time PCR still have not reached a definitive preference for use of either genomic or plasmid DNA as quantitative standards. Type A, C and D standards were prepared using genomic DNA and, therefore, contained a large excess of non-target sequences, as opposed to type B where plasmid DNA was used and may have contained a relatively small amount of non-target DNA. Yun *et al.* (2006) noted that besides the inherent properties of the primer pair, the complexity and nature of the DNA sample containing the amplicon has itself an influence on the amplification efficiency. However, the efficiency values defined by the LightCycler® 4.1. Software for type A and C standard curves were satisfactory, which may suggest that the DNA complexity was not an issue in these two quantification standard types. According to Pfaffl (2004), real-time PCR efficiency varies with high linearity from 1.60 or 60% to maximal values up to 2.10 or 110%. The best efficiency was obtained for type C standards (101.6%) whereas satisfactory efficiencies were associated with plasmid

DNA standards (101.3%) and genomic DNA standards (99.2%). Satisfactory coefficients of determination ($R^2$) for standard curves type A, B and C ranged from 0.991 to 0.998. However, the efficiency of 1.682 obtained using type D standards showed a poor amplification of the target DNA. Hodek *et al.* (2009) explained that the efficiency of real-time PCR procedures have an essential impact on DNA quantification. In the case of poor efficiency values such as those obtained using the type D standards, an unsatisfactory slope was observed with a coefficient of determination ($R^2$) of 0.951 for the calibration curve and, correspondingly, the interpolation of DNA quantity may be misinterpreted. The presence of PCR inhibitors in type D standards may be the cause of low efficiency, as DNA was not purified for this standard type. The most significant inhibitors of PCR are endogenous contaminants present in insufficiently purified target DNA samples (Mackay *et al.*, 2007a; Wilson, 1997). Matrices containing high amounts of lipids such as those that constitute the bacterial membrane are inclined to be inhibitory.

## 3.3. Evaluation using FCI Software

FCI Software uses an algorithm that follows a linear regression model according to an ANOVA that includes regression, error, lack of fit and pure error values. Efficiency is calculated based on the regression coefficient of the standard curve, whereas the precision is given by the pure error as calculated by FCI Software. Efficiency and pure error are two distinct features and a standard curve with a good efficiency can be imprecise and *vice versa*. When the lack of fit test was applied under FCI Software to standard curve data, only standard type A curves were reported as having optimal efficiency; a lack of fit was reported for types B, C and D standards. According to Verderio *et al.* (2008) the "lack of fit" message is purported to be a warning message that should be considered for DNA quantification, whereas a lack of fit was clearly observed for type D standard curves. This was not obvious for type B or C generated standard curves, as seen on Figure 4.20, especially given that both the standard curves had efficiencies close to 2 (Table 4.21B).

Commercial software such as Roche LightCycler® 4.1. generally do not investigate the fitting of the data to the linear regression as well as the FCI algorithm. As regards to a "lack of fit" message appearing in the FCI output, communication with the manufacturer on this issue elicited a response which outlined that a standard

curve should not be systematically discarded if the pure error is small because the "lack of fit" warning may be due to the presence of near identical Cp values within each standard or a low standard deviation, as it was in the case for type B and C standards. Therefore, it is reasonable to consider that an improvement of the FCI algorithm may be required when formulating the "lack of fit" message in the context of PCR data that actually fit the linear regression.

## 3.4. Stability of the DNA standards

The stability of the diluted standards depended on the storage duration at -20°C. As shown on Figure 4.21, Type D standards were excluded from the study on the basis of previous data regarding efficiency.

Overall, the CV did not exceed 9% on day 0 for each dilution standard. Type B standards were the most stable over time when stored over 30 days at -20°C. Despite the slight increase of variance noticed at 30 days, circular plasmid DNA standards appeared to be more stable over 1 month at -20°C. These results were in agreement with Dhanasekaran *et al.* (2010) who noted that diluted circular plasmid standards were more stable than linear plasmid DNA or PCR products when stored at -20°C. However, the use of circular plasmid DNA is still controversial. In contrast, type A and C diluted standards had similar profiles to each other, but with higher CVs compared to type B diluted standards. At lower dilutions, it appeared that the CVs were increased by 2-fold from 14 days of storage at -20°C ; the maximum CVs recorded for type A and C standards were ≥10% and ≤18%. Dhanasekaran *et al.* (2010) considered as acceptable diluted standards that showed a CV ≤ 10%.

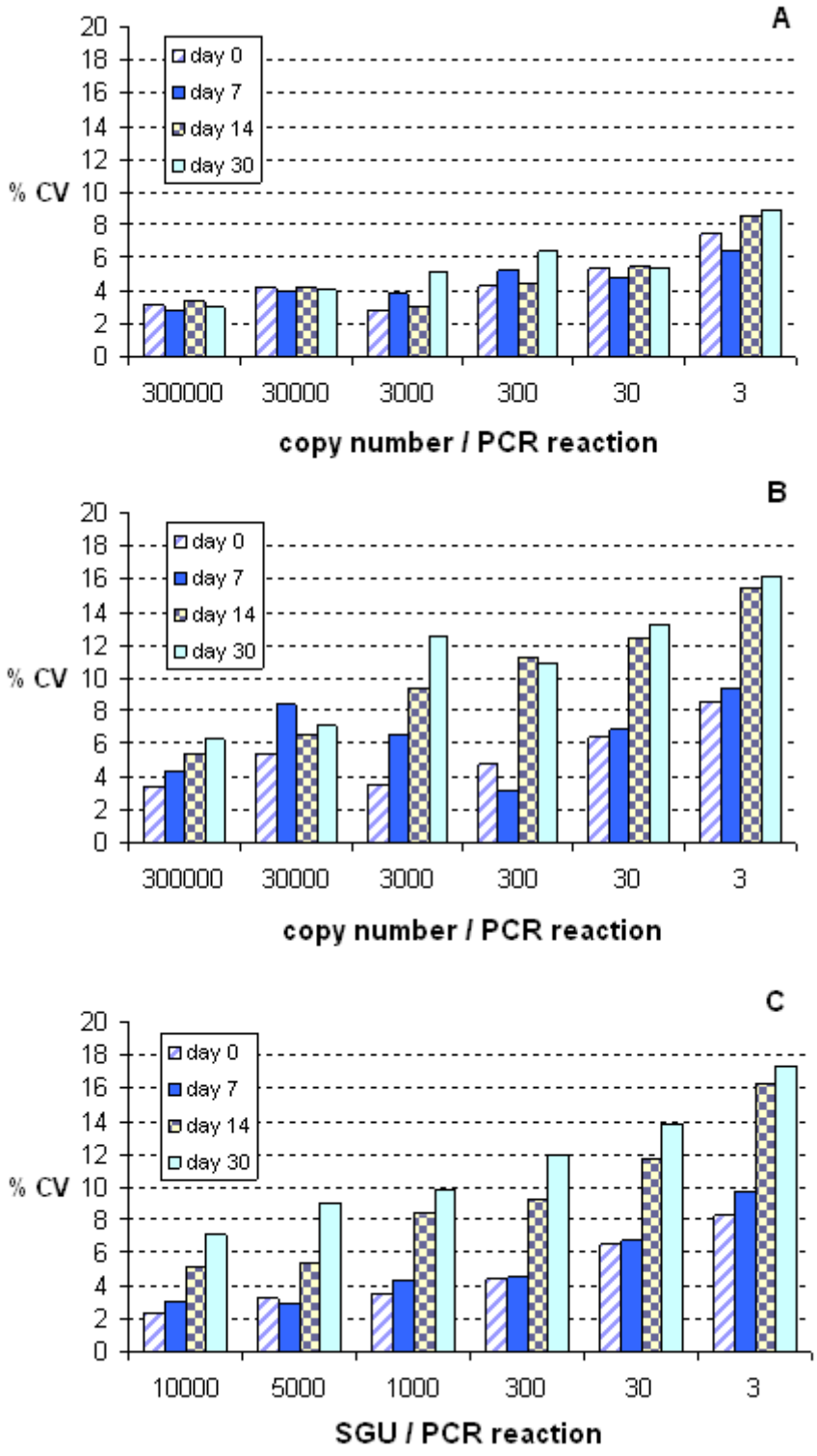


**Figure 4.21.** Coefficients of variation (CVs) of Cp values for real-time PCR quantification of the *nuc* gene using type A, B or C standards stored for 0, 7, 14 and 30 days at -20°C.

The handling of DNA standards with multiple freezing and thawing procedures exposes DNA to conditions that may affect stability and integrity

(Schaudien *et al.*, 2007). Consequently, we would recommend keeping such diluted standards for up to seven days for re-use. Otherwise, diluted standards should be freshly prepared from an aliquoted DNA stock solution kept at -20°C. As an alternative to the inclusion of diluted standards for absolute quantification analysis, a previously saved standard curve may be imported under the LightCycler® 4.1. Software. However, one dilution point must be included into the PCR run with the same analysis method, channel settings and concentration units. This dilution point has to fit to the external standard so that it can be applied for quantification. This option offered by the LightCycler® device allows the user to spare reagents, consumables and time. Nevertheless, it should not replace the preference for construction of a new standard curve for quantification purposes. However, aliquots corresponding to one dilution point may be prepared in advance and conserved at -20°C.

To improve the stability of types A and C standards, it is intended to further optimise storage conditions. Generally, DNA is conserved in solution in distilled water, such as in the current study, or in Tris:$Na_2$EDTA. Tris has a buffering capacity whereas the sodium salt stabilizes the DNA double helix and EDTA inhibits possible action of nucleases (Farkas *et al.*, 1996). In contrast, DNA samples that contain EDTA can chelate divalent cations like $Mg^{2+}$ and act as a PCR inhibitor (Wiedbrauk *et al.*, 1995). Consequently, storage conditions in the presence of EDTA should be further evaluated. Shaudien *et al.* (2007) preserved DNA standards in 50% glycerol and found that 16 cycles of freezing and thawing did not affect DNA quantification in cDNA samples in contrast to DNA standards conserved in distilled water. Therefore, this method may be a good option to conserve type B and C standards at -20°C for up to 14 days. As an alternative, Visvikis *et al.* (1998) were in favour of DNA lyophilisation provided that better methods of DNA hydration were found. However it is not clear whether DNA degradation of lyophilised DNA is due to disruption during storage or to difficulties in rehydration. In this context, further experiments are needed to optimize DNA lyophilisation and rehydration procedures.

# 4. CONCLUSION

In this study, the use of FACS-generated standards with optimal temperature and storage stability was shown to significantly improve the quantification of *S. aureus* using real-time PCR. This novel molecular tool provides a valuable alternative to quantification standards expressed in "copy number" or "CFU" as defined quantities of microorganisms are generated. Subsequent PCR assays using such standards had the best reaction efficiency. It was demonstrated that FACS-generated standards may be maintained for up to seven days for re-use or alternatively fresh diluted standards from an aliquoted DNA stock solution kept at -20°C may be prepared. Storage conditions may be further optimised in order to improve stability over time. Using FCI Software, a "lack of fit" warning message was generated for plasmid and FACS-generated standard curves. This may be due to close or identical Cp values within each standard or a low standard deviation for plasmid and FACS-generated standard curves, which does not question the quality of both quantification processes. The FCI algorithm may need to be modified for such cases. In order to optimise similarities with the analysed sample, accurate quantities of viable bacteria may also be sorted and resuspended into diluent containing the sample matrix or a synthetic formulation to mimic the biochemical background. Overall, the main advantages of this study may be to allow in-house real-time PCR methods to become more compatible and enable progress towards data standardization among laboratories worldwide. The use of FACS as a method to prepare standards for real-time PCR appears to have a great commercial potential for diagnostic companies. Despite the high cost of purchase of a cell sorter and, therefore, a limited access to such equipment for many microbiological laboratories, it is conceivable in the future to optimise and/or dedicate external FACS-equipped laboratories to the unique production of precise microbiology standards. Moreover, it is possible to include ready-to-amplify FACS generated standards into commercial real-time PCR detection kits or to provide them as separate components for use in real-time PCR systems.

# 5. REFERENCES

Alarcon, B., Vicedo, B. and Aznar, R. (2006) 'PCR-based procedures for detection and quantification of *Staphylococcus aureus* and their application in food', *Journal of Applied Microbiology,* 100(2), 352-364.

Aldea, C., Alvarez, C. P., Folgueira, L., Delgado, R. and Otero, J. R. (2002) 'Rapid detection of herpes simplex virus DNA in genital ulcers by real-time PCR using SYBR green I dye as the detection signal', *Journal of Clinical Microbiology,* 40(3), 1060-1062.

Anonymous (2003) 'Applied Biosystems - Creating Standard Curves with Genomic DNA or Plasmid DNA Templates for Use in Quantitative PCR.', [online], available: www6.appliedbiosystems.com/support/tutorials/pdf/quant_pcr.pdf [accessed

Anonymous (2006) 'TOPO TA Cloning® Kit for Sequencing - Five-minute cloning of Taq polymerase-amplified PCR products for sequencing, Invitrogen Corporation ',

Anonymous (2007) 'LightCycler® 2.0 Instrument Operators' Manual - Software version 4.1 - Manual B: for General Laboratory Use - Roche Diagnostics GmbH',

Beld, M. G. H. M., Birch, C., Cane, P. A., Carman, W., Claas, E. C. J., Clewley, J. P., Domingo, E., Druce, J., Escarmis, C., Fouchier, R. A. M., Foulongne, V., Ison, M. G., Jennings, L. C., Kaltenboeck, B., Kay, I. D., Kubista, M., Landt, O., Mackay, I. M., Mackay, J., Niesters, H. G. M., Nissen, M. D., Palladino, S., Papadopoulos, N. G., Petrich, A., Pfaffl, M. W., Rawlinson, W., Reischl, U., Saunders, N. A., Savolainen-Kopra, C., Schildgen, O., Scott, G. M., Segondy, M., Seibl, R., Sloots, T. P., Tang, Y.-W., Tellier, R. and Woo, P. C. Y. (2007) 'Experts' Roundtable: Real-time PCR and Microbiology' in Mackay, I. M., ed. *Real-time PCR in Microbiology from Diagnosis to Characterization*, Norwich, U.K.: Caister Academic Press, 357-443.

Berrada, H., Soriano, J. M., Manes, J. and Pico, Y. (2006a) 'Real-time quantitative PCR of *Staphylococcus aureus* and application in restaurant meals', *Journal of Food Protection,* 69(1), 106-111.

Berrada, H., Soriano, J. M., Pico, Y. and Manes, J. (2006b) 'Quantification of *Listeria monocytogenes* in salads by real-time quantitative PCR', *International Journal of Food Microbiology,* 107(2), 202-206.

Bowers, H. A., Brutemark, A., Carvalho, W. F. and Graneli, E. (2010) 'Combining Flow Cytometry and Real-Time PCR Methodology to Demonstrate Consumption by *Prymnesium parvum*', *Journal of the American Water Resources Association,* 46(1), 133-143.

Braga, P. C., Bovio, C., Culici, M. and Dal Sasso, M. (2003) 'Flow cytometric assessment of susceptibilities of *Streptococcus pyogenes* to erythromycin and rokitamycin', *Antimicrobial Agents and Chemotherapy,* 47(1), 408-412.

Brakstad, O. G., Aasbakk, K. and Maeland, J. A. (1992) 'Detection of *Staphylococcus aureus* by Polymerase Chain Reaction, Amplification of the *nuc* gene', *Journal of Clinical Microbiology,* 30(7), 1654-1660.

Chen, X., Wang, J. and Liu, J. (2008) 'Development of real-time PCR approach for cellulolytic bacteria quantification in the rumen', *Chinese Journal of Animal Science,* 44(11), 36-40.

Dhanasekaran, S., Doherty, T. M., Kenneth, J. and Grp, T. B. T. S. (2010) 'Comparison of different standards for real-time PCR-based absolute quantification', *Journal of Immunological Methods,* 354(1-2), 34-39.

Farkas, D. H., Kaul, K. L., Wiedbrauk, D. L. and Kiechle, F. L. (1996) 'Specimen collection and storage for diagnostic molecular pathology investigation', *Archives of Pathology & Laboratory Medicine,* 120(6), 591-596.

Fernandez, F., Gutierrez, J., Sorlozano, A., Romero, J. M., Soto, M. J. and Ruiz-Cabello, F. (2006) 'Comparison of the SYBR Green and the hybridization probe format for real-time PCR detection of HHV-6', *Microbiological Research,* 161(2), 158-163.

Furet, J.-P., Quénée, P. and Tailliez, P. (2004) 'Molecular quantification of lactic acid bacteria in fermented milk products using real-time quantitative PCR', *International Journal of Food Microbiology,* 97(2), 197-207.

Guilbaud, M., de Coppet, P., Bourion, F., Rachman, C., Prevost, H. and Dousset, X. (2005) 'Quantitative detection of *Listeria monocytogenes* in biofilms by real-time PCR', *Applied and Environmental Microbiology,* 71(4), 2190-2194.

Guillebault, D., Laghdass, M., Catala, P., Obernosterer, I. and Lebaron, P. (2010) 'An improved method of bacterial cell capture after flow cytometry cell sorting', *Applied and Environmental Microbiology*, AEM.00621-10.

Hein, I., Jorgensen, H. J., Loncarevic, S. and Wagner, M. (2005) 'Quantification of *Staphylococcus aureus* in unpasteurised bovine and caprine milk by real-time PCR', *Research in Microbiology,* 156(4), 554-563.

Hein, I., Lehner, A., Rieck, P., Klein, K., Brandl, E. and Wagner, M. (2001) 'Comparison of different approaches to quantify *Staphylococcus aureus* cells by real-time quantitative PCR and application of this technique for examination of cheese', *Applied and Environmental Microbiology,* 67(7), 3122-3126.

Hodek, J., Ovesna, J. and Kucera, L. (2009) 'Interferences of PCR Effectivity: Importance for Quantitative Analyses', *Czech Journal of Food Sciences,* 27, 42-49.

Hoffmann, P. R., Gurary, A., Hoffmann, F. W., Saux, C. J. L., Teeters, K., Hashimoto, A. C., Tam, E. K. and Berry, M. J. (2007) 'A new approach for analyzing cellular infiltration during allergic airway inflammation', *Journal of Immunological Methods,* 328(1-2), 21-33.

Ikeda, T., Tamate, N., Yamaguchi, K. and Makino, S. I. (2005) 'Quantitative analysis of *Staphylococcus aureus* in skimmed milk powder by real-time PCR', *Journal of Veterinary Medical Science,* 67(10), 1037-1041.

Ishii, S., Tago, K. and Senoo, K. (2010) 'Single-cell analysis and isolation for microbiology and biotechnology: methods and applications', *Applied Microbiology and Biotechnology,* 86(5), 1281-1292.

Kao, C. L., King, C. C., Chao, D. Y., W.u., H. L. and Chang, G. J. (2005) 'Laboratory Diagnosis of Dengue Virus Infection: Current and Future Perspectives in Clinical Diagnosis and Public Health', *J. of Microbiol. Immunol and Infect.,* 38, 5-16.

Kim, C. H., Khan, M., Morin, D. E., Hurley, W. L., Tripathy, D. N., Kehrli, M., Oluoch, A. O. and Kakoma, I. (2001) 'Optimization of the PCR for detection of *Staphylococcus aureus nuc* gene in bovine milk', *Journal of Dairy Science,* 84(1), 74-83.

Krishnapillai, V. (1995) 'Comparative Genome Architecture and Dynamics in Bacteria', *Journal of Genetics,* 74(1-2), 61-76.

Loddenkotter, B., Becker, K., Hohoff, C., Brinkmann, B. and Bajanowski, T. (2005) 'Real-time quantitative PCR assay for the detection of *Helicobacter pylori*: no association with sudden infant death syndrome', *International Journal of Legal Medicine,* 119(4), 202-206.

Loewen, P. C. and Switala, J. (1995) 'Template Secondary Structure can increase the Error Frequency of the DNA Polymerase from *Thermus aquaticus*', *Gene,* 164(1), 59-63.

Mackay, I. M., Bustin, S. A., Andrade, J. M., Kubista, M. and Sloots, T. P. (2007) 'Quantification of Microorganisms: not Human, not simple, not Quick' in Mackay, ed. *Real-Time PCR in Microbiology*, Norfolk, UK: Caister Academic Press, 133-182.

Martinon, A. and Wilkinson, M. G. (2011) 'Selection of optimal primer sets for use in a duplex SYBR Green-based real-time PCR protocol for the detection of *L. monocytogenes* and *S. aureus* in foods', *Journal of Food Safety*, , DOI 10.1111/j.1745-4565.2011.00301.x.

Mlynarczyk, A., Mlynarczyk, G. and Jeljaszewicz, J. (1998) 'The genome of *Staphylococcus aureus*: A review', *Zentralblatt Fur Bakteriologie-International Journal of Medical Microbiology Virology Parasitology and Infectious Diseases,* 287(4), 277-314.

Nebe-von-Caron, G., Stephens, P. J., Hewitt, C. J., Powell, J. R. and Badley, R. A. (2000) 'Analysis of bacterial function by multi-colour fluorescence flow cytometry and single cell sorting', *Journal of Microbiological Methods,* 42(1), 97-114.

Ott, S. J., Musfeldt, M., Ullmann, U., Hampe, J. and Schreiber, S. (2004) 'Quantification of Intestinal Bacterial Populations by Real-Time PCR with a Universal Primer Set and Minor Groove Binder Probes: a Global Approach to the Enteric Flora', *J. Clin. Microbiol.,* 42(6), 2566-2572.

Peters, R. P. H., van Agtmael, M. A., Gierveld, S., Danner, S. A., Groeneveld, A. B. J., Vandenbroucke-Grauls, C. and Savelkoul, P. H. M. (2007) 'Quantitative detection of *Staphylococcus aureus* and *Enterococcus faecalis* DNA in blood to diagnose bacteremia in patients in the intensive care unit', *Journal of Clinical Microbiology,* 45, 3641-3646.

Pfaffl, M. W. (2004) 'Quantification Strategies in Real-time PCR' in Bustin, S. A., ed. *A-Z of Quantitative PCR*, La Jolla, CA, USA: International University Line, 87-112.

Pinto, B., Chenoll, E. and Aznar, R. (2005) 'Identification and typing of food-borne *Staphylococcus aureus* by PCR-based techniqnes', *Systematic and Applied Microbiology,* 28(4), 340-352.

Queipo-Ortuno, M. I., Colmenero, J. D. D., Macias, M., Bravo, M. J. and Morata, P. (2008) 'Preparation of bacterial DNA template by boiling and effect of immunoglobulin G as an inhibitor in real-time PCR for serum samples from patients with brucellosis', *Clinical and Vaccine Immunology,* 15(2), 293-296.

Rossmanith, P., Krassnig, M., Wagner, M. and Hein, I. (2006) 'Detection of *Listeria monocytogenes* in food using a combined enrichment/real-time PCR method targeting the *prfA* gene', *Research in Microbiology,* 157(8), 763-771.

Sails, A. D., Fox, A. J., Bolton, F. J., Wareing, D. R. A. and Greenway, D. L. A. (2003) 'A real-time PCR assay for the detection of *Campylobacter jejuni* in foods after enrichment culture', *Applied and Environmental Microbiology,* 69(3), 1383-1390.

Schaudien, D., Baumgartner, W. and Herden, C. (2007) 'High preservation of DNA standards diluted in 50% glycerol', *Diagnostic Molecular Pathology,* 16(3), 153-157.

Shapiro, H. M. (2003a) 'Overture' in Wiley-Liss, ed. *Practical Flow Cytometry - Fourth Edition*, Hoboken, New Jersey.: John Wiley and Sons Inc., 1-60.

Shapiro, H. M. (2003b) 'Parameters and probes' in Wiley-Liss, ed. *Practical Flow Cytometry - Fourth Edition*, Hoboken, New Jersey.: John Wiley and Sons Inc., 273-410.

Siguret, V., Ribba, A. S., Cherel, G., Meyer, D. and Pietu, G. (1994) 'Effect of Plasmid Size on Transformation Efficiency by Electroporation of *Escherichia coli* DH5 Alpha', *Biotechniques,* 16(3), 422-426.

Szostkova, M. and Horakova, D. (1998) 'The effect of plasmid DNA sizes and other factors on electrotransformation of *Escherichia coli* JM109', *Bioelectrochemistry and Bioenergetics,* 47(2), 319-323.

Takahashi, H., Iwade, Y., Konuma, H. and Hara-Kudo, Y. (2005) 'Development of a quantitative real-time PCR method for estimation of the total number of *Vibrio parahaemolyticus* in contaminated shellfish and seawater', *Journal of Food Protection,* 68(5), 1083-1088.

Tan, Z. J. and Chen, S. J. (2006) 'Nucleic acid helix stability: Effects of salt concentration, cation valence and size, and chain length', *Biophysical Journal,* 90(4), 1175-1190.

Tse, C. and Capeau, J. (2002) 'Quantification des acides nucléiques par PCR quantitative en temps réel', *Annales de Biologie Clinique,* 61(3), 279-293.

Vaerman, J. L., Saussoy, P. and Ingargiola, I. (2004) 'Evaluation of real-time PCR data', *Journal of Biological Regulators and Homeostatic Agents,* 18(2), 212-214.

Verderio, P., Pizzamiglio, S., Gallo, F. and Ramsden, S. C. (2008) 'FCI: an R-based algorithm for evaluating uncertainty of absolute real-time PCR quantification', *Bmc Bioinformatics,* 9, 7.

Visvikis, S., Schlenck, A. and Maurice, M. (1998) 'DNA extraction and stability for epidemiological studies', *Clinical Chemistry and Laboratory Medicine,* 36(8), 551-555.

Wallner, G., Fuchs, B., Spring, S., Beisker, W. and Amann, R. (1997) 'Flow sorting of microorganisms for molecular analysis', *Applied and Environmental Microbiology,* 63(11), 4223-4231.

Wang, Z. and Spadoro, J. (1998) 'Determination of target Copy Number of Quantitative Standards Used in PCR-Based Diagnostic Assays' in Ferré, F., ed. *Gene Quantification*, Carlsbad, USA: Birkhäuser, 31-44.

Wiedbrauk, D., Werner, J. and Drevon, A. (1995) 'Inhibition of PCR by aqueous and vitreous fluids', *J. Clin. Microbiol.,* 33(10), 2643-2646.

Wilson, I. G. (1997) 'Inhibition and facilitation of nucleic acid amplification', *Applied and Environmental Microbiology,* 63(10), 3741-3751.

Xu, W., Li, S.-f., Liu, J. and Hu, D. (2008) 'The development of duplex real-time PCR for detection of *Listeria monocytogenes* and *Shigella*', *Zhonghua Weishengwuxue He Mianyixue Zazhi,* 28(10), 946-950.

Yang, H., Qu, L. W., Wimbrow, A. N., Jiang, X. P. and Sun, Y. P. (2007) 'Rapid detection of *Listeria monocytogenes* by nanoparticle-based immunomagnetic separation and real-time PCR', *International Journal of Food Microbiology,* 118, 132-138.

Yun, J. J., Heisler, L. E., Hwang, I. I. L., Wilkins, O., Lau, S. K., Hyrcza, M., Jayabalasingham, B., Jin, J., McLaurin, J., Tsao, M.-S. and Der, S. D. (2006) 'Genomic DNA functions as a universal external standard in quantitative real-time PCR', *Nucl. Acids Res.,* 34(12), e85-.

# CHAPTER FIVE

## Swab sample preparation and viable real-time PCR methodologies for the recovery of *E. coli*, *S. aureus* or *L. monocytogenes* from artificially contaminated food processing surfaces[4]

[4] The work described in this chapter has been submitted to publication in *Food Control* (Martinon A., Cronin U., Quealy J., Stappleton A. and Wilkinson M.), "Swab sample preparation and viable real-time PCR methodologies for the recovery of *E. coli*, *S. aureus* or *L. monocytogenes* from artificially contaminated food processing surfaces".

## ABSTRACT

This study was undertaken to quantify viable *E. coli*, *S. aureus* or *L. monocytogenes* cells in swab samples using modified real-time PCR procedures and novel DNA standards generated by fluorescence activated cell sorting (FACS). Initially, the hygienic status of food contact surfaces within a commercial frozen meal factory was assessed resulting in low Total Viable (<10 $CFU.cm^{-2}$) and Enterobacteriaceae (<1 $CFU.cm^{-2}$) counts detected. From the same premises, swab samples were pooled into a single suspension which, when applied to stainless steel coupons at various levels, simulated a range of contaminated food processing environments. These artificial surfaces were inoculated with the above pathogens, held at ambient temperature and swabbed after various storage times. Samples were prepared using Swab Extraction Tube Systems and treated with Reagent D or PMA to eliminate DNA from non-viable cells prior to DNA extraction. SYBR Green or LUX™-based real-time PCR for quantitative measurement of pathogen populations were subsequently performed. Inclusion of Reagent D into the PCR protocol appeared unsuitable for enumeration of viable *S. aureus* or *L. monocytogenes*; real-time PCR counts were almost 3 times lower than plate counts for immediately swabbed surfaces and no DNA amplification occurred after 30 and 60 min of storage. A significant difference was not found between PMA real-time PCR and plate counts from the artificial food processing surfaces. The PMA real-time PCR method may find applications for enumeration of viable bacteria from food contact surfaces having high contaminating bacterial loads or for monitoring of disinfection efficacy of process surfaces.

# 1. INTRODUCTION

In the food industry, European legislation requires that surfaces should be "in a sound condition and must be easy to clean" (Anonymous, 1993). Microbiological surface control is ensured in the food process environment provided it is hygienically designed and that adequate hygiene procedures are implemented (Verran *et al.*, 2001). However, insufficient cleaning and disinfection of food contact surfaces represent a potential risk of cross-contamination to food (Mattila *et al.*, 1990; Scott and Bloomfield, 1990). Therefore, any lack of hygiene management may lead to a reduction in food shelf-life with adverse economic and public health consequences (Moore and Griffith, 2002a). Several studies have shown that various foodborne pathogens such as *Escherichia coli*, *Staphylococcus aureus* or *Listeria monocytogenes* can survive for hours or even days on hands, sponge/cloths, or utensils (Kusumaningrum *et al.*, 2003; Wilks *et al.*, 2005; Wilks *et al.*, 2006). To date, no standard protocol has been accepted by the food industry for surface hygiene monitoring (Moore and Griffith, 2002c). However, various methods of detection and enumeration of microorganisms from surfaces are currently available. Microbiological methods including hygiene swabbing or agar contact methods are traditionally employed to assess the cleanliness of surfaces. However, despite the simplicity of use, microbiological testing is limited by incubation steps of 24 to 48 h whereas results may be needed earlier, ideally prior to the release of the food product to market. The adenosine triphosphate (ATP) bioluminescence method is another technique which provides an immediate estimate of total surface cleanliness, including the presence of organic debris and microbial contamination. However, it has been shown that microbiological methods may recover microorganisms from surfaces whereas an ATP test may have indicated a clean surface, which therefore poses the issue of detection limit for such bioluminescence tests (Davidson *et al.*, 1999). According to Moore and Griffith (2002c), "the use of hygiene swabs to sample food contact surfaces remains an important means of measuring the effectiveness of sanitation procedures". In combination with swabbing techniques, rapid results may be obtained using real-time PCR protocols. Nevertheless, DNA can remain intact after cell death and may persist from a few days to 3 weeks; therefore the presence of living microorganisms may be overestimated or false positive results may occur in cell detection using this technique (Nocker *et al.*, 2006). Several attempts in detecting and quantifying mRNA from cells using reverse transcriptase real-time PCR provided the first demonstration that

enumeration of viable cells was possible. However, limitations remain in for reproducibility when using mRNA as a target (McKillip *et al.*, 1998; Sheridan *et al.*, 1998). In parallel, viable and dead cell methods based on microscopy and flow cytometry with SYTO 9 / propidium iodide (PI) staining are currently applied, following the criterion of membrane integrity (Nocker *et al.*, 2006). Live cells with intact membranes exclude DNA-binding agents that penetrate dead or damaged cells. Recently, the inclusion of photoactivable DNA-intercalating dyes that inhibit PCR is becoming more popular for the quantification of viable cells and the elimination of non-viable cells. Such dyes in combination with PCR have been reported to be easy-to-use alternatives to microscopic or flow cytometric applications (Nogva *et al.*, 2003; Rudi *et al.*, 2005a; Wang and Levin, 2006). Ethidium monoazide (EMA) and propidium monoazide (PMA) have been tested over a range of live/dead Gram-positive and Gram-negative bacteria (Nocker and Camper, 2006; Nocker *et al.*, 2006; Nocker *et al.*, 2007a; Nocker *et al.*, 2007b; Nogva *et al.*, 2003; Rudi *et al.*, 2005b). Both molecules include an azide group allowing covalent binding to DNA or RNA upon exposure to bright visible light (Bolton and Kearns, 1978; Nocker *et al.*, 2006). However, it has been demonstrated that EMA is a poor indicator of cell viability (Flekna *et al.*, 2007; Nocker and Camper, 2006; Nocker *et al.*, 2006). PMA has become more popular for combination with real-time PCR protocols as PMA is purported to penetrate dead cells with permeabilized cell membranes only (Bae and Wuertz, 2009; Garcia-Cayuela *et al.*, 2009; Josefsen *et al.*, 2010; Kobayashi *et al.*, 2009b; Nocker *et al.*, 2007b; Varma *et al.*, 2009). Another reagent has been recently released by Biotecon Diagnostics under the name “Reagent D” and is purported to act in a similar manner to EMA or PMA in eliminating DNA from dead cells by preventing its amplification. However, little published information is available concerning the identity of this reagent.

To date, we have been working on a number of optimised real-time PCR protocols to enable the detection of Enterobacteriaecae (Martinon *et al.*, 2011b), *S. aureus* or *L. monocytogenes* in a range of foods (Martinon and Wilkinson, 2011b). The objectives of this study were to develop sample preparation and viable real-time PCR procedures for the quantitative recovery of foodborne pathogens such as *E. coli*, *S. aureus* or *L. monocytogenes* in swabs collected from contaminated food contact surfaces. First, samples were collected from naturally contaminated surfaces to evaluate the hygienic status of a commercial ready meal production plant in respect of

the presence of the target microorganisms. Secondly, in the laboratory, surfaces were contaminated with the target bacteria on stainless steel surfaces pre-inoculated with organic matter that created an artificial food processing environment. Thereafter the efficiency of inclusion of Reagent D or PMA in the real-time PCR procedure was evaluated for detection of viable *E. coli*, *S. aureus* and *L. monocytogenes* in swab samples. A novel use for sample preparation was also tested using the Swab Extraction Tube System (SETS). The selected viable real-time PCR procedure was then assessed for bacterial quantification in swab samples collected from artificially contaminated surfaces in simulated food processing environment. The applicability of the viable real-time PCR procedure was also evaluated in order to define the limitations of the method.

# 2. MATERIALS AND METHODS

## 2.1. Naturally contaminated surfaces

### 2.1.1. Sampling procedure

Naturally contaminated food contact surfaces were selected from a leading commercial manufacturer of frozen ready-to-eat meals (Dawn Fresh Food Company, Fethard, Co. Tipperary, Ireland). For surface sampling, SRK Rayon swabs (Copan Italia S.p.a, Bovezzo, Italy) were provided with SRK Rinse Solution, which is an isotonic salt solution containing additional substances to neutralize and inactivate disinfectants and sanitizing agents. In parallel, 34 cotton swab (Copan Italia S.p.a) samples were collected from the same food contact surfaces in the manufacturing plant. Each swab was mixed to 0.1% peptone water (Oxoid Ltd, Basingstoke, UK) directly after sampling.

Flat and curved surfaces (containers, working tables, conveyor belts, knives, pieces of machine and pipes) were sampled. For flat surfaces, a sterile square sampling 10 x 10 cm template was used to define the sample size and the areas were swabbed vertically and horizontally. The surfaces were swabbed from top to bottom, then from left to right of the template.

### 2.1.2. Bacterial identification from the swab samples

The sampling procedures followed the standard ISO method 18593:2004 (Anonymous, 2004d). All the samples were transported to the laboratory in a cool box at 4°C within 4 hours. Upon arrival at the laboratory, the SRK and cotton swab samples were vortexed thoroughly in order to release material from the swab. While the SRK suspensions were analysed separately, the 34 cotton swab suspensions were pooled and mixed with the same volume of 50% glycerol (Sigma Aldrich Inc, Saint Louis, USA). The suspension denoted as suspension A was divided into 1 ml aliquots in microfuge tubes that were stored at - 80°C.

In SRK suspensions and suspension A, a total viable count was determined by the Miles and Misra method (Miles *et al.*, 1938) and by plate counting on Plate Count Agar (PCA; Oxoid Ltd) following a dilution series of suspension A. A particular focus of the analysis was *L. monocytogenes, S. aureus* and Enterobacteriaceae.

For detection of *L. monocytogenes*, 1 ml of suspension A was dispensed into 9 ml of Buffered Peptone Water (BPW; Oxoid Ltd) and incubated at 37ºC for 18 h. Then, 10 ml of pre-enrichment culture were transferred into 90 ml of *Listeria* Enrichment broth (Oxoid Ltd) and incubated for 48 h at 30ºC. A loopful of enriched suspension was streaked on a *Listeria* selective agar (LSA; Oxoid Ltd) plate and incubated for 48 h at 30ºC. Presumptive *L. monocytogenes* colonies on LSA appeared as black surrounded by a black halo. For comparison, 3M™ Petrifilm™ Environmental *Listeria* Plates (3M, Dublin, Ireland) were used following manufacturer's instructions.

For detection of *S. aureus*, 100 µl of suspension A were plated onto Baird Parker agar (Oxoid Ltd) and incubated at 37ºC for 48 h. Characteristic colonies of *S. aureus* on Baird Parker agar were grey-black, shiny, convex and surrounded by a zone of clearing. Catalase activity was tested by the addition of $H_2O_2$ (Sigma Aldrich Inc, Saint Louis, USA). To reveal the presence of coagulase, agglutination was tested using Pastorex® Staph Plus test. (Biorad, Hercules, USA). In parallel, 100 µl of each SRK suspension was mixed with 900 µl of Ringer solution ¼ strength and pipetted onto a Petrifilm™ Staph Express (3M) following manufacturer's instructions.

For detection of Enterobacteriaceae, 1 ml of a neat and 1 ml of a 1.0 x $10^{-1}$ dilution of the suspension A were plated on Violet Red Bile Glucose Agar (VRBGA; Oxoid Ltd). The plates were incubated at 37°C for 24 h. Characteristic colonies on VRBGA plates were pink to red or purple, with or without precipitation haloes. At

least one representative colony from each VRBGA plate was sub cultured onto Nutrient Agar (NA; Oxoid Ltd) with plates incubated for 24 h at 37°C. An oxidase test was performed on each subculture and a colony from each plate was stabbed into a glucose agar tube for incubation at 24 h at 37°C. Colonies testing oxidase negative and positive for fermentation of glucose were identified using an API ID32E (bioMerieux S.A., Craponne, France) kit according to manufacturer's instructions and bioMerieux Apiweb Software. In parallel, the 3M™ Petrifilm™ Enterobacteriaceae Count Plates were used for analysis.

## 2.2. Artificially contaminated surfaces

### 2.2.1. Bacterial strains and culture conditions

Type strains of *E. coli* (ATCC 11775) and *Listeria monocytogenes* (ATCC 19115) were obtained from MicroBioLogics Inc, Saint Cloud, USA. *Staphylococcus aureus* (NCTC 8325) was obtained from the National Collection of Type Cultures (Health Protection Agency Culture Collections, Salisbury, UK). All strains were stored on Protect™ beads 109 (LangenBach Services Ltd, Dublin, Ireland) at – 20°C until cultivation.

For the preparation of inocula, *E. coli*, *L. monocytogenes* and *S. aureus* were incubated overnight on nutrient agar (NA; Oxoid Ltd, Basingstoke, UK) at 37˚C. A preculture of each strain was made by inoculating one loopful of each culture into a flask of 30 ml of nutrient broth (NB; Oxoid Ltd) with overnight shaking at 37˚C for *S. aureus* and *E. coli*. Tryptic Soya Broth (TSB; Oxoid Ltd) was used for growth of *L. monocytogenes* (37˚C). Following growth, 300 µl of the preculture was transferred into 30 ml of fresh broth. A culture of each strain was obtained under the conditions outlined above and grown to late exponential growth phase in order to obtain maximum cell viability and limit large amounts of metabolites and dense cell walls that would decrease the quality of subsequent DNA extraction. The bacterial concentrations used for artificial contamination of the surfaces were of the order of 1.0 x $10^8$ CFU.ml$^{-1}$ (based on data not shown and generated from the exponential phase of a growth curve) and further diluted to 1.0 x $10^7$ and 1.0 x $10^6$ CFU.ml$^{-1}$.

### 2.2.2. Inoculation of surfaces and recovery of pathogens using SETS

Test surfaces were food grade stainless steel surfaces (10 x 10 cm, type AISI-304 standard Mirror polished, Shortt Stainless Steel Ltd, Limerick, Ireland). The coupons were prepared as per Poimenidou *et al.* (2009). A set of coupons were generated simulating food processing environments which varied in contamination using swab suspension A: high (h; undiluted suspension A), moderate (m; 1/10 dilution of suspension A in 0.85% saline water) and low (l; 1/100 dilution of suspension A in 0.85% saline water). The coupons were first inoculated with 100 µl of suspension A over a 10 x 10 cm surface using a spreader with a sampling template and air-dried at room temperature for 30 min. Secondly, 100 µl of either *L. monocytogenes*, *S. aureus* or *E. coli* inoculum were spread over the same 100 $cm^2$ surface. The coupons were stored for 0, 30 and 60 min at room temperature.

For the purposes of sampling, each coupon was swabbed horizontally and vertically using a cotton swab (Copan Italia S.p.a) pre-moistened with 100 µl of Ringer ¼ strength solution. Each swab was cut close to the tip and placed into the inner tube of a SETS (Roche Diagnostics, Mannheim, Germany). Then, the collection tubes were centrifuged at 6000 x *g* for 10 min in a Sigma centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). The inner tubes and the supernatant were discarded. Pellets were re-suspended in 250 µl of Ringer ¼ strength solution (Oxoid Ltd). The obtained bacterial suspension was aliquoted in 50 µl-volumes that were analysed according to the nature of the experiment: quantification by plate counting on NA after serial dilutions, by direct real-time PCR, by real-time PCR following treatment to inactivate DNA from dead cells and DNA extraction.

A swab control was included to assess the bacterial release from the bud of the swab. Cotton swabs (Copan Italia S.p.a) were directly inoculated with 100 µL of an 18 h bacterial suspension of *L. monocytogenes, S. aureus* or *E. coli* which was equivalent to 1.0 x $10^8$ CFU per / swab and denoted as swab controls. The SETS protocol was identical to that described above.

### 2.2.3. Elimination of DNA from dead cells and DNA extraction

Reagent D (Biotecon Diagnostics, Potsdam, Germany) was assessed using samples from surfaces inoculated with *E. coli*, *S. aureus* or *L. monocytogenes* without the inclusion of the artificial food processing environment. DNA from dead cells (50 µl of SETS suspension) was eliminated by adding 300 µl of Reagent D.

PMA (phenanthidium, 3-amino-8-azido-5[3-(diethylmethylammonio)]-6-phenyl dichloride Biotium Inc, Hayward, USA) was also assessed in the real-time PCR procedure. The reagent was prepared following Nocker *et al* (2007b). The treated samples were then centrifuged at 10,000 x *g* for 10 minutes and the supernatants discarded.

DNA was prepared using a DNeasy Blood and Tissue Kit (Qiagen, Crawley, UK) based on DNA purification through chromatography columns. Each PMA-treated SETS pellet was resuspended in 180 µl of enzymatic lysis buffer for *L. monocytogenes* and *S. aureus* and 180 µl of buffer ATL for *E. coli*. Then, the DNA extraction procedures were carried out by following the manufacturer's instructions for Gram negative or Gram positive bacteria.

In this study, Reagent D or PMA were combined with real-time PCR for the detection of *L. monocytogenes*, *S. aureus* and *E. coli* in swab samples collected after 0, 30 and 60 min following inoculation of the surfaces.

### 2.2.4. Real-time PCR conditions

Three primer sets were used (Table 5.22) for the detection of bacteria previously spiked on surfaces. All primers were purchased from MWG Eurofins Operon (Ebersberg, Germany).

**Table 5.22.** Primer sets used for the detection of *E. coli* (A), *L. monocytogenes* (B) or *S. aureus* (C).

| Genetic target | Set | Primer | Primer sequence | a | b | Reference |
|---|---|---|---|---|---|---|
| 16S rRNA | A | LUX - F[c] | 5'-CGGTGTACCCGCAGAAGAAG CAC[FAM]G-3' | 55 | 368 | D-LUX™ Software, Martinon *et al.* (2011), Nakano *et al.* (2003) |
| | | ENT - R[d] | 5'-GCCTCAAGGGCACAACCTCC AAG-3' | | | |
| Listeriolysine O | B | *hly A* - F | 5'-TGCAAGTCCTAAGACGCCA-3' | 60 | 112 | Nogva *et al.* (2000) |
| | | *hly A* - R | 5'-CACTGCATCTCCGTGGTATA CTAA-3' | | | |
| Thermonuclease | C | *nuc* - F | 5'-GCGATTGATGGTGATACGGT-3' | 62 | 279 | Brakstad *et al.* (1992) |
| | | *nuc* - R | 5'-AGCCAAGCCTTGACGAACTA AAGC-3' | | | |

[a] Annealing temperature (°C)
[b] Amplicon size (bp)
[c] Forward
[d] Reverse

The detection of the Enterobacteriaceae using LUX™ primers was assayed on the LightCycler® 480 in channel FAM/SYBR Green with a 10 µl-reaction of PCR mix containing 5 µl of Platinum® qPCR SuperMix-UDG (Invitrogen), 5 mM $MgCl_2$ (final concentration); 500 nM concentration of each primer; 0.5 µl of Bovine Serum Albumin; 0.06 µl of FastStart *Taq* DNA Polymerase (Roche Diagnostics GmbH); and 1 µl of DNA template. Real-time PCR conditions are shown in Table 5.23.

**Table 5.23.** Real-time PCR conditions used for detection of foodborne pathogens.

| Program | LUX™-based real-time PCR | SYBR Green-based real-time PCR |
|---|---|---|
| Pre-Incubation | 50°C, 2 min | 95°C, 10 min |
| | 95°C, 2 min | |
| Amplification | 35 cycles | 35 cycles |
| | 94°C, 5 s | 95°C, 5 s |
| | 55°C, 10 s (Single) | [a]°C, 10 s |
| | 72°C, 10s | 72°C, 20 s (Single) |
| Melting | 95°C, 0 s | 95°C, 0 s |
| | 55°C, 15 s | 65°C, 10 s |
| | 95°C, 0s | 95°C, 0 s |
| | 0.1°C/s (Continuous) | 0.2°C/s (Continuous) |
| Cooling | 30°C, 30 s | 40°C, 30 s |

[a] specific to each primer set used

*L. monocytogenes* and *S. aureus* were detected using a real-time procedure on the LightCycler® 1.2 (Roche Diagnostics GmbH), using LightCycler® FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH). In each capillary, the 20-µl reaction mix contained 1X concentration LightCycler-FastStart DNA Master SYBR Green (Roche Diagnostics GmbH); 4 mM $MgCl_2$; 500 nM concentration of each primer; and 2 µl of the template. Times and temperatures applied are shown in Table 5.23 and DNA amplification was followed in real-time in channel F1 or 530 nm. Standard curves were calculated using DNA from *E. coli*, *L. monocytogenes* or *S. aureus* cells using Fluorescence Activated Cell Sorting (FACS) described below.

### 2.2.5. Preparation of novel PCR quantification standards by FACS

For quantification purposes using real-time PCR, cell standards were prepared as per Martinon *et al.* (2011c) using a FACS methodology. After DNA purification, tubes contained 1.0 x $10^6$ equivalent cells or 1.0 x $10^6$ Signal Generating Units (SGU) / 200 µl of elution buffer. Wang and Spadoro (1998) introduced the SGU to measure the concentration of template available for PCR amplification. An SGU corresponds to the smallest unit that generates a positive signal by PCR amplification or one particle containing at least one amplifiable molecule. This unit was used in the present study when dealing with standards generated by FACS. Subsequently, serial dilutions

were performed in DNA grade water over 9 cell concentrations: $10^4$, 5 x $10^3$, 3 x $10^3$, $10^3$, 300, 100, 30, 10 and 3 equivalent cells. Standard curves were generated for the quantification of each of the three bacterial species. Mean Ct values from triplicates and standard deviations (SD) were calculated in three separate assays. A mean standard curve was created from each species.

### 2.2.6. Statistical analysis

Statistical analysis was applied to bacterial quantification by direct real-time PCR (without Reagent D or PMA), viable real-time PCR (including Reagent D or PMA) and plate counting in the absence or the presence of the artificial food-processing environment, based on triplicate assays carried out on the same swab sample. The LightCycler® Software provided the Ct values that were converted to cell number per 100 $cm^2$ surface. One-way analysis of variance (ANOVA) with post-hoc Tukey test was used to compare data obtained by the real-time PCR procedures and the plate counts at different storage times or in swab control samples. The SPSS 17.0 Software for Windows was used to determine the statistical significance.

# 3. RESULTS AND DISCUSSION

## 3.1. Microbial ecology of naturally contaminated surfaces in a food processing plant

Bacterial recovery from surfaces using swabbing techniques may be influenced by several factors such as the ability of the swab to remove the micro-organisms from the surface, their effective release from the swab and subsequent recovery using plating or other techniques (Moore and Griffith, 2002a). The removal of bacteria from dry surfaces can be improved using a pre-moistened swab as confirmed by Moore and Griffith (2002c). In the present study, swabs were impregnated with a commercial solution (SRK) or Ringer ¼ strength solution. The SRK solution contained in a swab tube is a non-nutrient phosphate buffered solution purporting to include substances that neutralize and inactivate disinfectants and sanitizing agents. This non-nutrient, phosphate buffered solution enables quantification of micro-organisms when transported at ambient temperature. Bazaco *et al.* (2007) advised the use of Copan

SRK solution for quantifying microbial recoveries as confirmed by Moore and Griffith (2007) for the recovery of *E. coli* and *S. aureus* Moreover, the use of cotton swabs associated with Ringer ¼ strength solution has been demonstrated to be advantageous (Moore and Griffith, 2002c). Analysis of naturally contaminated surfaces generally involves the use of traditional plate counting or 3M Petrifilm™. The latter is frequently used by the food industry for the enumeration of *L. monocytogenes*, *S. aureus* and the Enterobacteriaceae in foods or from surfaces with bacterial recoveries comparable to traditional plating methods (Fedio *et al.*, 2008; Ferraz *et al.*; Nyachuba and Donnelly, 2007; Paulsen *et al.*, 2008; Silva *et al.*, 2005; Tassinari *et al.*, 2006; Vicosa *et al.*). In the present study, data showed that *L. monocytogenes* was not detected but some non-presumptive colonies were found on 3M Petrifilm™ *Listeria* Count Plate and LSA for 4 samples collected inside a food filling dispenser within the factory. It is reasonable to suggest that the microorganism involved may be *Listeria* species but not *L. monocytogenes*. *S. aureus* was not detected either, despite the presence of black colonies on Baird Parker; however these tested coagulase negative, and originated from a sample collected inside a large empty food container undergoing a cleaning process. However, the presence of Enterobacteriaceae, using plating and Petrifilm™ methods, was confirmed from surface samples. Characteristic colonies of Enterobacteriaceae were observed in samples collected from hoppers, exposed machine surfaces or cylinders of machines. The tests performed on the subcultures showed that the bacteria were oxidase negative and positive for glucose fermentation. ID32E systems subsequently identified them as *Enterobacter cloacae* (99.7%), *Citrobacter braakii* (92.4%), *Citrobacter freundii* (99.9%), *Enterobacter amnigenus* (62.3%).

Microbiological specifications for post cleaning populations on surfaces in food premises generally disagree in the literature. A variety of total viable count (TVC) levels have been recommended: <2.5 CFU.cm$^{-2}$ (Dancer, 2004; Griffith, 2005; Griffith *et al.*, 2000; Mossel *et al.*, 1999), <10 CFU/cm$^{-2}$ (Anonymous, 2001; Brown and Baird-Parker, 1982), and <100 CFU.cm$^{-2}$ (Holah, 2003). At the time of sample collection in the present study, a number of flat stainless steel surfaces were available for analysis. Enterobacteriaceae and TVC enumeration were only possible on two hopper surfaces, as a flat 10 x 10 cm template must be used when swabbing, and the counts obtained were 0.25 or 0.4 CFU.cm$^{-2}$ for Enterobacteriaceae and 2.4 or 4.2 CFU.cm$^{-2}$ for TVC. This data indicated that satisfactory cleaning procedures of the

process surfaces were in place at these industrial premises. Acceptable contamination levels were also evident for Enterobacteriaceae as levels were <1 CFU.cm$^{-2}$ (Anonymous, 2001). The quantification of Enterobacteriaceae was not available for a majority of naturally contaminated samples, as parts of machinery were not flat so a template could not be applied. Some studies have related issues associated bacterial recoveries from dry surfaces using swabbing techniques (Davidson *et al.*, 1999; Moore and Griffith, 2002a, 2002b). Moore and Griffith (2002c) explained that the efficiency of the swabbing technique was lower when sampling dry surfaces compared with wet surfaces as microbial viability may be affected by drying. Moreover, the same authors (2007) hypothesized that cellular damage, probably caused by the swabbing action itself, may have reduced recoverability from a dry surface. As the bacterial load of a surface sample appeared to be very low at the commercial premises, quantification by real-time PCR could not be applied for analysis of these naturally contaminated surfaces, which was also compounded by potential bacterial losses while swabbing, DNA losses from DNA extraction and preparation of dilutions for PCR reactions.

## 3.2. Linearity of the quantitative PCR using standard curves generated by FACS

Standard curves were generated using DNA extracted from cells prepared by FACS (Figure 5.22). Logarithmic values of fluorescence (y axis) for each dilution were plotted against Cycle Threshold ($C_T$; x axis).

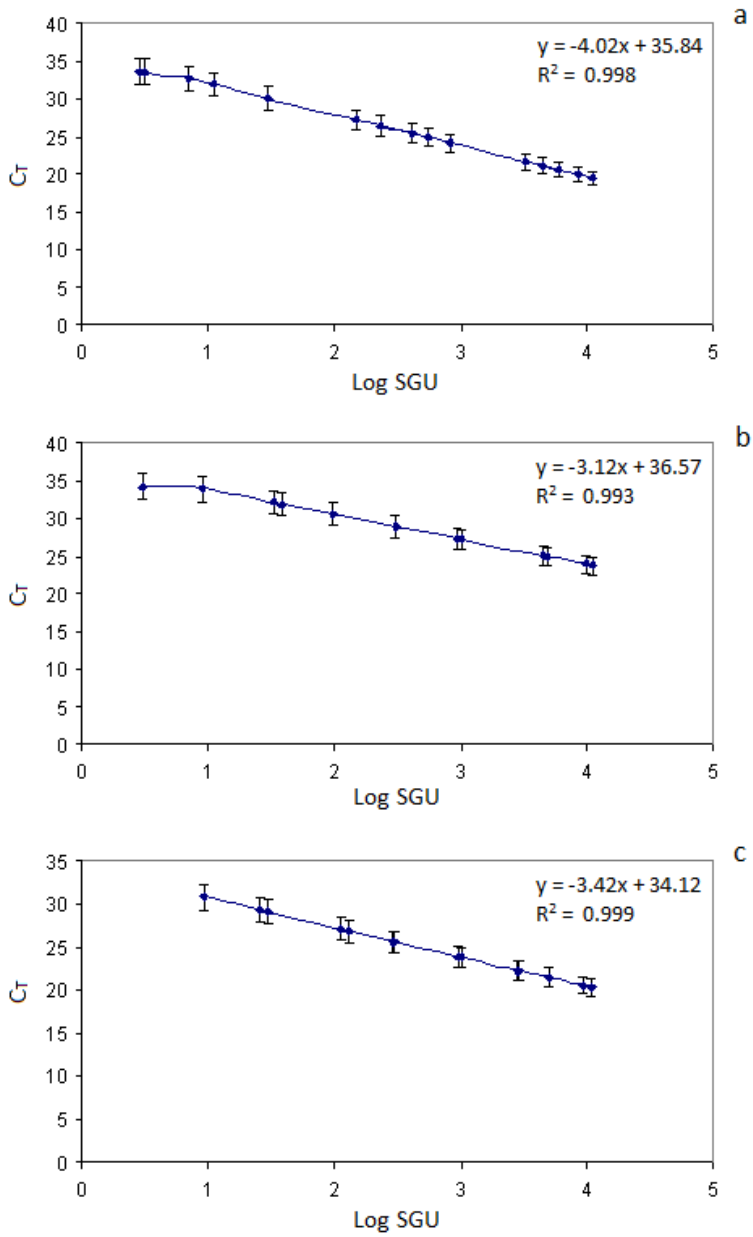


**Figure 5.22.** Standard curves generated by SYBR Green and LUX™-based real-time PCR of serially diluted DNA extracted from (a) *E. coli*, (b) *S. aureus* or (c) *L. monocytogenes* sorted cells, represented as Ct (crossing point) as a function of log of SGU.

Figure 5.22a corresponds to the standard curve obtained from the LUX™-based real-time PCR data for the amplification of E. *coli* DNA. The standard curves in Figure 5.22b and c were constructed from data retrieved from SYBR Green-based

real-time PCR procedures for the amplification of *S. aureus* and *L. monocytogenes* DNAs. A direct relationship was observed from $10^4$ to 3 equivalent cells / reaction between $C_T$ and the log concentration (log SGU) of *E. coli*, *S. aureus* or *L. monocytogenes* initially present in the real-time PCR reaction. $R^2$ values were calculated between 0.993 and 0.999, which indicated that the quantitative PCR assays were highly linear. For each species, detection limits were defined as 3 SGU / reaction.

## 3.3. Assessment of inclusion of Reagent D or PMA in real-time PCR method

According to the instructions manual (Anonymous, 2008b), Reagent D is optimised for elimination of DNA from dead Enterobacteriaceae in up to 100 µl of bacterial enrichment cultures for analysis of instant milk formula. Biotecon Diagnostics, the manufacturer, state that the reagent may not work for other species or in samples other than instant milk formula. In contrast, an increasing number of researchers appear to favour use of PMA for quantitative detection of viable cells by real-time PCR (Bae and Wuertz, 2009; Fittipaldi *et al.*, 2010; Garcia-Cayuela *et al.*, 2009; Josefsen *et al.*, 2010; Kobayashi *et al.*, 2009b; Luo *et al.*; Nocker *et al.*; Nocker *et al.*, 2007b; Varma *et al.*, 2009). Table 5.24 shows a comparative analysis between direct and viable (Reagent D or PMA-based) real-time PCR data including plate counting of SETS suspensions.

**Table 5.24.** Comparison of direct (without Reagent D or PMA) and viable (including Reagent D or PMA) real-time PCR with plate counting from SETS samples obtained from food-grade stainless-steel surfaces inoculated with an 18 h culture of (a), *E. coli,* (b) *S. aureus* or (c) *L. monocytogenes*. Samples were stored for the following times and then swabbed: 0 min (sample 1), 30 min (sample 2), 60 min (sample 3); swab control (sample 4).

| Bacterial load | Sample | Plate count ($log_{10}$ CFU.100cm$^{-2}$) | Real-time PCR quantification ($log_{10}$ cells.100cm$^{-2}$) | | | Significant difference |
|---|---|---|---|---|---|---|
| | | | direct | viable | | |
| | | | | Reagent D | PMA | |
| *E. coli* | 1 | 7.02 | 7.46 | 7.42 | 7.38 | NS (0.058) |
| | 2 | 4.92 | 5.07 | 5.42 | 5.27 | NS (0.065) |
| | 3 | 3.87 | 4.02 | 3.99 | 3.81 | NS (0.068) |
| | 4 | 8.03 | 8.25 | 8.23 | 8.17 | NS (0.110) |
| *S. aureus* | 1 | 6.25 | 6.42 | 3.46 | 6.36 | [b, c] |
| | 2 | 4.02 | 4.74 | 0 | 4.38 | [b, c] |
| | 3 | 3.98 | 4.28 | 0 | 4.16 | [b, c, d] |
| | 4 | 8.07 | 8.33 | 0 | 8.10 | [b] |
| *L. monocytogenes* | 1 | 6.08 | 6.25 | 3.12 | 6.09 | [b] |
| | 2 | 3.47 | 3.86 | 0 | 3.68 | [b, c] |
| | 3 | 3.06 | 3.57 | 0 | 3.37 | [b, c] |
| | 4 | 7.98 | 8.44 | 0 | 8.25 | [b, c] |

[a] Non Significant. One way ANOVA significance levels are indicated in brackets.

Significant differences were defined after Post-hoc Tukey test between data obtained:

[b] with Reagent D-based real-time PCR and all the other methods

[c] with plate counting and direct real-time PCR

[d] with direct real-time PCR and PMA real-time PCR

Plate counts correspond to the total cell count which is essentially a quantification of the live cells within a bacterial population. This population will also include some injured cells that can resume growth under appropriate conditions (Bogosian and Bourneuf, 2001). The difference between plate counts and enumeration using molecular methods may be explained by the presence of viable but non-culturable bacteria in a sample (Knight, 2000; Szewzyk *et al.*, 2000).

Direct real-time PCR has been reported to overestimate the bacterial count as DNA from viable non viable cells is also amplified (Birch *et al.*, 2001; Josephson *et al.*, 1993; Scheu *et al.*, 1998; Wolffs *et al.*, 2005). As shown on Table 5.24, bacterial counts by direct real-time PCR, without any inclusion of Reagent D or PMA, were

higher than for plate counting, which gives an indication regarding cell damage after inoculation on the coupons. For instance, a 3 log difference was observed between plate counting and direct real-time PCR in the case of a sample of *L. monocytogenes* collected after 60 min of holding at ambient temperature. *L. monocytogenes* appeared to be the most sensitive strain to storage among the strains tested. Cox *et al.* (1989) demonstrated that dry conditions and the lack of food residues could decrease the survival of *Listeria* spp. Fuster-Valls *et al.* (2008) showed that *S. aureus* remained viable on stainless steel at least 72 h.

Data obtained from real-time PCR analysis with inclusion of Reagent D were almost 3 log lower than for plate counting in the case of *S. aureus* and *L. monocytogenes* samples collected immediately after inoculation or in swab controls. After 30 or 60 min of storage, real-time PCR data for *S. aureus* and *L. monocytogenes* samples treated with Reagent D showed no DNA amplification. This would appear to be false negative results as colonies were found on NA. For *S. aureus* and *L. monocytogenes*, the inclusion of Reagent D was shown not to be appropriate for these strains, as it may have bound to DNA from both viable as well as dead cells. Elizaquível *et al.* (2010) also evaluated Reagent D and obtained similar results to this study for *L. monocytogenes*. These workers found that Reagent D was toxic to *L. monocytogenes* and that the dye may bind to any DNA present. It is reasonable to suggest that the Reagent D may have similar toxic effects on *S. aureus* cells. Assuming that Reagent D is similar to EMA, these findings are in agreement with Kobayashi *et al.* (2009c) and Nocker *et al.* (2006) who showed that EMA was poorly efficient in terms of revealing the viability of *Staphylococcus* species. In the case of *L. monocytogenes*, Flekna *et al.* (2007) and Nocker *et al.* (2006) showed that EMA was also unsuitable for differentiating between DNA from live or dead cells. An important consideration is the loss of DNA from EMA-treated live cells which has been observed for different bacterial species and may be influenced by the efficiency of the efflux mechanisms and permeability of the bacterial cell (Nocker *et al.*, 2006). According to Nocker *et al.* (2006), PMA appeared to be more suitable for differentiation of live and dead cells as this agent does not penetrate live cells, which has been attributed to the higher charge of the molecule. Data in Table 5.24 indicates that PMA did not affect live cells in the swab samples.

When cells were swabbed immediately after inoculation on the surface, data obtained from plate counting, direct and PMA-based real-time PCR treatment were

comparable. After 30 and 60 min storage of the coupons before swabbing, 2 to 3 log less viable cells were counted compared with immediate sampling after inoculation for all bacterial species. Bacterial counts decreased in the order: direct real-time PCR > PMA real-time PCR > plate count. PMA-based real-time PCR allows counting of viable cells; however it is reasonable to speculate that PMA may also bind to DNA from injured cells. These damaged cells may repair their membranes and may be present as Viable But Not Culturable (VBNC) cells in samples collected after 30 and 60 min of storage. Overall, PMA appeared to be more suitable than Reagent D as the dye does not seem to affect live cells. However, further experiments based on Reagent D and PMA real-time PCR procedures on sub-lethally heat treated samples may be required in order to assess the detection of viable versus non viable cells by exclusion of DNA from dead cells.

## 3.5. Bacterial quantification in an artificially contaminated food process environment

Bacterial quantification from real-time PCR of PMA-treated samples collected from an artificial food processing environment was compared with plate counts using a one-way ANOVA stability test (Table 5.25). For the 3 bacterial species swabbed immediately after inoculation for all organic loads, no significant differences ($\alpha$=0.05) were evident among the various enumeration methods, which indicates that the methods were comparable for quantification in the present study.

**Table 5.25.** Analysis of bacterial populations expressed as $\log_{10}$ CFU.100cm$^{-2}$ on stainless coupons inocuated with suspension A at different concentrations and with a bacterial load of $10^8$ CFU.100 cm$^{-2}$ as a function of storage time (0, 30 or 60 min) in the presence of high (h), medium (m) or low (l) organic loads, using plate counts, real-time PCR and PMA-based real-time PCR methods.

| Bacterial load | Storage time (min) | Organic Load | Plate count | Real-time PCR | PMA real-time PCR | One way ANOVA Significance level ($p \leq 0.05$) |
|---|---|---|---|---|---|---|
| *E. coli* | 0 | h | 6.23 | 6.28 | 6.19 | 0.016; NS[a] |
| | | m | 6.31 | 6.34 | 6.26 | 0.101; NS |
| | | l | 6.21 | 6.29 | 6.23 | 0.086; NS |
| | 30 | h | 6.12 | 6.15 | 6.02 | 0.096; NS |
| | | m | 4.53 | 4.65 | 4.39 | 0.006; S[b, c] |
| | | l | 4.15 | 4.46 | 4.21 | 0.003; S[d] |
| | 60 | h | 5.88 | 5.98 | 5.89 | 0.119; NS |
| | | m | 3.47 | 4.50 | 3.43 | 0; S[c, d] |
| | | l | 3.07 | 4.19 | 3.00 | 0; S[c, d] |
| *S. aureus* | 0 | h | 5.80 | 5.92 | 5.71 | 0.075; NS |
| | | m | 5.49 | 5.51 | 5.50 | 0.809; NS |
| | | l | 5.35 | 5.47 | 5.40 | 0.127; NS |
| | 30 | h | 5.41 | 5.60 | 5.53 | 0.680; NS |
| | | m | 5.89 | 4.97 | 4.92 | 0.415; NS |
| | | l | 3.68 | 3.80 | 3.75 | 0.073; NS |
| | 60 | h | 5.40 | 5.54 | 5.47 | 0.081; NS |
| | | m | 4.31 | 4.43 | 4.38 | 0.024; S[d] |
| | | l | 4.46 | 3.78 | 3.57 | 0.003; S[c, d] |
| *L. monocytogenes* | 0 | h | 5.71 | 5.72 | 5.67 | 0.102; NS |
| | | m | 5.64 | 5.70 | 5.69 | 0.143; NS |
| | | l | 5.61 | 5.71 | 5.61 | 0.084; NS |
| | 30 | h | 5.14 | 5.28 | 5.20 | 0.029; S[d] |
| | | m | 3.70 | 3.91 | 3.78 | 0; S[c, d] |
| | | l | 3.57 | 3.94 | 3.67 | 0; S[c, d] |
| | 60 | h | 5.07 | 5.29 | 5.18 | 0.135; NS |
| | | m | 3.05 | 3.66 | 3.61 | 0; S[c, d, e] |
| | | l | 3.03 | 3.38 | 3.31 | 0; S[c, d, e] |

[a] No significant difference, [b] significant difference.

Significant differences were defined after Post-hoc Tukey test between data obtained:

[c] with direct and PMA-real-time PCR

[d] with plate counting and direct real-time PCR

[e] with plate counting and PMA real-time PCR

Data agreement between the various enumeration methods may be explained by the quantification of mainly viable cells. However, after storage times of 30 or 60

min on the contaminated surfaces, calculated ANOVA levels for most of the samples were lower than 0.05, which indicated a statistically significant difference between plate counts, direct real-time PCR and PMA real-time PCR. The subsequent *post-hoc* Tukey test confirmed that, in regard to the bacterial counts, there were some statistically significant differences between direct real-time PCR and plating, as well as between direct real-time PCR and PMA real-time PCR as p values obtained were less than 0.05. It is reasonable to suggest that parameters such as duration of storage on the stainless steel coupons may have had an influence on data agreement among the 3 counting methods. This may be explained by the presence of a heterogeneous population of live, dead, non-viable or VBNC cells within a swab sample. However, there was no statistically significant difference for bacterial enumeration using plate counts and PMA real-time PCR, which confirms that PMA real-time PCR appears to be suitable for bacterial quantification in swab samples.

PMA-based real-time PCR has the advantage to be rapid, to quantify viable bacteria and finally to provide data on the VBNC fraction that is not detected by cultural methods. However, such viable real-time PCR has limitations in the quantification of viable bacteria as outlined by Fittipaldi *et al.* (2010). False negative results may be generated as PMA can have access to the DNA in viable cells with reversibly damaged cells. False positive data may be obtained in the presence of suspended solids or biomass that would prevent the dye or the light to penetrate membrane-compromised cells.

The survival and growth of microorganisms on surfaces is ensured by the food soil (Sharma and Anand, 2002), also known as organic challenge. To validate real-time PCR methods for bacterial quantification from swab samples, the ideal methodology is their use on naturally contaminated surfaces. However, such samples remain difficult to obtain in sufficient numbers from a food processing environment, as most food manufacturing plants have efficient cleaning procedures in place as shown in the present study. Therefore, several studies have used an artificial food processing environment using food mixes (Pan *et al.*, 2006; Poimenidou *et al.*, 2009) under laboratory conditions to obtain sample contaminated batches. In the present study, suspension A was applied to stainless steel coupons at 3 different concentrations (h, m, l) in order to evaluate the influence of organic matter on the survival of *E. coli*, *S. aureus* or *L. monocytogenes* (as shown on Table 5.25).In this study, bacterial survival appeared to be influenced by 3 parameters: initial bacterial

load, storage time and the concentration of the organic load applied on the surfaces. Generally, bacterial densities decreased with storage time. For all species inoculated at $10^8$ CFU.100 $cm^{-2}$, there was a 1 log difference between 0 and 60 min storage with high organic load whereas a 3 log difference was observed in the presence of a low organic load (Table 5.25). For surfaces inoculated with bacteria at $10^7$ or $10^6$ CFU.100 $cm^{-2}$, bacterial counts by PMA real-time PCR were zero after 30 min storage when low organic loads were applied to surfaces or even medium organic loads (Table 5.25). However, such data may be considered as false negative results as cell or DNA losses may have occurred during sample preparation and/or dilution while preparing the PCR reaction. Therefore, this method may only be applied for enumeration at high bacterial densities such as $10^6$, $10^7$ or $10^8$ CFU.100 $cm^{-2}$ which are not found on cleaned food contact surfaces in the food industry. Under the conditions of the study, the sensitivity over 60 min storage was $10^8$ CFU.100 $cm^{-2}$, $10^7$ CFU.100 $cm^{-2}$ with a medium organic load and $10^6$ CFU.100 $cm^{-2}$ with a high organic load.

## 4. CONCLUSION

In summary, the PMA-based real-time PCR method was preferred to the inclusion of Reagent D in real-time PCR procedures as it appeared that PMA became bound to only dead cells of *E. coli, S. aureus* and *L. monocytogenes*. PMA discriminates dead from live cells and appears to be promising for the enumeration of foodborne pathogens from surfaces. In order to minimize erroneous bacterial survival using the developed PMA-real-time PCR method, PMA concentration may be optimized by performing a heat-treatment at different temperatures of bacterial suspensions and data should be compared with plate counts. Comparable data to plate counts were obtained using PMA-real-time PCR. This method allows rapid quantification assays using DNA standards generated by FACS. This novel methodology requires further development to enable a greater range of bacterial population numbers to be generated for standard curves. However, this report has shown that absolute bacterial quantification using PMA-based real-time PCR was limited to occasions when high bacterial concentrations are present in swab samples. We attribute this lack of sensitivity to cell and DNA loss during sample preparation. However, the method may find an application in factories where highly contaminated

raw materials are processed, in slaughterhouses for instance. The method may then allow the quantification of biofilms in such premises. Another application may be in the assessment of detergents or disinfectants efficiency by assessing the logarithmic bacterial decrease.

## 5. REFERENCES

Anonymous. (1993) Council Directive 93/43/EEC of 14 June 1993 on the hygiene of foodstuffs In E. Commission (Ed.). Official Journal of the European Communities, Brussels, Belgium.

Anonymous. (2001) EC Decision 2001/471/EC - Commission decision of 8 June 2001 laying down rules for the regular checks on the general hygiene carried out by the operators in establishments according to Directive 64/433/EEC on health conditions for the production and marketing of fresh meat and Directive 71/118/EEC on health problems affecting the production and placing on the market of fresh poultry meat. In E. Commission (Ed.). Official Journal of the European Communities, Brussels, Belgium.

Anonymous. (2004) ISO 18593:2004 - Microbiology of food and animal feeding stuffs - Horizontal methods for sampling techniques from surfaces using contact plates and swabs. In. Geneva, Switzerland.

Anonymous. (2008) Reagent D - version 2.0 - For the Elimination of DNA from Dead Cells to Avoid False-positive PCR Results, Biotecon Diagnostics, Potsdam, Germany. In.

Bae, S., and Wuertz, S. (2009) Rapid decay of host-specific fecal Bacteroidales cells in seawater as measured by quantitative PCR with propidium monoazide. *Water Research,* **43**(19), 4850-4859.

Bazaco, M. C., Eifert, J. D., Williams, R. C., and Kathariou, S. (2007) Quantitative recovery of *Listeria monocytogenes* and select *Salmonella* serotypes from environmental sample media. *Journal of Aoac International,* **90**(1), 250-257.

Birch, L., Dawson, C. E., Cornett, J. H., and Keer, J. T. (2001) A comparison of nucleic acid amplification techniques for the assessment of bacterial viability. *Letters in Applied Microbiology,* **33**(4), 296-301.

Bogosian, G., and Bourneuf, E. V. (2001) A matter of bacterial life and death. *Embo Reports,* **2**(9), 770-774.

Bolton, P. H., and Kearns, D. R. (1978) Spectroscopic Properties of Ethidium Monoazide - Fluorescent Photoaffinity Label for Nucleic Acids. *Nucleic Acids Research,* **5**(12), 4891-4903.

Brown, M. H., and Baird-Parker, A. C. (1982) The microbiological examination of meat. In M. H. Brown (Ed.), *Meat Microbiology*.London, England: Applied Science Publishers.

Cox, L. J., Kleiss, T., Cordier, J. L., Cordellana, C., Konkel, P., Pedrazzini, C., Beumer, R., and Siebenga, A. (1989) *Listeria* spp. in food processing, non-food and domestic environments. *Food Microbiology,* **6**(1), 49-61.

Dancer, S. J. (2004) How do we assess hospital cleaning? A proposal for microbiological standards for surface hygiene in hospitals. *Journal of Hospital Infection,* **56**(1), 10-15.

Davidson, C. A., Griffith, C. J., Peters, A. C., and Fielding, L. M. (1999) Evaluation of two methods for monitoring surface cleanliness - ATP bioluminescence and traditional hygiene swabbing. *Luminescence,* **14**(1), 33-38.

Elizaquível, P., Sánchez, G., Salón, N., Cerveró, J., and Aznar, R. (2010) Specific Detection of Viable Foodborne Pathogenic Bacteria in Fresh-Cut Vegetables. In *foodInnova 2010*. Universidad Politechnica de Valencia.

Fedio, W. M., Wendakoon, C. N., Zapata, R., Carrillo, C., and Browning, P. (2008) Comparison of Petrifilm (TM) Staph Express Count System with the Bacteriological Analytical Manual Direct-Plating Method for Enumeration of *Staphylococcus aureus* in Artificially Contaminated Hard Cheese. *Journal of Aoac International,* **91**(5), 1138-1141.

Ferraz, M. A., Cerqueira, M., and Souza, M. R. Evaluation of Enterobacteriaceae in the powdered milk production chain using both traditional (ISO 21528:2) and rapid (3M (TM) Petrifilm (TM)) methods. *Annals of Microbiology,* **60**(2), 373-376.

Fittipaldi, M., Codony, F., Adrados, B., Camper, A., and Morató, J. (2010) Viable Real-Time PCR in Environmental Samples: Can All Data Be Interpreted Directly? *Microbial Ecology,* **61**(1), 7-12.

Flekna, G., Stefanic, P., Wagner, M., Smulders, F. J. M., Mozina, S. S., and Hein, I. (2007) Insufficient differentiation of live and dead *Campylobacter jejuni* and *Listeria monocytogenes* cells by ethidium monoazide (EMA) compromises EMA/real-time PCR. *Research in Microbiology,* **158**(5), 405-412.

Fuster-Valls, N., Hernadez-Herrero, M., Marin-De-Mateo, M., and Rodriguez-Jerez, J. J. (2008) Effect of different environmental conditions on the bacteria survival on stainless steel surfaces. *Food Control,* **19**(3), 308-314.

Garcia-Cayuela, T., Tabasco, R., Pelaez, C., and Requena, T. (2009) Simultaneous detection and enumeration of viable lactic acid bacteria and bifidobacteria in fermented milk by using propidium monoazide and real-time PCR. *International Dairy Journal,* **19**(6-7), 405-409.

Griffith, C. (2005) Improving surface sampling and detection of contamination. In H. L. M. Lelieveld, M. A. Mostert & J. Holah (Eds.), *Handbook of hygiene control in the food industry*.Cambridge, England: Woodhead Publishing in Food Science and Technology.

Griffith, C. J., Cooper, R. A., Gilmore, J., Davies, C., and Lewis, M. (2000) An evaluation of hospital cleaning regimes and standards. *Journal of Hospital Infection,* **45**(1), 19-28.

Holah, J. (2003) Cleaning and disinfection. In H. L. M. Lelieveld, M. A. Mostert & J. Holah (Eds.), *Hygiene in Food Processing*.Cambridge, England: J. Woodhead Publishing Ltd.

Josefsen, M. H., Lofstrom, C., Hansen, T. B., Christensen, L. S., Olsen, J. E., and Hoorfar, J. (2010) Rapid Quantification of Viable *Campylobacter* Bacteria on Chicken Carcasses, Using Real-Time PCR and Propidium Monoazide Treatment, as a Tool for Quantitative Risk Assessment. *Applied and Environmental Microbiology,* **76**(15), 5097-5104.

Josephson, K. L., Gerba, C. P., and Pepper, I. L. (1993) Polymerase Chain Reaction Detection of Non Viable Bacterial Pathogens. *Applied and Environmental Microbiology,* **59**(10), 3513-3515.

Knight, I. T. (2000) Molecular Genetic Methods for detection and Identification of Viable but Nonculturable Microorganisms. In C. R.R. & G. D.J. (Eds.), *Noncultivable microorganisms in the Environment*.Washington DC, USA: ASM Press, 77-86.

Kobayashi, H., Oethinger, M., Tuohy, M. J., Hall, G. S., and Bauer, T. W. Distinction between Intact and Antibiotic-Inactivated Bacteria by Real-Time PCR after Treatment with Propidium Monoazide. *Journal of Orthopaedic Research,* **28**(9), 1245-1251.

Kobayashi, H., Oethinger, M., Tuohy, M. J., Hall, G. S., and Bauer, T. W. (2009a) Improving Clinical Significance of PCR: Use of Propidium Monoazide to Distinguish Viable from Dead *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Journal of Orthopaedic Research,* **27**(9), 1243-1247.

Kobayashi, H., Oethinger, M., Tuohy, M. J., Hall, G. S., and Bauer, T. W. (2009b) Unsuitable distinction between viable and dead *Staphylococcus aureus* and *Staphylococcus epidermidis* by ethidium bromide monoazide. *Letters in Applied Microbiology,* **48**(5), 633-638.

Kusumaningrum, H. D., Riboldi, G., Hazeleger, W. C., and Beumer, R. R. (2003) Survival of foodborne pathogens on stainless steel surfaces and cross-contamination to foods. *International Journal of Food Microbiology,* **85**(3), 227-236.

Luo, J. F., Lin, W. T., and Guo, Y. Method to detect only viable cells in microbial ecology. *Applied Microbiology and Biotechnology,* **86**(1), 377-384.

Martinon, A., Cronin, U. P., and Wilkinson, M. G. (2011a) Comparison of In-house and Commercial Real-time PCR Systems for the Detection of Enterobacteriaceae and their Evaluation Within an Interlaboratory Study Using Infant Formula Samples. *Food Analytical Methods*, DOI 10.1007/s12161-12010-19188-12167

Martinon, A., Cronin, U. P., and Wilkinson, M. G. (2011b) Development of Defined Microbial Population Standards Using Fluorescence Activated Cell Sorting for the Absolute Quantification of *S. aureus* Using Real-Time PCR. *Molecular Biotechnology*, DOI 10.1007/s12033-12011-19417-12033.

Martinon, A., and Wilkinson, M. G. (2011) Selection of optimal primer sets for use in a duplex SYBR Green-based real time PCR protocol for the detection of *L. monocytogenes* and *S. aureus* in foods. *Journal of Food Safety*, DOI 10.1111/j.1745-4565.2011.00301.x.

Mattila, T., Manninen, M., and Kylasiurola, A. L. (1990) Effect of Cleaning-in-place Disinfectants on Wild Bacterial Strains Isolated from a Milking Line. *Journal of Dairy Research,* **57**(1), 33-39.

McKillip, J. L., Jaykus, L. A., and Drake, M. (1998) rRNA stability in heat-killed and UV-irradiated enterotoxigenic *Staphylococcus aureus* and *Escherichia coli* O157 : H7. *Applied and Environmental Microbiology,* **64**(11), 4264-4268.

Miles, A. A., Misra, S. S., and Irwin, J. O. (1938) The estimation of the bactericidal power of the blood. *Journal of Hygiene,* **38**, 732-749

Moore, G., and Griffith, C. (2002a) A comparison of surface sampling methods for detecting coliforms on food contact surfaces. *Food Microbiology,* **19**(1), 65-73.

Moore, G., and Griffith, C. (2002b) A comparison of traditional and recently developed methods for monitoring surface hygiene within the food industry: an industry trial. *International Journal of Environmental Health Research,* **12**(4), 317-329.

Moore, G., and Griffith, C. (2002c) Factors Influencing Recovery of Microorganisms from Surfaces by use of Traditional Hygiene Swabbing. *Dairy, Food and Env. Sanitation,* **22**(6), 410-421.

Moore, G., and Griffith, C. (2007) Problems associated with traditional hygiene swabbing: the need for in-house standardization. *Journal of Applied Microbiology,* **103**(4), 1090-1103.

Mossel, D. A. A., Jansen, J. T., and Struijk, C. B. (1999) Microbiological safety assurance applied to smaller catering operations world-wide. From angst through ardour to assistance and achievement - the facts. *Food Control,* **10**(3), 195-211.

Nocker, A., and Camper, A. K. (2006) Selective removal of DNA from dead cells of mixed bacterial communities by use of ethidium monoazide. *Applied and Environmental Microbiology,* **72**(3), 1997-2004.

Nocker, A., Cheung, C. Y., and Camper, A. K. (2006) Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *Journal of Microbiological Methods,* **67**(2), 310-320.

Nocker, A., Richter-Heitmann, T., Montijn, R., Schuren, F., and Kort, R. Discrimination between live and dead cells in bacterial communities from environmental water samples analyzed by 454 pyrosequencing. *International Microbiology,* **13**(2), 59-65.

Nocker, A., Sossa-Fernandez, P., Burr, M. D., and Camper, A. K. (2007a) Use of propidium monoazide for live/dead distinction in microbial ecology. *Applied and Environmental Microbiology,* **73**(16), 5111-5117.

Nocker, A., Sossa, K. E., and Camper, A. K. (2007b) Molecular monitoring of disinfection efficacy using propidium monoazide in combination with quantitative PCR. *Journal of Microbiological Methods,* **70**(2), 252-260.

Nogva, H. K., Dromtorp, S. M., Nissen, H., and Rudi, K. (2003) Ethidium monoazide for DNA-based differentiation of viable and dead bacteria by 5 '-nuclease PCR. *Biotechniques,* **34**(4), 804-+.

Nyachuba, D. G., and Donnelly, C. W. (2007) Comparison of 3M (TM) Petrifilm (TM) environmental Listeria plates against standard enrichment methods for the detection of *Listeria monocytogenes* of epidemiological significance from environmental surfaces. *Journal of Food Science,* **72**(9), 346-354.

Pan, Y., Breidt, F., and Kathariou, S. (2006) Resistance of *Listeria monocytogenes* biofilms to sanitizing agents in a simulated food processing environment. *Applied and Environmental Microbiology,* **72**(12), 7711-7717.

Paulsen, P., Borgetti, C., Schopf, E., and Smulders, F. J. M. (2008) Enumeration of Enterobacteriaceae in various foods with a new automated most-probable-number method compared with Petrifilm and international organization for standardization procedures. *Journal of Food Protection,* **71**(2), 376-379.

Poimenidou, S., Belessi, C. A., Giaouris, E. D., Gounadaki, A. S., Nychas, G. J. E., and Skandamis, P. N. (2009) *Listeria monocytogenes* Attachment to and Detachment from Stainless Steel Surfaces in a Simulated Dairy Processing Environment. *Applied and Environmental Microbiology,* **75**(22), 7182-7188.

Rudi, K., Moen, B., Dromtorp, S. M., and Holck, A. L. (2005a) Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples. *Applied and Environmental Microbiology,* **71**(2), 1018-1024.

Rudi, K., Naterstad, K., Dromtorp, S. M., and Holo, H. (2005b) Detection of viable and dead *Listeria monocytogenes* on gouda-like cheeses by real-time PCR. *Letters in Applied Microbiology,* **40**(4), 301-306.

Scheu, P. M., Berghof, K., and Stahl, U. (1998) Detection of pathogenic and spoilage micro-organisms in food with the polymerase chain reaction. *Food Microbiology,* **15**(1), 13-31.

Scott, E., and Bloomfield, S. F. (1990) The Survival and Transfer of Microbial Contamination via Cloths, Hands and Utensils. *Journal of Applied Bacteriology,* **68**(3), 271-278.

Sharma, M., and Anand, S. K. (2002) Bacterial biofilm on food contact surfaces: A review. *Journal of Food Science and Technology-Mysore,* **39**(6), 573-593.

Sheridan, G. E. C., Masters, C. I., Shallcross, J. A., and Mackey, B. M. (1998) Detection of mRNA by reverse transcription PCR as an indicator of viability in *Escherichia coli* cells. *Applied and Environmental Microbiology,* **64**(4), 1313-1318.

Silva, B. O., Caraviello, D. Z., Rodrigues, A. C., and Ruegg, P. L. (2005) Evaluation of Petrifilm for the Isolation of *Staphylococcus aureus* from Milk Samples. *Journal of Dairy Science,* **88**(8), 3000-3008.

Szewzyk, U., Szewzyk, R., Manz, W., and Schleifer, K. H. (2000) Microbiological safety of drinking water. *Annual Review of Microbiology,* **54**, 81-127.

Tassinari, A. R., Noda, P. K., Franco, G. M., Landgraf, M., and Destro, M. T. (2006) Express Evaluation - An evaluation of 3M Petrifilm Staph Express Count System for enumerating coagulase-positive *Staphylococcus*. *Food Quality magazine*.

Varma, M., Field, R., Stinson, M., Rukovets, B., Wymer, L., and Hauglanda, R. (2009) Quantitative real-time PCR analysis of total and propidium monoazide-resistant fecal indicator bacteria in wastewater. *Water Research,* **43**(19), 4790-4801.

Verran, J., Boyd, R. D., Hall, K., and West, R. H. (2001) Microbiological and chemical analyses of stainless steel and ceramics subjected to repeated soiling and cleaning treatments. *Journal of Food Protection,* **64**(9), 1377-1387.

Vicosa, G. N., Moraes, P. M., Yamazi, A. K., and Nero, L. A. Enumeration of coagulase and thermonuclease-positive *Staphylococcus* spp. in raw milk and fresh soft cheese: An evaluation of Baird-Parker agar, Rabbit Plasma Fibrinogen agar and the Petrifilm (TM) Staph Express count system. *Food Microbiology,* **27**(4), 447-452.

Wang, S. S., and Levin, R. E. (2006) Discrimination of viable *Vibrio vulnificus* cells from dead cells in real-time PCR. *Journal of Microbiological Methods,* **64**(1), 1-8.

Wang, Z., and Spadoro, J. (1998) Determination of target Copy Number of Quantitative Standards Used in PCR-Based Diagnostic Assays. In F. Ferré (Ed.), *Gene Quantification*.Carlsbad, USA: Birkhäuser, 31-44.

Wilks, S. A., Michels, H., and Keevil, C. W. (2005) The survival of *Escherichia coli* O157 on a range of metal surfaces. *International Journal of Food Microbiology,* **105**(3), 445-454.

Wilks, S. A., Michels, H. T., and Keevil, C. W. (2006) Survival of *Listeria monocytogenes* Scott A on metal surfaces: Implications for cross-contamination. *International Journal of Food Microbiology,* **111**(2), 93-98.

Wolffs, P., Norling, B., and Radstrom, P. (2005) Risk assessment of false-positive quantitative real-time PCR results in food, due to detection of DNA originating from dead cells. *Journal of Microbiological Methods,* **60**(3), 315-323.

# CHAPTER SIX

# General Discussion

This research thesis was undertaken to develop and apply novel methods based on PCR and fluorescence activated cell sorting (FACS) for the detection of the foodborne pathogens: the Enterobacteriaceae, *S. aureus* and *L. monocytogenes*. These microorganisms are important indicators of quality and safety in the food industry and their presence is routinely monitored using traditional agar-based methods following ISO standards with up to 1 week for a confirmatory result. However, the use of rapid methods such as PCR for routine microbial analysis is becoming more frequent in food quality laboratories. On the other hand, FACS is an emerging technology that allows the concentration and the generation of microbial populations of value for the optimisation of PCR methods (see Chapter 1). Alternative methods such as real-time PCR have the potential to generate results in a shorter time than traditional methods, and may also allow direct identification of the foodborne pathogen, using specific detection formats such as probes or primers. Therefore, prior to their widespread application in industry or research food scientists must be provided with clear information regarding their advantages, but also their limitations. In this project, the focus was primarily on the development of real-time PCR procedures for the detection of the above foodborne pathogens in food samples. The objective was to develop simple, lower-cost qualitative detection methods for the target microorganisms and establish their limitations in food analysis as described in chapters 2 and 3. Thereafter in Chapter 4, FACS methodology was developed to determine whether this innovative cytometric technology could be used to improve quantitative real-time PCR methods and also establish their analytical limitations. Finally, in Chapter 5, FACS-based real-time PCR methods were applied and evaluated for the enumeration of viable foodborne pathogens collected from industrial food process surfaces and artificially contaminated model process surfaces.

In Chapter 2 (Martinon and Wilkinson, 2011b), a preliminary evaluation of the main commercial real-time PCR systems for the detection of *S. aureus* and *L. monocytogenes* was carried out to give a critical overview on the performance of such detection kits. These kits included: the foodproof® *Listeria monocytogenes* Detection Kit and the Hygiene Screening System kit supplied by Biotecon Diagnostics (Potsdam, Germany). Overall, the tested commercial molecular kits provided rapid satisfactory data for the separate detection of *L. monocytogenes* and *S. aureus*. Overall, the foodproof® *Listeria monocytogenes* Detection Kit appeared to be more suitable for the food industry as it was confirmed as highly specific for the detection

of *L. monocytogenes* in foods. In contrast, the Hygiene Screening Detection kit allows the detection in multiplex of *Staphylococcus*, *Micrococcus* and *Corynebacterium* species that are mostly found in pharmaceutical environments. This particular real-time PCR system appears to require a high degree of operator experience in the analysis of melting profiles. In this study, two *S. aureus* strains were used to validate the melting curve profiles and compared with the positive control provided by the Hygiene Screening Detection kit using the LightCycler® 1.2. at 640 nm. *S. aureus* strains are theoretically identified with a single peak/shoulder > 64°C according to the manufacturer. This profile was observed for the positive control whereas the tested strains of *S. aureus* were discriminated with difficulty by melting curve analysis. This finding illustrated the potential issues for the operator in the identification of *S. aureus* species. It would appear that more extensive testing of the Hygiene Screening Detection kit may be required using a range of *S. aureus* strains on various LightCycler® 1.2 or 2.0 real-time PCR devices to enable further validation of this system. In addition, the high cost per single reaction for both kits needs to be taken into consideration for routine analysis or research activities.

As an alternative, in-house SYBR Green-based real-time PCR protocols were developed which potentially enable lower cost and simplified real-time PCR assays for the detection of *S. aureus* and *L. monocytogenes*. The highly flexible SYBR Green I was preferred to probes for the development of real-time PCR methods to detect *S. aureus* and *L. monocytogenes* in food samples as it is less expensive and can be detected by all real-time PCR instruments (Nitsche, 2007). Another advantage of SYBR Green I is that the PCR products can be subjected to melting curve analysis (Ririe *et al.*, 1997). However, the intercalating dye binds to any double stranded DNA, notably primer dimers. Therefore, highly specific primers sets were selected, as suggested by Ririe *et al.* (1997), to obtain a single PCR product that was detected by observing a single melting peak for each species. The primer sets were evaluated with a range of foodborne pathogens by PCR, *in-silico* PCR using Primer Blast and real-time PCR analysis. The *hlyA* primers that anneal to a 112-bp fragment of the listeriolysin O gene of *L. monocytogenes* and the *nuc* primers designed to amplify a 279-bp fragment from the gene encoding for a thermonuclease of *S. aureus* were selected. Melting profiles of both PCR products allowed the separate identification of both foodborne pathogens with simplex real-time PCR. A highly reproducible duplex real-time PCR system, including both primer sets, for the simultaneous detection of *S.*

*aureus* and *L. monocytogenes*, provided satisfactory results and melting temperatures were sufficiently different for identification. This duplex real-time PCR method allowed the detection of *S. aureus* or *L. monocytogenes* in food samples enriched only in BPW for 18h. Indeed, BPW may be used as an alternative to selective enrichment media which require hazardous supplements and additional costs in consumables (Duffy *et al.*, 2001). Using the in-house assay, the optimal detection limits were 7 CFU/g in coleslaw for *L. monocytogenes* and 2 CFU/g in raw minced meat for *S. aureus*, as confirmed using commercial kits and plate counting.

However, the main technical issue for this duplex real-time PCR method arises in the case of food samples simultaneously contaminated by *S. aureus* and *L. monocytogenes* where pathogen detection is limited by particular SYBR Green I properties which preferentially binds it to *S. aureus* PCR products. The basis of this result was predictable as SYBR Green I favourably binds to amplicons with the highest Guanine + Cytosine percentage (G+C %) and the largest of the two PCR products (Giglio *et al.*, 2003a). As stated by Giglio *et al*. (2003a) and Gundry *et al.* (2003), SYBR Green may be redistributed from PCR products corresponding to *L. monocytogenes* with lowest melting temperature ($T_M$) to a higher $T_M$ attributed to *S. aureus* PCR products during melting curve analysis. The SYBR Green concentration in the PCR reaction may also influence the redistribution kinetics of the dye but this requires further research. While such data misinterpretation may only appear when food samples are contaminated with both microorganisms, this is unlikely as such a multiple contamination and subsequent food poisoning outbreak have not been described to date.

This duplex real-time PCR method was developed using the LightCycler® FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany). It may be of interest in the future to modify the PCR master mix using other SYBR Green kits in order to evaluate the robustness of the method. Indeed, commercial SYBR Green kits often include PCR additives or preservatives (Chakrabarti and Schutt, 2001) that may modify the binding characteristics of SYBR Green I (Giglio *et al.*, 2003a). As an alternative, the in-house SYBR Green mixes developed in Chapter 2 may be further optimised for duplex real-time PCR, by varying the dye concentration. Giglio *et al.* (2003a) showed that SYBR Green concentration may be an important parameter for the detection of multiple amplicons in multiplex PCR reactions. Other DNA-associating dyes could replace SYBR Green I

for the developed duplex method, SYBR Gold is one such example as it appears to be more stable (Lee *et al.*, 1999) and more sensitive than SYBR Green I (Edwards and Logan, 2009). However, cost may be a factor for consideration as the use of SYBR Gold is not common. In the future, the inclusion of SYBR Green I in the developed method may also be compared to the inclusion of BEBO (M. Bengtsson *et al.*, 2003), YOYO® (Ogura *et al.*, 1994; Srinivasan *et al.*, 1993), YO-PRO®, or BOXTO® (Lind *et al.*, 2006) as detection formats.

In Chapter 3 (Martinon *et al.*, 2011a), an evaluation of the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit – 5'Nuclease was firstly undertaken in order to assess its specificity over a range of Enterobacteriaceae and non-Enterobacteriaceae. The commercial system was subsequently compared to a lower cost in-house method developed to detect the Enterobacteriaceae in foods. It was confirmed that the commercial rapid detection kit was indeed highly specific for the Enterobacteriaceae.

To design the method based on the LUX™ technology, the optimal primer set was first selected among four primer pairs after PCR, *in-silico* PCR using Primer Blast and real-time PCR analysis. The best primer set, selected for its specificity and its sensitivity, was created by associating two primers from two different sets: the forward labelled LUX™ primer from one set and the reverse primer from another, both initial sets targeting a 368 bp-fragment of the *Escherichia coli* 16S ribosomal RNA gene. The main limitation from the newly developed method appears to be the poor species identification by melting curve analysis. Indeed, species discrimination was not feasible due to the low variability of $T_M$ from one species to another. The undesired detection of the Vibrionaceae species also imposes a limitation based on specificity. However, as enrichment of food samples before DNA extraction is of importance, further evaluation of a range of enrichment broths may allow improved specificity by excluding Vibrionaceae should be evaluated. Examples of enrichment broth include: Brain Heart Infusion (BHI) (Nakano *et al.*, 2003), Buffered Peptone Water (BPW) (Joosten *et al.*, 2008b) or BPW supplemented with 40 µM 8-hydroxyquinoline, 0.5 g/L ammonium iron (III) citrate, 0.1 g/L sodium deoxycholate and 0.1 g/L sodium pyruvate (Weber *et al.*, 2009).

The developed in-house method was subsequently evaluated in the analysis of 40 infant milk formula samples provided within the framework of an inter-laboratory study. This study was carried out as part of the MicroVal EN ISO 16140:2003

validation of the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit – 5'Nuclease as an alternative detection method for *Cronobacter sakazakii* and other Enterobacteriaceae. The developed LUX™-based real-time PCR appeared to be as rapid and as sensitive as the commercial detection system and may be a lower cost alternative to the commercial kit. Therefore, the new method allows the food analyst to choose either a lower specificity test at low cost or a higher specificity test at higher cost with the guarantee to identify *C. sakazakii* from other Enterobacteriaceae. No other in-house methods or other commercial real-time PCR systems are available on the food market with the specificity of the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit for safety control in milk samples. However the method developed in Chapter 3 could be a screening system to directly discard negative samples in a first analytical step, while presumptive positive samples could be further analysed using the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit for Enterobacteriaceae confirmation, or even identification of *C. sakazakii*.

Real-time PCR systems such as LUX™-based assays have only been described using a single labelled primer. In Chapter 3, the forward primer was FAM-labelled (carboxyfluorescein). However, it is feasible to label both primers in order to obtain a stronger, if more costly, fluorescent signal (Mackay *et al.*, 2007d), which may increase the sensitivity of the LUX™-based real-time PCR. Martin *et al.* (2010) developed a SYBR-Green-based system for the detection of lactose fermenting Enterobacteriaceae. Therefore, it may be of future interest to design a new LUX™-based primer set targeting a segment of the β-galactosidase gene (*lacZ*) and evaluate this primer set by PCR, *in-silico* PCR and real-time PCR analysis over a range of Enterobacteriaceae and non-Enterobacteriaceae.

In Chapters 2 and 3, the developed real-time PCR methods are simple, low cost and rapid screening solutions for the analysis of food samples in quality and safety laboratories as a first indication of the contamination by the Enterobacteriaceae, *S. aureus* or *L. monocytogenes*. However, the inclusion of internal amplification controls in each method may be advised in further assay developments in respect of the criteria for the validation of real-time PCR procedures. Integrated into quality and safety procedures, these methods may be applied for validation and evaluation of food samples as part of the Hazard Analysis Critical Control Plan and the Good Manufacturing Practices implemented in a food factory. It may be of interest to evaluate the newly developed methods for the analysis of a larger range of food

samples. Indeed, such DNA-based methods must be evaluated according to standardized protocols before being used at large industrial scale. As shown in Chapter 3, interlaboratory assays using defined food samples are necessary to evaluate the suitability and the feasibility of the method to the laboratory. The ISO 16140:2003 procedure is normally used for the validation of an alternative methodology. In regard to this, the inclusivity and exclusivity of both methods developed in Chapters 2 and 3 may be evaluated over a wider range of strains. The developed methods should also be tested over a range of real-time PCR platforms to evaluate their compatibility.

Several technical issues were identified with both the developed detection methods when performing melting curve analysis for identification of PCR products. As an alternative, High Resolution Melting (HRM) analysis has recently been developed for the detection of variations in DNA and applied to species identification or characterization (Choi *et al.*, 2010; Odell *et al.*, 2005; Omiccioli *et al.*, 2009; Robertson *et al.*, 2009; Won *et al.*, 2010). HRM follows the principle of melting curve analysis. However, it usually requires brighter fluorescent dyes at higher concentrations, real-time PCR platforms that collect data at a finer temperature resolution and suitable software which uses new fluorescent scaling algorithms and plots in order to distinguish small differences in melt curve data (Anonymous, 2009b). The PCR products are then identified according to their sequence, length, Guanine – Cytosine (GC) content or strand complementarity (Anonymous, 2009b). In practice, the use of SYBR Green I for HRM has been discouraged by Wittwer *et al.* (2003) and Reed *et al.* (2007). However, Price *et al.* (2007) and Pornprasert *et al.* (2008) support the use of SYBR Green I combined with HRM analysis. Mitchell *et al.* (2009) have developed a real-time PCR method using FAM-labelled LUX™ primers to detect and genotype *Chlamydophila psittaci* by HRM. Therefore, the qualitative detection methods developed in the thesis may in the future be combined with HRM to identify PCR amplicons.

In Chapter 4 (Martinon *et al.*, 2011c), a novel molecular tool was developed for the absolute quantification of microorganisms in samples by real-time PCR. As an alternative to quantification standards expressed in “copy number” or “Colony Forming Unit” per sample volume or mass, known quantities of microorganisms were generated using single-cell isolation by FACS. A comparative analysis of four standards was performed in a SYBR Green-based real-time PCR procedure for the quantification of *S. aureus* cells. In this chapter the following DNA standards were

evaluated: purified *S. aureus* genomic DNA, circular plasmid DNA containing a *nuc* gene fragment, DNA extracted from defined populations of *S. aureus* cells generated by FACS using a MoFlo cell sorter (Beckman Coulter Inc, Fullerton, USA) with or without purification of DNA by boiling. The optimal PCR efficiency of 2.016 was obtained for FACS generated standards. The quality of each quantification process was evaluated using Fieller's Confidence Interval (FCI) Software, an R-based algorithm software developed by Verderio *et al.* (2008) that evaluates PCR efficiencies. However, using this software a "lack of fit" warning message was generated which may have indicated close or identical Crossing points (Cp) values within each standard or a low standard deviation for plasmid and FACS-generated standard curves, as discussed in a personal communication with the software manufacturer. Therefore the FCI algorithm may need to be revised for replicates having a low standard deviation in Ct values when formulating the criteria for "lack of fit" in the specific case where PCR data fit the linear regression. The developed methodology for FACS-generated standards is a novel potential option for quantitative real-time PCR as defined populations are generated prior to DNA extraction and the construction of standard curves. Such standards may avoid inaccurate data arising from use of a UV spectrophotometer that may overestimate the DNA concentration in a sample (Queipo-Ortuno *et al.*, 2008). It is conceivable to imagine the inclusion of ready-to-amplify FACS generated standards into commercial real-time PCR detection kits or their provision as separate components for use in real-time PCR systems. The main advantages may be in the removal of incompatible in-house methods and the subsequent progress towards data standardization among laboratories worldwide (Mackay *et al.*, 2007a). However, this novel methodology requires further development to enable a greater range of bacterial population numbers to be generated for standard curves.

However, it may be of interest to standardize large scale production of FACS-generated standards for inclusion into commercial or in-house quantitative real-time PCR systems. FACS platforms may be optimised and/or dedicated to the production of precise microbiology standards and, inter-laboratory assays may be performed to confirm the accuracy of the technique. The quality of these quantification standards may be compared with commercial BioBall™ spheres containing viable microorganisms produced by a cell sorting cytometer (Morgan *et al.*, 2004). In Chapter 4, the FACS-generated standards were dispensed in Phosphate Buffer Saline

into tubes. In order to obtain DNA standards that get closer to the analysed sample as much as possible, accurate quantities of viable bacteria may be sorted and resuspended into diluent containing sample matrix or a synthetic food formulation to mimic the biochemical background.

The stability of the four standard types in sterile DNA grade water from 0 to 1 month at -20°C was investigated. From the data, it is recommended to maintain the FACS-generated standards for up to seven days for re-use or to prepare fresh diluted standards from an aliquoted DNA stock solution kept at -20°C. The storage conditions may be optimised in order to improve stability over time. Future studies may involve an evaluation of different storage conditions including: preservation of DNA standards in 50% glycerol (Schaudien *et al.*, 2007) 50% glycerol-double-distilled water (Roder *et al.*, 2010), Tris:$Na_2$EDTA (Farkas *et al.*, 1996) or lyophilisation (Morgan *et al.*, 2004; Visvikis *et al.*, 1998).

In Chapter 5, the SYBR Green and LUX™-based real-time PCR procedures developed in chapters 2 and 3 including novel DNA standards generated by FACS (see Chapter 4) were modified to include a photoactivable dye in order to eliminate DNA from dead cells. The methods were then applied to the quantification of viable *E. coli*, *S. aureus* or *L. monocytogenes* cells in swab samples. It appeared that, among the two photoactivable DNA-intercalating dyes, Reagent D was unsuitable for enumeration of viable *S. aureus* or *L. monocytogenes* and could only be applied to *E. coli*. Indeed, this chemical agent may have toxic effects on *S. aureus* and *L. monocytogenes* cells as suggested by Elizaquível *et al.* (2010). In the future, Reagent D may be tested over a larger range of Enterobacteriaceae and non-Enterobacteriaceae to increase the specificity of the LUX-based method for Enterobacteriaceae developed in Chapter 3. The use of PMA was more appropriate as this DNA-binding agent allowed the enumeration of the three foodborne pathogens in the viable state only, with no significant difference from plate counts. To optimise the PMA-based real-time PCR procedures, PMA concentration may be subsequently adjusted by performing a heat-treatment at different temperatures of bacterial suspensions and evaluating various PMA concentrations. Methodologies including PMA real-time PCR should always involve data comparison with plate counting.

Before implementing the real-time PCR procedures on naturally contaminated surfaces, it was of interest to assess the hygienic status of food contact surfaces within a commercial frozen meal factory (Dawn Fresh Foods, Fethard) with a particular

focus on the Enterobacteriaceae, *S. aureus* and *L. monocytogenes*. From the surfaces collected by swab technique, neither *S. aureus*, neither *L. monocytogenes* were detected while low Total Viable (<10 CFU.cm$^{-2}$) and Enterobacteriaceae (<1 CFU.cm$^{-2}$) counts were detected. Therefore, a real-time PCR method could not be evaluated on these industrial surfaces as low bacterial levels were indicated using traditional plating methods and therefore DNA loss may occur after extraction and dilution of the sample in the PCR reaction. Hence, the real-time PCR systems were applied to artificially contaminated surfaces. Stainless steel coupons were manufactured on site and then contaminated with a single suspension prepared by pooling of swab samples collected in the plant to create an artificial food process environment and inoculated with *E. coli*, *S. aureus* or *L. monocytogenes*. After different allocated times of storage at room temperature, the coupons were swabbed, the cells were recovered from each bud using a Swab Extraction Tube System (SETS). To date, SETS has been mainly used for virus sample collection and in this study it appeared to be a rapid and novel tool for bacterial recovery from surfaces and may find further application in the food industry. However, additional studies on this system may be required in order to optimise the recovery as a function of the species by adjusting the centrifugation step. It may be conceivable to design a new system combining SETS and a DNA extraction column. Following a centrifugation step, the recovered cells would settle at the top of the column and would avoid additional pipetting. Bacterial recoveries using SETS and sonication should also be compared in order to assess cell viability.

It appeared that the PMA-based methods combined with SETS could be only applied for enumeration from surfaces with high bacterial densities >$10^6$ CFU.100 cm$^{-2}$. Such contamination levels are not routinely found on cleaned food contact surfaces in the food industry and at lower levels, the PMA-based real-time PCR may not be applied as false negative may be obtained. The developed PMA-based real-time PCR may be limited to surfaces with high bacterial densities. However, this method may find an application in factories where highly contaminated raw materials are processed, in slaughterhouses for instance on animal carcasses. Bacterial quantification by PMA real-time PCR is feasible at least on artificially made biofilms as on the contaminated stainless steel coupons (Guilbaud *et al.*, 2005; Michu *et al.*, 2010). Another application may be the assessment of the efficiency of detergents or disinfectants or assessing bactericidal activity by the logarithmic bacterial decrease.

In summary, the innovative PCR and FACS-based methodologies developed in this thesis were shown to be highly useful for the detection of foodborne pathogens such as the Enterobacteriaceae, *S. aureus* and *L. monocytogenes*. In qualitative pathogen detection, rapid, simple and low cost real-time PCR methodologies were generated as alternative solutions to commercial molecular detection systems. The development of highly reproducible and sensitive simplex and duplex SYBR Green-based methods allowed the simultaneous detection of *S. aureus* and *L monocytogenes* in food samples whereas a screening LUX™-based method was designed for the detection of the Enterobacteriaceae. The principle of combining PCR with FACS was demonstrated as a novel quantification strategy in real-time PCR. The improved accuracy of DNA standards from sorted cells demonstrated possible applications in bacterial enumeration. Bacterial recoveries of the three pathogens were then studied using the above methods with a cell pre-treatment using PMA, with good agreement found between plate counts and real-time PCR data. While the PCR / FACS quantification scheme requires further development to ascertain it's potential, this novel approach has been shown to be highly promising for microbial analysis. Overall, this thesis has further developed the connection between the emerging technology of FACS with that of an established molecular biology PCR to improve quantitative detection of a range of microorganisms of interest to the food industry.

## REFERENCES

Anonymous (2009) 'A Guide to High Resolution Melting (HRM) Analysis, Applied Biosystems, Life Technologies Corporation.'

Bengtsson, M., Karlsson, H. J., Westman, G. and Kubista, M. (2003) 'A new minor groove binding asymmetric cyanine reporter dye for real-time PCR', *Nucleic Acids Research,* 31(8).

Chakrabarti, R. and Schutt, C. E. (2001) 'The enhancement of PCR amplification by low molecular-weight sulfones', *Gene,* 274(1-2), 293-298.

Choi, G. E., Lee, S. M., Yi, J., Hwang, S. H., Kim, H. H., Lee, E. Y., Cho, E. H., Kim, J. H., Kim, H.-J. and Chang, C. L. (2010) 'High-Resolution Melting Curve Analysis for Rapid Detection of Rifampin and Isoniazid Resistance in Mycobacterium tuberculosis Clinical Isolates', *J. Clin. Microbiol.,* 48(11), 3893-3898.

Duffy, G., Walsh, D., Sheridan, J. J., Logue, C. M., Harrington, D., Blair, I. S. and McDowell, D. A. (2001) 'Comparison of selective and non-selective enrichment media in the detection of *Listeria monocytogenes* from meat containing *Listeria innocua*', *Journal of Applied Microbiology,* 90(6), 994-999.

Edwards, K. J. and Logan, J. M. J. (2009) 'Performing Real-Time PCR' in Logan, J., Edwards, K. and Saunders, N., eds., *Real-Time PCR - Current Technology and Applications*, Norfolk, UK: Caister Academic press.

Elizaquível, P., Sánchez, G., Salón, N., Cerveró, J. and Aznar, R. (2010) 'Specific Detection of Viable Foodborne Pathogenic Bacteria in Fresh-Cut Vegetables', in *foodInnova 2010*, Universidad Politechnica de Valencia,

Farkas, D. H., Kaul, K. L., Wiedbrauk, D. L. and Kiechle, F. L. (1996) 'Specimen collection and storage for diagnostic molecular pathology investigation', *Archives of Pathology & Laboratory Medicine,* 120(6), 591-596.

Giglio, S., Monis, P. T. and Saint, C. P. (2003) 'Demonstration of preferential binding of SYBR Green I to specific DNA fragments in real-time multiplex PCR', *Nucleic Acids Research,* 31(22), 5.

Guilbaud, M., de Coppet, P., Bourion, F., Rachman, C., Prevost, H. and Dousset, X. (2005) 'Quantitative detection of *Listeria monocytogenes* in biofilms by real-time PCR', *Applied and Environmental Microbiology,* 71(4), 2190-2194.

Gundry, C. N., Vandersteen, J. G., Reed, G. H., Pryor, R. J., Chen, J. and Wittwer, C. T. (2003) 'Amplicon melting analysis with labelled primers: A closed-tube method for differentiating homozygotes and heterozygotes', *Clinical Chemistry,* 49(3), 396-406.

Joosten, H., Marugg, J., Stephan, R., Klijn, A., Jackson, T. and Iversen, C. (2008) 'A rapid and reliable alternative to ISO 21528-1: 2004 for detection of Enterobacteriaceae', *International Journal of Food Microbiology,* 125(3), 344-346.

Lee, M. A., Brightwell, G., Leslie, D., Bird, H. and Hamilton, A. (1999) 'Fluorescent detection techniques for real-time multiplex strand specific detection of *Bacillus anthracis* using rapid PCR', *Journal of Applied Microbiology,* 87(2), 218-223.

Lind, K., Stalberg, A., Zoric, N. and Kubista, M. (2006) 'Combining sequence-specific probes and DNA binding dyes in real-time PCR for specific nucleic acid quantification and melting curve analysis', *Biotechniques,* 40(3), 315-319.

Mackay, I. M., Bustin, S. A., Andrade, J. M., Kubista, M. and Sloots, T. P. (2007a) 'Quantification of Microorganisms: not Human, not simple, not Quick' in Mackay, ed. *Real-Time PCR in Microbiology*, Norfolk, UK: Caister Academic Press, 133-182.

Mackay, I. M., Mackay, J. F., Nissen, M. D. and Sloots, T. P. (2007b) 'Introduction to Real-time PCR' in Mackay, ed. *Real-Time PCR in Microbiology*, Norfolk, UK: Caister Academic Press, 1-39.

Martin, M. C., Martinez, N., del Rio, B., Ladero, V., Fernandez, M. and Alvarez, M. A. (2010) 'A novel real-time polymerase chain reaction-based method for the detection and quantification of lactose-fermenting Enterobacteriaceae in the dairy and other food industries', *Journal of Dairy Science,* 93(3), 860-867.

Martinon, A., Cronin, U. P. and Wilkinson, M. G. (2011a) 'Comparison of In-house and Commercial Real-time PCR Systems for the Detection of Enterobacteriaceae and their Evaluation Within an Interlaboratory Study Using Infant Formula Samples', *Food Analytical Methods*, DOI 10.1007/s12161-010-9188-7

Martinon, A., Cronin, U. P. and Wilkinson, M. G. (2011b) 'Development of Defined Microbial Population Standards Using Fluorescence Activated Cell Sorting for the Absolute Quantification of *S. aureus* Using Real-Time PCR', *Molecular Biotechnology*, DOI 10.1007/s12033-011-9417-3.

Martinon, A. and Wilkinson, M. G. (2011) 'Selection of optimal primer sets for use in a duplex SYBR Green-based real-time PCR protocol for the detection of *L. monocytogenes* and *S. aureus* in foods', *Journal of Food Safety*, DOI 10.1111/j.1745-4565.2011.00301.x.

Michu, E., Cervinkova, D., Babak, V., Kyrova, K. and Jaglic, Z. (2010) 'Biofilm formation on stainless steel by *Staphylococcus epidermidis* in milk and influence of glucose and sodium chloride on the development of ica-mediated biofilms', *International Dairy Journal,* 21(3), 179-184.

Mitchell, S. L., Wolff, B. J., Thacker, W. L., Ciembor, P. G., Gregory, C. R., Everett, K. D. E., Ritchie, B. W. and Winchell, J. M. (2009) 'Genotyping of *Chlamydophila psittaci* by Real-Time PCR and High-Resolution Melt Analysis', *J. Clin. Microbiol.,* 47(1), 175-181.

Morgan, C. A., Bigeni, P., Herman, N., Gauci, M., White, P. A. and Vesey, G. (2004) 'Production of precise microbiology standards using flow cytometry and freeze drying', *Cytometry Part A,* 62A(2), 162-168.

Nakano, S., Kobayashi, T., Funabiki, K., Matsumura, A., Nagao, Y. and Yamada, T. (2003) 'Development of a PCR assay for detection of Enterobacteriaceae in foods', *Journal of Food Protection,* 66(10), 1798-1804.

Nitsche, A. (2007) 'Oligonucleotide Design for In-house real-time PCR Applications in Microbiology' in Mackay, ed. *Real-Time PCR in Microbiology*, Norfolk, UK: Caister Academic Press, 41-69.

Odell, I. D., Cloud, J. L., Seipp, M. and Wittwer, C. T. (2005) 'Rapid species identification within the *Mycobacterium chelonae-abscessus* group by high-resolution melting analysis of hsp65 PCR products', *American Journal of Clinical Pathology,* 123(1), 96-101.

Ogura, M., Keller, C., Koo, K. and Mitsuhashi, M. (1994) 'Use of the Fluorescent Dye YOYO-1 to Quantify Oligonucleotides Immobilized on Plastic plates', *Biotechniques,* 16(6), 1032-1034.

Omiccioli, E., Amagliani, G., Brandi, G. and Magnani, M. (2009) 'A new platform for Real-Time PCR detection of *Salmonella spp*., *Listeria monocytogenes* and *Escherichia coli* O157 in milk', *Food Microbiology,* 26(6), 615-622.

Pornprasert, S., Phusua, A., Suanta, S., Saetung, R. and Sanguansermsri, T. (2008) 'Detection of alpha-thalassemia-1 Southeast Asian type using real-time gap-PCR with SYBR Green1 and high resolution melting analysis', *European Journal of Haematology,* 80(6), 510-514.

Price, E. P., Smith, H., Huygens, F. and Giffard, P. M. (2007) 'High-resolution DNA melt curve analysis of the clustered, regularly interspaced short-palindromic-repeat locus of *Campylobacter jejuni*', *Applied and Environmental Microbiology,* 73(10), 3431-3436.

Queipo-Ortuno, M. I., Colmenero, J. D. D., Macias, M., Bravo, M. J. and Morata, P. (2008) 'Preparation of bacterial DNA template by boiling and effect of immunoglobulin G as an inhibitor in real-time PCR for serum samples from patients with brucellosis', *Clinical and Vaccine Immunology,* 15(2), 293-296.

Reed, G. H., Kent, J. O. and Wittwer, C. T. (2007) 'High-resolution DNA melting analysis for simple and efficient molecular diagnostics', *Pharmacogenomics,* 8(6), 597-608.

Ririe, K. M., Rasmussen, R. P. and Wittwer, C. T. (1997) 'Product differentiation by analysis of DNA melting curves during the polymerase chain reaction', *Analytical Biochemistry,* 245(2), 154-160.

Robertson, T., Bibby, S., O'Rourke, D., Belfiore, T., Lambie, H. and Noormohammadi, A. H. (2009) 'Characterization of Chlamydiaceae species using PCR and high resolution melt curve analysis of the 16S rRNA gene', *Journal of Applied Microbiology,* 107(6), 2017-2028.

Roder, B., Fruhwirth, K., Vogl, C., Wagner, M. and Rossmanith, P. (2010) 'Impact of long-term storage on the stability of standard DNA for nucleic acid based methods', *J. Clin. Microbiol.*, JCM.01230-10.

Schaudien, D., Baumgartner, W. and Herden, C. (2007) 'High preservation of DNA standards diluted in 50% glycerol', *Diagnostic Molecular Pathology,* 16(3), 153-157.

Srinivasan, K., Morris, S. C., Girard, J. E., Kline, M. C. and Reeder, D. J. (1993) 'Enhanced detection of PCR products through use of TOTO and YOYO intercalating dyes with laser induced fluorescence: Capillary electrophoresis', *Applied and Theoretical Electrophoresis,* 3(5), 235-239.

Verderio, P., Pizzamiglio, S., Gallo, F. and Ramsden, S. C. (2008) 'FCI: an R-based algorithm for evaluating uncertainty of absolute real-time PCR quantification', *Bmc Bioinformatics,* 9, 7.

Visvikis, S., Schlenck, A. and Maurice, M. (1998) 'DNA extraction and stability for epidemiological studies', *Clinical Chemistry and Laboratory Medicine,* 36(8), 551-555.

Weber, C., Stephan, R., Druggan, P., Joosten, H. and Iversen, C. (2009) 'Improving the enrichment procedure for Enterobacteriaceae detection', *Food Microbiology,* 26(6), 565-572.

Wittwer, C. T., Reed, G. H., Gundry, C. N., Vandersteen, J. G. and Pryor, R. J. (2003) 'High-resolution genotyping by amplicon melting analysis using LCGreen', *Clinical Chemistry,* 49(6), 853-860.

Won, H., Rothman, R., Ramachandran, P., Hsieh, Y. H., Kecojevic, A., Carroll, K. C., Aird, D., Gaydos, C. and Yang, S. (2010) 'Rapid Identification of Bacterial Pathogens in Positive Blood Culture Bottles by Use of a Broad-Based PCR Assay Coupled with High-Resolution Melt Analysis', *Journal of Clinical Microbiology,* 48(9), 3410-3413.

# APPENDIX ONE

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# SELECTION OF OPTIMAL PRIMER SETS FOR USE IN A DUPLEX SYBR GREEN-BASED, REAL-TIME POLYMERASE CHAIN REACTION PROTOCOL FOR THE DETECTION OF *LISTERIA MONOCYTOGENES* AND *STAPHYLOCCOCUS AUREUS* IN FOODS

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## ABSTRACT

A low-cost duplex SYBR Green-based, real-time polymerase chain reaction (PCR) for the simultaneous detection of *Listeria monocytogenes* and *Staphyloccocus aureus* in foods was developed following selection of optimal primers. For *L. monocytogenes*, the set targeting the listeriolysin O gene (*hlyA* primers) was more specific than the one annealing to the metalloprotease gene (*mpl* primers). For *S. aureus*, the *nuc* primers targeting the thermonuclease gene were highly specific. Simplex SYBR Green-based, real-time PCR methods for the separate detection of *L. monocytogenes* and *S. aureus* were performed. Finally, the developed duplex real-time PCR was applied to foods spiked with these microorganisms using a simple enrichment step in buffered peptone water at 37C for 18 h. Melting temperatures were sufficiently different for identification with intra and inter-assay coefficients of variation in melting temperature of 0.08% and 0.20%, respectively. Detection limits were 7 colony-forming unit (cfu)/g in coleslaw for *L. monocytogenes* and 2 cfu/g in raw minced meat for *S. aureus*, as confirmed using the commercial kits and plate counting.

## PRACTICAL APPLICATIONS

This multiplex real-time polymerase chain reaction method provides a potential two-in-one screening enabling simultaneous detection of positive samples of *S. aureus* and *L. monocytogenes* in minimally enriched food samples. Considering the simplicity, low cost, rapidity and acceptable reproducibility shown for the detection of *S. aureus* and *L. monocytogenes*, this duplex method may be used in food analysis prior to further potentially more expensive investigations by giving an initial indication of contamination and discarding of negative samples. This method may contribute to the validation and the verification of hazard analysis critical control plans, good hygiene practices and the acceptability of batches in food industries. In regard to European Community microbiological criteria regulation, this alternative method may be applied especially for the analysis of *L. monocytogenes* in ready-to-eat foods and *S. aureus* in dairy products.

## INTRODUCTION

Most outbreaks of food poisoning are related to ingestion of contaminated foods arising from time and temperature abuse, inadequate preparation or insufficient hygiene during production. *Listeria monocytogenes* and *Staphyloccocus aureus* are of major concern in the food industry as their presence may indicate a failure of quality control systems during production. European Community regulations (Anon 2005) require conventional culture methods according to International Organization for Standardization (ISO) standards in official food control laboratories. In contrast, an increasing

number of commercial real-time polymerase chain reaction (PCR) kits for qualitative detection of such foodborne pathogens are becoming available (Choudhurry *et al.* 2006; Jasson *et al.* 2010; Oravcova *et al.* 2007) in either simplex or multiplex systems. Overall, ready-to-use PCR systems, while being convenient for qualitative detection, are still regarded by the food industry as being too expensive to be applied for routine analysis. The uptake of real-time PCR for routine testing by the food industry would therefore be advanced by the availability of standardized protocols enabling detection of multiple pathogens. Despite the increasing commercial availability of PCR and real-time PCR kits (Glynn *et al.* 2006), standardized and simplified real-time PCR methods are not in regular use in food quality applications.

Development of simplex real-time PCR methods applicable to food microbiology is ongoing with a range of in-house protocols reported for the detection of *L. monocytogenes* (O'Grady *et al.* 2009) or *S. aureus* (Chiang *et al.* 2007; Trncikova *et al.* 2009) in enriched food samples. Such methodology may be extended to the simultaneous detection of multiple target DNA sequences via multiplex real-time PCR (Fukushima *et al.* 2003; Wang *et al.* 2004; Gubala 2006; Elizaquivel and Aznar 2008; Lee *et al.* 2009; Omiccioli *et al.* 2009).

The purpose of multiplex PCR is to simultaneously amplify segments of target DNA, using more than one pair of primers in the assay, where time and reaction costs are minimized (Shi *et al.* 2010). Multiplex PCR methods have been recently reported for the simultaneous detection of *L. monocytogenes* and *S. aureus* in foods and broth cultures (Kim *et al.* 2007; Kobayashi *et al.* 2009; Kumar *et al.* 2009; Zhang *et al.* 2009). However, limitations include being time consuming and conventional multiplex PCR involves additional costs in chemicals and machine features when using probes for multiplex-based, real-time PCR procedures. In contrast, SYBRGreen I, a commonly used fluorescent dye, does not require specific and costly probe design, is easy to use (Kugelman *et al.* 2009), and binding is not affected by potential mutations of the target gene (Guilbaud *et al.* 2005). Aldea *et al.* (2002) and Fernandez *et al.* (2006) commented on the simpler and cheaper use of SYBR Green I in comparison with the increased handling and the possible loss of detection found for probes. Indeed probes include many fluorescent labels, instead of just one molecule, which is inserted into the amplicon. The SYBR Green I detection format is suitable for melting curve analysis where PCR products can be identified as a function of their melting temperature (Tm). However, as SYBR Green I binds to any double stranded DNA, notably primer-dimers, the primer sets used must be highly specific and generate a single PCR product (Ririe *et al.* 1997). To date, multiplex real-time PCR systems or commercial kits for the simultaneous detection of *L. monocytogenes* and *S. aureus*, using a low cost detection format, have yet to be developed.

This study was therefore undertaken to develop a duplex SYBR Green-based protocol as an alternative simple lower cost method for the simultaneous detection of *L. monocytogenes* and *S. aureus*. Two commercial real-time PCR systems were evaluated for the detection of both microorganisms. Then, suitable primer sets were selected for the detection of *L. monocytogenes* and *S. aureus*, using PCR and Primer Blast software. Subsequently, optimal primer sets were evaluated in a simplex SYBR Green-based, real-time PCR, and finally a duplex SYBR Green-based, real-time PCR method was developed and evaluated for the simultaneous detection of *L. monocytogenes* and *S. aureus* in minimally enriched food samples spiked with known bacterial levels and compared with the commercial PCR systems and traditional plate method.

## MATERIALS AND METHODS

### Bacterial Strains

Type strains of *L. monocytogenes* (ATCC 19115, 13451), *Vibrio parahaemolyticus* (ATCC 17802), *Aeromonas hydrophila* (ATCC 7966) and *Campylobacter jejuni* (ATCC 29428) and Enterobacteriaceae: *Escherichia coli* (ATCC 11775), *Serratia marcescens* (ATCC 13880), *Enterobacter aerogenes* (ATCC 13048), *Salmonella typhimurium* (ATCC 13311), *Erwinia persicina* (ATCC 1381), *Shigella flexneri* (ATCC 9199), *Klebsiella pneumoniae* (ATCC 700603), *Yersinia enterocolitica* (ATCC 9610), were obtained from Microbiologics Inc, Saint Cloud, MN. *S. aureus* (NCTC 8325) was obtained from the National Collection of Type Cultures (Health Protection Agency Culture Collections, Salisbury, U.K.). *S. aureus* UL (University of Limerick) isolate, *Cronobacter sakazakii*, *Enterobacter cloacae*, *Pseudomonas aeruginosa* and *Proteus mirabilis* were obtained from the culture collection of the Department of Life Sciences, University of Limerick. These strains were selected as most of them are foodborne pathogens themselves or they may be present in the background flora within food samples. Strains were stored on Protect beads 109 (Langen-Bach Services Ltd., Dublin, Ireland) at –20C until cultivation.

### Culture Conditions

Pure cultures were prepared for primer optimisation. *L. monocytogenes*, *S. aureus* and the *Enterobacteriaceae* were grown overnight on nutrient agar (NA; Oxoid Ltd., Basingstoke, U.K.) at 37C, except *Erwinia persicina*, which was incubated 30C. *V. parahaemolyticus* was grown overnight at 35C on tryptic soya agar (TSA; Oxoid Ltd.). *A. hydrophila* was grown overnight at 35C on Columbia blood agar (Oxoid Ltd.). *P. aeruginosa* was incubated overnight on nutrient agar at 25C. *C. jejuni* was grown on Columbia blood agar in a microaerophilic environment (CampyGen 2.5 L, Oxoid Ltd.)

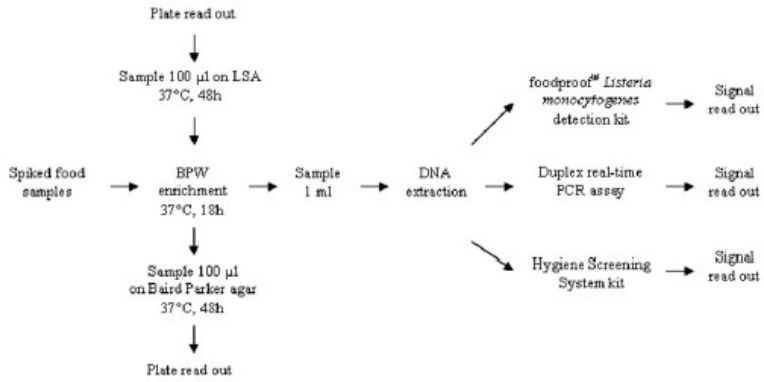



**FIG. 1.** ANALYSIS WORK FLOW OF THE ARTIFICIALLY INOCULATED FOOD SAMPLES

for 72 h at 37C. Prior to DNA extraction, a preculture of each strain was made by inoculating one loopful of each culture into a flask of 30 mL of nutrient broth (NB; Oxoid Ltd.) with overnight shaking at 37C for *S. aureus* and the Enterobacteriaceae, except for *E. percisina* that was cultured at 30C. Tryptic soya broth (TSB; Oxoid Ltd.) was used for growth of *L. monocytogenes* (37C), *Vibrionaceae* (35C) and *P. aeruginosa* (25C). Following this, 300 µL of preculture was transferred into 30 mL of fresh broth. A culture of each strain was obtained under the conditions outlined above and grown to exponential growth phase. *C. jejuni* colonies grown on a Columbia agar plate were suspended in 1 mL of 0.85% saline sterile water prior to DNA extraction.

For spiking of food samples, cultures were prepared by inoculating separately one loopful of *L. monocytogenes* in 30 mL of TSB and one loopful of *S. aureus* in 30 mL of NB with overnight shaking at 37C. Expected bacterial concentrations were ~$10^9$ colony forming unit (cfu)/mL. The inocula were serially diluted to obtain the required bacterial concentration for spiking.

## Analysis of Artificially Inoculated Food Samples

A range of food samples were purchased from local supermarkets in Limerick city including: ready-to-eat meals tagliatelle and ham, chicken supreme, vegetable soup, chicken soup, cottage pie, ice cream, coleslaw, cottage cheese and raw minced beef. The food samples were prepared in triplicate according to standard methods ISO 8261:2002 (milk and milk products), ISO 6887-2:2003 (meat and meat products), ISO 6887-4:2003 (products other than milk and milk products, meat and meat products and fish and fishery products), ISO 6887-1:1999 (preparation of test samples for microbiological examination), and were artificially inoculated according to ISO 16140:2003 Microbiology of food and animal feeding stuffs – Protocol for the validation of alternative methods) (Anon 1999, 2002, 2003a,b,d). The food samples were treated as described in Fig. 1. 25 g of each food were previously inoculated with 1 mL of overnight culture for *L. monocytogenes* or *S. aureus* to achieve final concentrations of the order of 1, 10, 100 or $1 \times 10^3$ cfu per 25 g of food. Negative samples consisted of preparing a food sample without any bacterial inoculation. Each food sample was transferred into 225 mL of buffered peptone water (BPW; Oxoid) and homogenized bags (Seward Medical, London, U.K.) using a Blender Stomacher 400 (Seward Medical). A simple enrichment consisted of incubating the contents of each stomacher bag for 18 h at 37C.

After incubation, the presence of *L. monocytogenes* and *S. aureus* was confirmed by plating out 100 µL of each enrichment culture on *Listeria* selective agar (LSA; Oxoid) for *L. monocytogenes* or Baird Parker agar (Oxoid) for *S. aureus*. Plates were incubated at 37C for 48 h. Typical *L. monocytogenes* colonies on LSA were black surrounded by a black halo. Characteristic colonies of *S. aureus* on Baird Parker agar were gray-black, shiny, convex and surrounded by a zone of clearing.

## DNA Extractions

DNA used for primer optimisation was extracted following manufacturer's instructions for Gram negative or Gram positive bacteria using a DNeasy Blood and Tissue Kit (Qiagen, Crawley, U.K.) based on DNA purification through chromatography columns. For each strain, DNA was extracted from pellets obtained from cultures in the exponential growth phase following centrifugation of 1 mL of a bacterial suspension at 5,000 × g for 10 min in a Sigma centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). DNA quantifications were performed by spectrophotometry at 260 nm using a Nanodrop Spectrophotometer ND-1000 (Thermo Scientific, Wilmington, DE). Finally, DNA concentrations were adjusted to 1 ng per volume depending on the

PCR protocol. A mix of *L. monocytogenes* and *S. aureus* DNA was also prepared at final DNA concentrations of 1 ng/2 µL for each DNA species.

For food sample analysis, the DNA isolation using DNeasy Blood and Tissue Kit was assessed on 1 mL of BPW enrichment culture, following the manufacturer's instructions.

The sensitivity of DNA detection in pure cultures was determined by serially diluting *L. monocytogenes* or *S. aureus* DNA to 3 copies per 2 µL. Assuming that the target gene was present as a single-copy in the 2.9-mbp-sized genome (Nelson *et al.* 2004), 1 ng of *L. monocytogenes* DNA therefore equals $3.1 \times 10^5$ copies of the entire genome. From the 2.7- to 2.8-mbp-sized genome of *S. aureus* (Mlynarczyk *et al.* 1998), 1 ng of *S. aureus* DNA corresponds to $6.10^5$ copies of the entire genome provided the target gene is present as a single copy.

## Commercial Real-Time PCR Systems

**foodproof® *Listeria monocytogenes* Detection Kit.** This commercial kit (Biotecon Diagnostics GmbH, Potsdam, Germany) uses sequence-specific primers and probes that amplify and simultaneously detect both a 149-bp fragment of a metalloprotease gene (*mpl*) located within the virulence cluster of *L. monocytogenes* and a fragment of the Internal Control (IC) (Anon 2007a). This IC contained chimeric non-relevant DNA added to the master mix amplifiable by the same primer set as the target DNA. Each reaction mix or glass capillary contained 2 µL of foodproof® *Listeria monocytogenes* Detection Mix, which included HybProbes probes 10X, 2 µL of foodproof® Enzyme Master Mix, 10X concentrated, and 1 µL foodproof® Uracil-DNA Glycosylase made up with PCR-grade water to 15 µL. The Enzyme Master Mix itself contains the foodproof® Enzyme Solution combined with foodproof® Reaction Mix including primer sets, dUTPs, DNA polymerase and $MgCl_2$. The foodproof® Uracil-DNA Glycosylase was included to prevent cross contaminations occurring with DNA residues containing uracil-glycosidic bonds at U-DNA sites, as dUTP instead of dNTP may have been used in previous runs for the synthesis of PCR products. Five microliters of sample DNA were added to each PCR reaction.

The positive control consisted of a PCR reaction where the template DNA is replaced by a control DNA, a stabilized plasmid solution, provided with the kit (foodproof® Control Template). In the case of the negative control, template DNA was replaced by PCR-grade water. Experiments were carried out using a LightCycler® 1.2 (Roche Diagnostics GmbH, Mannheim, Germany). Amplification conditions were as follows: one cycle of 37C for 2 min and 95C for 10 min, followed by 45 cycles of heating at 20C/s to 95C, holding at 95C for 0 s, cooling at 20C/s to 59C, holding at 59C for 30 s where fluorescence was acquired (single acquisition), heating to 72C, and holding at 72C for 5 s. After amplification, a melting curve analysis was performed by heating at 20C/s to 95C for 1 min, cooling at 20C/s to 40C for 1 min, and slowly heating at 0.1C/s to 80C with fluorescence collection in continuous acquisition mode. The final step involved cooling to 40C during 30 s at 20C/s. HybProbes probes, contained in the foodproof® Detection Mix, were designed to bind specifically to the IC, allowing detection in channel F3 or 705 nm, whereas the *L. monocytogenes* DNA was detected in channel F2 or 640 nm.

The use of this kit required the creation of a color compensation file or color compensation object with the LightCycler® 1.2. This was a prerequisite for the unambiguous discrimination of *L. monocytogenes* DNA and Internal Control DNA amplification in the dual-color experiment. As crosstalk may be observed between single channels when using differently labelled HybProbe probes in multiplex dual-color experiments the LightCycler® Color Compensation Set (Roche Diagnostics GmbH) was used to compensate for this event. The generated color-compensation file was used in all subsequent real-time PCR experiments with the foodproof® *Listeria monocytogenes* detection kit.

**Hygiene Screening System Kit for *S. aureus*.** The Hygiene Screening System kit (Anon 2007b) is designed for the rapid identification of *Staphylococcus* spp., *Micrococcus* spp. or *Corynebacterium* from environmental samples in pharmaceutical industries. A melting curve analysis is used to identify *S. aureus* from other species detected in a sample by this kit. A 20-µL standard reaction contains a ready-to-use primer and HybProbe (Biotecon Diagnostics) mix for the specific amplification and detection of DNA of *Staphylococcus* spp. (Hygiene Screening System Master Mix, 16 µL) to which 1 µL of Enzyme Mix (Taq Polymerase/UNG; Biotecon Diagnostics) is added separately. A stabilized solution of plasmid DNA acting as an internal control is provided to check the quality of amplification (Hygiene Screening System Internal Control, 1 µL). Finally, 2.5 µL of DNA sample is added. The positive control comprised of 2.5 µL Hygiene Screening System Positive Control, a stabilized plasmid solution that replaces the DNA template. The negative control consisted of 2.5 µL of DNA grade water instead of the template. Amplification conditions for 35 cycles were as follows: 95C for 2 s, 62C for 20 s (single acquisition) and 72C for 10 s. Melting curve analysis was performed at 95C for 0 s, 40C for 45 s and 80C for 0 s with temperature transition rate of 0.1C/s (continuous acquisition) followed by a cooling step of 40C for 30 s.

The HybProbes probes are designed to specifically bind to the IC, allowing detection in channel F3 or 705 nm, whereas the *Staphylococcus* DNA is detected in channel F2 or 640 nm.

In the case of positive results, a further melting curve analysis was performed to differentiate between the closely related

genera *Staphyloccocus* and *Micrococcus*, and also for a limited further characterization of detected *Staphylococcus* species. *S. aureus* was detected on channel F3 with a main peak at 57C (±1.5C).

Protocols have been developed and optimized by Biotecon on the LightCycler® 2.0 (Roche Diagnostics GmbH). However, the LightCycler® 1.2 used in the present study possesses the required channels for identification of *S. aureus*.

To our knowledge, no evaluation of these commercial kits has been reported in the literature. In consequence, it was of interest to assess these two kits and give a critical opinion on their use for food microbiology purposes.

## In-House Real-Time PCR Assays

**Primer Selection.** All primers were purchased from MWG Eurofins Operon (Ebersberg, Germany).

For the detection of *L. monocytogenes*, two primer sets were evaluated: (1) *mpl* primers targeting the metalloprotease gene of *L. monocytogenes* and previously tested by Scheu *et al.* (1999) in a PCR-ELISA – the *mpl* primers are purported to target a 149-bp fragment of the gene – the sequences of the primers were as follows: 5′-GAAAAAGCATTTGAAGCCAT-3′ (forward primer) and 5′-GCAACTTCCGGCTCAGC-3′ (reverse primer); and (2) *hly A* primers targeting the listeriolysin O of *L. monocytogenes* (Nogva *et al.* 2000). This set was designed according to the DNA sequence coding for the listeriolysin O gene. The sequence of the forward primer was 5′-TGCAAGTCCTAAGACGCCA-3′ and the sequence of the reverse primer was 5′-CACTGCATCTCCGTGGTATACTAA-3′. The expected PCR product size was 112 bp.

For the detection of *S. aureus*, a primer set targeting the *nuc* gene was assayed since the detection of *S. aureus* by PCR and real-time PCR using this primer set has been previously described in a number of reports (Brakstad *et al.* 1992; Hein *et al.* 2001; Kim *et al.* 2001; Ikeda *et al.* 2005; Pinto *et al.* 2005). The primers were designed to amplify a 279-bp fragment from the gene encoding for a thermonuclease. The forward primer was 5′-GCGATTGATGGTGATACGGT-3′ the reverse primer 5′-AGCCAA GCCTTGACGAACTAAAGC-3′.

The specificity of the primer sets was tested using *in silico* PCR analysis against complete genome sequences of *S. aureus*, *L. monocytogenes* and other species. All sequences were provided by the National Centre for Biotechnology Information (NCBI) nucleotide database. The Primer BLAST (Basic Local Alignment Search Tool) program, available on http://www.ncbi.nlm.nih.gov/tools/primer-blast/, was used for simulated PCR with the primer sets.

The specificity of primers for the detection of *L. monocytogenes* and *S. aureus* was tested separately as a first step. Then primer sets were combined in order to create a detection method in duplex using real-time PCR.

## PCR Conditions for Detection of *L. monocytogenes* and *S. aureus*

For conventional PCR, mixes were prepared with the ready-to-use kit from Roche Diagnostics GmbH: FastStart Taq DNA Polymerase, dNTPack 5 U/µL. On the basis of a final 50 µL reaction volume, the master mixture contained 48 µL of 4 mM $MgCl_2$ (2 mM from 10X PCR buffer and 2 mM from $MgCl_2$ solution), 500 nM of forward primer, 500 nM of reverse primer, dNTP mixture and the FastStart Taq DNA Polymerase to which 2 µL of DNA sample was added to each reaction.

PCR was performed on a G-Storm GS2 Thermal Cycler (Genetic Research Instrumentation, Braintree, U.K.) for the detection of *L. monocytogenes* or *S. aureus*. The PCR programmes were carried out as shown in Table 1, using annealing temperatures of 59C for *mpl* primers, 60C for *hly A* primers and 62C for *nuc* primers. Each PCR product was subsequently run on a 2% agarose electrophoresis gel and stained using SYBR Safe™ (Molecular Probes, Eugene, OR) and visualized with a transilluminator (Syngen, Frederick, MD) under ultraviolet (UV) light. The Hyperladder II, 50–2,000 bp (Bioline Ltd., London, U.K.) was used as a molecular marker.

## Simplex and Duplex SYBR Green-Based, Real-Time PCR

When testing the primer sets, conventional PCR procedures were adapted to provide a simplex real-time procedure on the LightCycler® 1.2 (Roche Diagnostics GmbH), using LightCycler® FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH).

In each capillary, the 20-µL reaction mix contained 1X concentration LightCycler-FastStart DNA Master SYBR Green (Roche Diagnostics GmbH); 4 mM $MgCl_2$; 500 nM concentration of each primer; and 2 µL of the template. A duplex real-time PCR was then evaluated based on combining the two primer sets in the same reaction mix with the same

**TABLE 1.** PCR AND REAL-TIME PCR CONDITIONS USING THE *MPL*, *HLY A* AND *NUC* PRIMER SETS

| Program | PCR | SYBR Green-based, real-time PCR |
|---|---|---|
| Preincubation | 95C, 6 min | 95C, 10 min |
| Amplification | 28 cycles | 35 cycles |
| | 95C, 30 s | 95C, 5 s |
| | C*, 15 s | C*, 10 s |
| | 72C, 30 s | 72C, 20 s (Single) |
| Melting | N/A† | 95C, 0 s |
| | | 65C, 10 s |
| | | 95C, 0 s 0.2C/s (Continuous) |
| Cooling | N/A | 40C, 30 s |

* Specific to each primer set used, similar to conventional PCR annealing temperatures.
† Not applicable.

concentrations of reagents and same volume of template, applying the Tm average from two primer sets.

Times and temperatures applied are shown in Table 1 and DNA amplification was followed in real time in channel F1 or 530 nm.

The intra and inter-assay reproducibility of the duplex PCR system was evaluated as described by Fan *et al.* (2007). The intra-assay evaluation corresponded to a unique PCR run where triplicates per sample were analyzed. The inter-assay evaluation was equivalent to three separate PCR runs where for each assay with data retrieved from triplicates for each sample. The coefficients of variation (CVs) for Ct and Tm values were calculated by dividing the standard deviation (SD) by the mean Ct or Tm for intra and inter-assays.

The duplex-based, real-time PCR was evaluated in the inoculated food samples and compared with the commercial real-time PCR systems and the traditional microbiological detection methods described earlier (Fig. 1).

## RESULTS AND DISCUSSION

### Evaluation of the Commercial Real-Time PCR Systems

**foodproof® *Listeria monocytogenes* Detection Kit.** PCR kinetic profiles revealed that extracted *L. monocytogenes* DNAs and the positive control were amplified and detected at 640 nm. A mean Ct of 21.21 (SD equal to 0.28) for *L. monocytogenes* ATCC 19115 and *L. monocytogenes* ATCC 13451 DNA was determined (Table 2A). The positive control had a Ct equal to 24.61 for the assay.

**TABLE 2.** INTRA-ASSAY CROSSING POINT VALUES AND SENSITIVITIES OBTAINED FROM TRIPLICATE SAMPLES ANALYZED USING VARIOUS REAL-TIME POLYMERASE CHAIN REACTION (PCR) SYSTEMS FOR THE DETECTION OF (A) *L. MONOCYTOGENES* ATCC 19115 AND 13451 AND (B) *S. AUREUS* NCTC 8325 AND UL ISOLATE

A

| Real-time PCR system | Ct* (SD) | Sensitivity (copies) |
|---|---|---|
| foodproof® *Listeria monocytogenes* detection kit | 18.97 (0.17) | 3 |
| Simplex SYBR-green with *hly A* primer set | 19.65 (0.2) | 30 |
| Duplex SYBR-green based | 18.67 (0.01) | 30 |

B

| Real-time PCR system | Ct* (SD) | Sensitivity (copies) |
|---|---|---|
| Hygiene screening detection kit | 18.51 (0.17) | 60 |
| Simplex SYBR-green with *nuc* primer set | 20.57 (0.19) | 60 |
| Duplex SYBR-green based | 17.18 (0.01) | 6 |

* Mean Ct value corresponded to the amplification of 1 ng of DNA (pure culture) per PCR reaction.

In contrast, the negative control was not amplified. Visualisation at 705 nm showed that the IAC was amplified in each sample, including the negative control. This commercial real-time PCR system, described by Junge and Berghof-Jager (2006) has been validated by the Association of Official Agricultural Chemists. Scheu *et al.* (1998) suggested that the increasing uptake of commercial PCR systems would eventually lead to standardized procedures, which is a prerequisite to routine analysis by the food industry using these kits. Malorny *et al.* (2003) included an IAC to PCR methods to monitor the robustness of diagnostic PCR, which is a criterion for standardization. As regards the frequency of when IACs are included in PCR, various real-time PCR methods have been recently developed with the inclusion of an IAC (Rossmanith *et al.* 2006; O'Grady *et al.* 2009; Rip and Gouws 2009). Some researchers include an IAC in order to overcome the possibility of false negative results (Hoorfar 2004) and to monitor the presence of PCR inhibitors (Malorny *et al.* 2003). However, a significant number of PCR-related publications do not include an IAC in their procedures. Barkham (2004) suggested that an IAC should not be mandatory, at least in clinical diagnostics, mainly because of the risk of increasing the cost of PCR testing. Another criterion mentioned by Malorny *et al.* (2003) was the analytical and diagnostic accuracy where "a selective PCR-based method comprises inclusivity (detection of the target pathogen from a wide range of strains) and exclusivity (lack of response from a relevant range of closely related but nontarget strains)." Biotecon claim to have evaluated the inclusivity of this kit using 102 *L. monocytogenes* isolates and the exclusivity using 60 non-*L. monocytogenes* bacteria. The company reported that a relative detection limit of 1 to 10 cells per 25 g sample could be achieved in various food samples. Biotecon stated that the kit can detect down to $10^3$–$10^4$ cfu/mL in enrichment cultures, depending on the sample preparation kit used (Anon 2007a). In the present study, 1 ng of *L. monocytogenes* DNA or $3.5.10^5$ copies per reaction was detected at a mean Ct equal to 18.97 (SD equal to 0.17) in the case of a pure culture. The LightCycler® instrument can detect an x-fold difference in template concentration, according to a statistical variability of Ct in multiple PCR reactions (Anon 2009). For instance, to detect a 10-fold change, the determined ΔCt must statistically confirm a 3.32-cycle change, assuming that a reaction has an efficiency of 2, which means that at each 10-fold dilution the Ct value is increased by adding 3.32. Therefore, 1 fg of *L. monocytogenes* DNA or three copies can theoretically be detected at a mean Ct equal to 35.57. In parallel, serial dilutions of DNA copy numbers amplified by real-time PCR confirmed that three copies could be detected at a mean Ct equal to 37.56 (SD equal to 0.5) with an efficiency of 1.91 (Table 2A). While the foodproof® *Listeria monocytogenes* detection kit appeared to have a high specificity and low detection limit, a single kit for 96 real-time PCR reactions

equates to a high cost per single reaction for routine analysis or research. Therefore, in-house real-time PCR protocols were developed to obtain alternative lower cost, real-time PCR assays.

**Hygiene Screening System Kit – *S. aureus*.** This commercial real-time PCR system is a typical example of multiplex real-time PCR system using a combination of primer sets and hybridization probes. At 640 nm, *S. aureus* DNAs and the positive control were amplified, whereas the negative control was not. *S. aureus* NCTC and UL isolate were detected at a mean Ct equal to 18.51 (SD equal to 0.17). In this study, the sensitivity of this commercial kit was found to be 60 copies for a mean Ct equal to 35.91 (SD equal to 0.20) (Table 2B). The positive control was detected later at a Ct equal to 19.27. When switching detection channel to 750 nm, amplification curves were observed even for the negative control confirming the reliability of the PCR using the internal control. To confirm the amplification of *S. aureus*, a melting curve analysis was performed. At 640 nm, a single peak/shoulder > 64C was observed for the positive sample (Fig. 2A). The presence of a shoulder on the melting curve may arise as an internal melting domain because of long PCR products, as described by Ririe *et al.* (1997), which can be a fingerprint for species identification. However, this shoulder was quite difficult to define for *S. aureus* NCTC 8325 and UL isolate in comparison with the positive control. Therefore, a highly experienced analyst would appear to be required to discriminate such a region on the melting curve using the commercial kit.

At 705 nm, *S. aureus* NCTC 8325 and UL isolate could be identified in both test DNA samples, where the mean Tm was 57.57C (SD equal to 0.09), which confirmed the identification of *S. aureus* (Fig. 2B).

The Hygiene Screening System kit allows rapid qualitative detection of the presence of *S. aureus* in contaminated samples while identifying the species using melting curve analysis. The kit has also been designed for performing a fast and reliable multiplex PCR system that detects other *Staphylococcus*, *Micrococcus* and *Corynebacterium* species.

***mpl* Primers.** The *mpl* gene was characterized by Domann *et al.* (1991) as a gene unique to *L. monocytogenes* species and physically linked to the listeriolysin gene of these strains. The *mpl* primers were purported to target a 149-bp fragment of the *mpl* gene, according to Scheu *et al.* (1999), whose work has been referenced in the manual instructions of the foodproof® *Listeria monocytogenes* detection kit. *In silico* PCR analysis using Primer Blast, as shown on Table 3 indicated poor specificity for *L. monocytogenes*. Complementary PCR analysis showed nonspecific detection for selected species such as *C. sakazakii*, *E. cloacae*, *S. aureus* or *V. parahaemolyticus*. The PCR system developed by Scheu *et al.* (1999) appeared to be

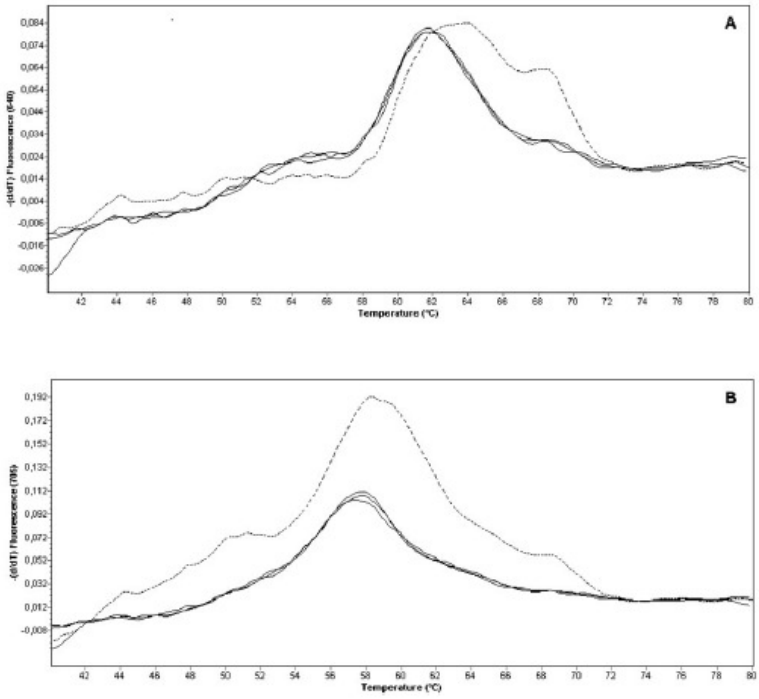



**FIG. 2.** MELTING CURVES OF *S. AUREUS* NCTC 8325, UL ISOLATE AND POSITIVE CONTROL ANALYZED USING THE HYGIENE SCREENING SYSTEM KIT AND DETECTED IN (A) CHANNEL F2 AT 640 nm OR (B) CHANNEL F3 AT 705 nm

**TABLE 3.** *IN SILICO* POLYMERASE CHAIN REACTION ANALYSIS USING THE *MPL*, *HLYA* AND *NUC* PRIMER SETS WITH PRIMER BLAST SOFTWARE

| | Primer sets | | |
|---|---|---|---|
| Bacterial species | *mpl* | *hlyA* | *nuc* |
| *Budvicia aquatica* | – | – | – |
| *Cedecea davisae* | – | – | – |
| *Citrobacter braakii* | – | – | – |
| *Citrobacter farmeri* | – | – | – |
| *Citrobacter freundii* | – | – | – |
| *Citrobacter koseri* | + | – | – |
| *Cronobacter sakazakii* | + | – | – |
| *Enterobacter aerogenes* | – | – | – |
| *Enterobacter cloacae* | + | – | – |
| *Erwinia persicina* | – | – | – |
| *Escherichia coli* | + | – | – |
| *Escherichia coli 0157:H7* | + | – | – |
| *Ewingella americana* | – | – | – |
| *Hafnia alvei* | – | – | – |
| *Klebsiella pneumoniae* | + | – | – |
| *Klebsiella oxytoca* | + | – | – |
| *Kluyvera ascorbata* | – | – | – |
| *Kluyvera intermedia* | – | – | – |
| *Pantoea agglomerans* | – | – | – |
| *Plesiomonas shigelloides* | – | – | – |
| *Proteus mirabilis* | + | – | – |
| *Proteus vulgaris* | – | – | – |
| *Salmonella typhimurium* | – | – | – |
| *Salmonella enteritidis* | + | – | – |
| *Serratia liquefaciens* | – | – | – |
| *Serratia marcescens* | + | – | – |
| *Shigella flexneri* | + | – | – |
| *Shigella sonnei* | + | – | – |
| *Yersinia enterocolitica* | + | – | – |
| *Yersinia pseudotuberculosis* | + | – | – |
| *Yersinia rohdei* | – | – | – |
| *Acinetobacter baumanii* | + | – | – |
| *Aeromonas hydrophila* | + | – | – |
| *Aeromonas punctata* | – | – | – |
| *Aeromonas sobria* | – | – | – |
| *Alcaligenes faecalis* | – | – | – |
| *Bacillus cereus* | + | – | – |
| *Bacillus subtilis* | + | – | – |
| *Campylobacter coli* | – | – | – |
| *Campylobacter jejuni* | – | – | – |
| *Enterococcus faecalis* | + | – | – |
| *Listeria innocua* | + | – | – |
| *Listeria monocytogenes* | + | + | – |
| *Micrococcus* spp. | + | – | – |
| *Pseudomonas aeruginosa* | + | – | – |
| *Pseudomonas fluorescens* | + | – | – |
| *Pseudomonas putida* | + | – | – |
| *Staphylococcus aureus* | + | – | + |
| *Staphyloccoccus capitis* | – | – | – |
| *Staphyloccoccus lentus* | – | – | – |
| *Staphyloccoccus xylosus* | + | – | – |
| *Vibrio parahaemolyticus* | + | – | – |
| *Vibrio vulnificus* | + | – | – |

+, target templates found; –, target templates not found.

specific for *L. monocytogenes* when combined with probes. The inclusion of probes can circumvent nonspecific amplifications by providing an additional layer of specificity (Nakken *et al.* 2009). However, based on the current data, this primer set would not appear to be applicable for the subsequent development of a SYBR Green-based method for detection of *L. monocytogenes*.

***hlyA* Primers.** The *hlyA* primer set anneals to the listeriolysin O gene of *L. monocytogenes*. This targeted sequence has been reported in several PCR and real-time PCR applications and uses different primer sequences targeting the same gene (Bansal 1996; Amagliani *et al.* 2004; Guilbaud *et al.* 2005). Aznar and Alarcón (2002) compared primer set specificities, notably primers that target the listeriolysin O, for the detection of *L. monocytogenes*. According to these workers, only one primer set was shown to be specific among the primer sets targeting the listeriolysin O gene, which confirmed that nonspecific amplification can occur. Nogva *et al.* (2000) selected a region with 100% homology between the *L. monocytogenes* sequences and with little homology to the sequences reported from other species with a gene encoding for the same type of protein using a TaqMan®-based PCR assay. However, a Primer Blast evaluation over a range of species showed a high specificity of the primer set for *L. monocytogenes* (Table 3). Data obtained in this study following PCR indicated that *L. monocytogenes* DNA was detected as a 112-bp fragment, which could be identified on the agarose electrophoresis gel under UV (Fig. 3). Therefore, the *hlyA* primer set appeared to be a better candidate for the detection of *L. monocytogenes* using real-time PCR methods on account of its specificity.

***nuc* Primers.** *S. aureus* DNAs were amplified using *nuc* gene primers. The DNA fragments were observed on SYBR Safe-stained 2% agarose electrophoresis gel under UV, which showed that a 279-bp PCR product was present (Fig. 4).

Among all the bacterial DNAs tested, only *S. aureus* was detected using the *nuc* primers whereas other *Staphylococcus* and *Micrococcus* species gave negative responses for this primer set. Data obtained confirmed findings reported by Alarcon *et al.* (2006), which support the use of PCR-based procedures using the same primer set and combined with enrichment in different types of food. Consequently, the *nuc* primer set was considered to be a suitable candidate for the detection of *S. aureus* in a SYBR Green real-time PCR method.

## Simplex Real-Time PCR

Conventional PCR was converted to real-time PCR protocols using a universal PCR mix where only the primer sets were modified in respect of the particular pathogen to be detected.

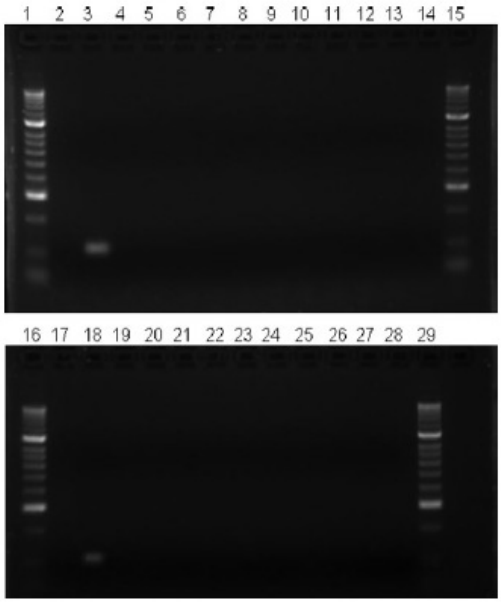



**FIG. 3.** AMPLIFICATION RESULTS OBTAINED BY PCR USING THE *HLY A* PRIMERS
Lanes 1, 15, 16, 29, Hyperladder II, 50–2,000 bp; lane 2, 17, negative control (no DNA template in the PCR reaction); lane 3, *L. monocytogenes* ATCC 19115, lane 18 *L. monocytogenes* ATCC 13451; lane 4, *E. coli*; lane 5, *E. aerogenes*; lane 6, *E. cloacae*; lane 7, *C. sakazakii*; lane 8, S. *typhimurium*; lane 9, *S. flexneri*; lane 10 *K. pneunomiae*; lane 11 *Y. enterocolitica*; lane 12, *E. percisina*; lane 13, *S. marcescens*; lane 14, *P. mirabilis*; lane 19, *V. parahaemolyticus*; lane 20, *A. hydrophila*; lane 21, *P. aeruginosa*; lane 22, *S. aureus*; lane 23, *S. capitis*; lane 24, *S. lentus*; lane 25, *S. xylosus*; lane 26, *Micrococcus spp.*; lane 27, *B. cereus*; lane 28, *C. jejuni*.

For the detection of *L. monocytogenes*, real-time PCR assays using mixes prepared with *hlyA* primers were performed. The amplification curves obtained confirmed that both *L. monocytogenes* ATCC 19115 and ATCC 13451 strains were detected at a mean Ct of 19.65 (SD equal to 0.20), as shown on Table 2. After 35 cycles, nonspecific amplifications appeared. In the case of *S. aureus*, data obtained indicated that the primer set was highly specific as *S. aureus* DNA was detected only at a mean Ct of 20.57 (SD equal to 0.19) for *S. aureus* NCTC 8325 and UL isolate. After 38 cycles, nonspecific amplifications occurred as an included *L. monocytogenes* DNA sample and the negative control displayed some fluorescence.

Fernandez *et al.* (2006) outlined the main limitation of SYBR Green I as being the likelihood of false positive results. This may occur in cases where the dye binds to double-stranded DNA other than the target, such as primer-dimers that decrease the specificity. As all double-stranded DNA are detected, SYBR Green becomes less sensitive than fluorogenic probes, in the presence of primer-dimers (Ciglenecki *et al.* 2008). The risk of formation of primer-dimers is usually related to a suboptimal concentration of primers, a low-target copy number or if the target is absent in the sample (Fernandez *et al.* 2006). In order to differentiate specific PCR products from potential artefacts, a melting curve analysis was conducted following both real-time PCR procedures that confirmed the reliability of the methods, as shown on Fig. 5.

For each amplified DNA sample, generation of a single and sharply defined melting curve with a narrow peak indicated that the PCR products were pure and homogenous without the presence of primer-dimers, which melt at relatively low temperatures and have broader peaks. The identity of *L. monocytogenes* was confirmed when the Tm of the PCR product approached 77.99C (SD equal to 0.07) whereas the *S. aureus* PCR product had a mean Tm of 80.48C (SD equal to 0.02) (Fig. 5).

The sensitivity of the SYBR Green-based, real-time PCR methods was assessed using a series of dilutions of genomic

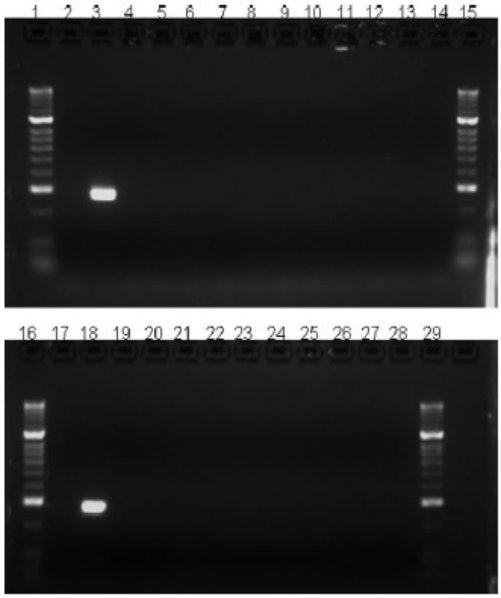



**FIG. 4.** AMPLIFICATION RESULTS OBTAINED BY PCR USING THE *HLY A* PRIMERS
Lanes 1, 15, 16, 29, Hyperladder II, 50–2,000 bp; lane 2, 17, negative control (no DNA template in the PCR reaction); lane 3, *S. aureus* NCTC 8325, lane 18, *S. aureus* UL isolate; lane 4, *E. coli*; lane 5, *E. aerogenes*; lane 6, *E. cloacae*; lane 7, *C. sakazakii*; lane 8, S. *typhimurium*; lane 9, *S. flexneri*; lane 10 *K. pneumoniae*; lane 11 *Y. enterocolitica*; lane 12, *E. percisina*; lane 13, *S. marcescens*; lane 14, *P. mirabilis*; lane 19, *V. parahaemolyticus*; lane 20, *A. hydrophila*; lane 21, *P. aeruginosa*; lane 22, *L. monocytogenes* ATCC 19115; lane 23, *S. capitis*; lane 24, *S. lentus*; lane 25, *S. xylosus*; lane 26, *Micrococcus spp.*; lane 27, *B. cereus*; lane 28, *C. jejuni*.

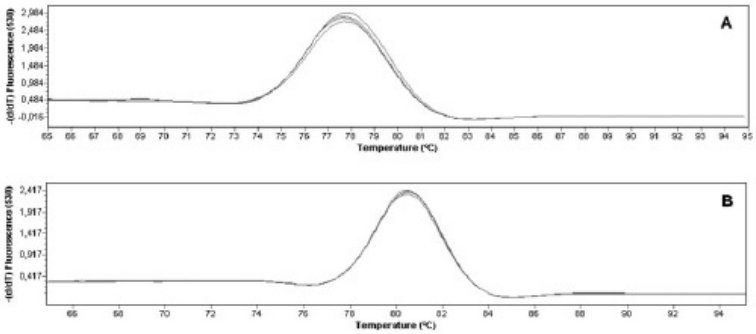



**FIG. 5.** MELTING CURVE PROFILES OF SIMPLEX SYBR GREEN-BASED PCR CHANGING THE PRIMERS SEQUENCE FOR THE IDENTIFICATION OF (A) *L. MONOCYTOGENES* AND (B) *S. AUREUS*

DNA subsequently converted into copy numbers as described previously for the assessment of the commercial real-time PCR systems. For both real-time PCR methods, using pure cultures, the detection limit was 30 copies (Table 2). Each simplex real-time PCR appeared to be highly specific but still required a separate run on the LightCycler® because of the difference in annealing temperatures. For this reason, a combination of both primer sets was assayed for the development of a duplex real-time PCR as a two-in-one solution in a single PCR run.

## Development of a Duplex SYBR Green-Based, Real-Time PCR

DNA samples extracted from pure culture of *S. aureus* and *L. monocytogenes* were amplified using the newly developed duplex system. Mean melting Tm and SD for each PCR product were compared with Tm data obtained using the TMUtility_1.5 software (Idaho Technology Inc., Salt Lake City, UT), as shown in Table 4. Positive data were confirmed in the range Tm ± 3SD for each targeted gene.

The specificity of the newly developed duplex real-time PCR method was assessed using a range of bacterial species, including *L. monocytogenes* and *S. aureus*. Amplification curves were observed for the samples containing *L. monocytogenes* DNA with a mean Ct of 18.67 (SD equal to 0.01) and *S. aureus* DNA with a mean Ct equal to 17.18 (SD equal to 0.01) (Table 2B) and the mix of *L. monocytogenes/S. aureus DNAs* with mean intra-assay Ct of 16.75 (SD equal to 0.12). The sensitivities of the duplex system were 30 copies (mean Ct of 32.15, SD equal to 0.09) for *L. monocytogenes* and 6 copies (mean Ct of 33.78, SD equal to 0.07) for *S. aureus*, in the case of samples containing either one microorganism or the other.

The melting curve profiles (Fig. 6A) showed that no primer-dimers were formed during the PCR runs, which would suggest that no excessive regions of complementarity existed between the two primers.

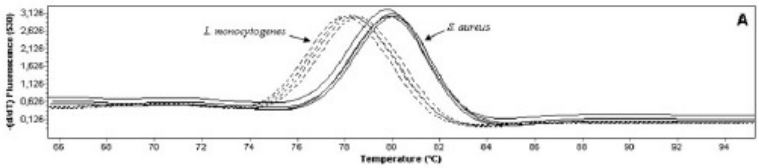
In the current study, the real-time PCR assays in duplex consisted of combining the *hly A* and *nuc* primer sets for the simultaneous detection of *L. monocytogenes* and *S. aureus*. According to Wu *et al.* (2007), the performance of multiplex PCR systems depends first on the primer design and the difference in Tm within one primer pair should not exceed 5C, which is in agreement with the two primer sets used in this study (ΔTm equal to 2C). PCR products should ideally be between 100 and 2,000 bp-long with a difference in size of more than 50 bp. Based on the data obtained for the duplex PCR methodology developed in this study these criteria appeared to be met (Figs. 6A and 7).

Distinct Tm values were obtained for *L. monocytogenes* and *S. aureus strains* when analyzed individually or in a mixture. When *L. monocytogenes* DNA was amplified, the Tm was between 77.9 and 78.2C, whereas the observed Tm for *S. aureus* was within the range 79.7C–80.1C (Table 4). This data would satisfy the criterion of Ririe *et al.* (1997) who suggested that PCR product Tms should differ by 2C. However, in this study similar melting curves profiles were obtained for *S. aureus* DNA and *L. monocytogenes/S. aureus* DNA mixes, where the mean Tm value was 80.10C (SD equal to 0.01) (Fig. 6B), which suggested the presence of a single PCR product instead of two. In contrast, analysis of multiplex PCR by agarose gel electrophoresis (Fig. 7) showed amplification of both gene targets.

However, these data were in agreement with Giglio *et al.* (2003) regarding the preferential binding of SYBR Green I to

| | *nuc* gene (*S. aureus*) | *hlyA* gene (*L. monocytogenes*) |
|---|---|---|
| Calculated Tm | 81.76 | 78.95 |
| Mean Tm (SD) (C) | 79.90 (0.07) | 78.05 (0.05) |
| Tm range (mean ± 3SD) (C) | 79.7–80.1 | 77.9–78.2 |

**TABLE 4.** MELTING TEMPERATURES (ACTUAL AND CALCULATED) OF THE POLYMERASE CHAIN REACTION (PCR) PRODUCTS OBTAINED BY DUPLEX REAL-TIME PCR



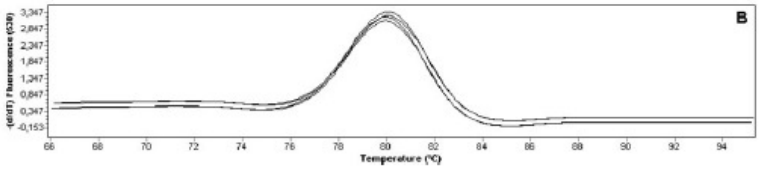




**FIG. 6.** MELTING CURVE PROFILES OBTAINED FROM (A) SAMPLES CONTAINING *L. MONOCYTOGENES* DNA ONLY OR *S. AUREUS* DNA ONLY AND (B) SAMPLES CONTAINING BOTH *L. MONOCYTOGENES* AND *S. AUREUS* DNAS IN A DUPLEX REAL-TIME PCR

amplicons with the highest Guanine + Cytosine percentage (G + C %) and hence the preferential binding to the largest of the two PCR products. The G + C content calculated for each PCR product was determined using the TMUtility_1.5 software. For *L. monocytogenes* PCR product, G + C% was equal to 38.4% whereas in the case of *S. aureus*, the G + C% was 36.2%. Because G + C% are close to each other, it is reasonable to speculate that SYBR Green I may preferentially bind to *S. aureus* amplicon because of its larger size. Therefore, the main technical issue for this duplex real-time PCR method would arise if food samples were simultaneously contaminated with *L. monocytogenes* and *S. aureus*, which may cause a misinterpretation of data. Kumar *et al.* (2009) developed a multiplex PCR system for the simultaneous detection of *B. cereus*, *S. aureus* and *L. monocytogenes* in case of coexistence of these pathogens in spiked food/milk samples and their similarities in terms of symptoms and incubation period. It was claimed that this assay could identify all the three species individually or in combination using conventional multiplex PCR on an agarose electrophoresis gel. While data analysis was simpler than for multiplex real-time PCR procedure, it was more time consuming and less sensitive. Wang *et al.* (2004) reported detection by duplex SYBR Green real-time PCR of *S. enteritidis* DNA and *L. monocytogenes* independently or combined in the same raw sausage sample. In their study, two distinct melting peaks were observed for samples containing both *S. enteritidis* and *L. monocytogenes*, one peak corresponded to *S. enteritidis* and the other was attributed to *L. monocytogenes*. Overall, the identification by melting curve analysis of microorganisms in combination in a sample depends on the assay. The causes of variation in melting curve profiles have not been fully explained. However, Giglio *et al.* (2003) and Gundry *et al.* (2003) attributed this to the redistribution of SYBR Green from PCR products with a lower Tm to a higher Tm during melting curve analysis. While it is not credible to exclude the possibility of samples being contaminated simultaneously by *S. aureus* and *L. monocytogenes* no such food poisoning outbreaks caused by the ingestion of foods contaminated by both microorganisms have been reported to date.

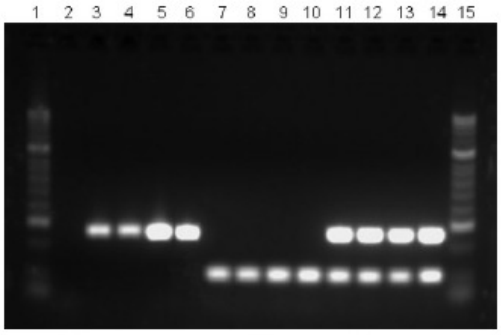



**FIG. 7.** PCR PRODUCTS OBTAINED AFTER SYBR GREEN-BASED DUPLEX REAL-TIME PCR USING *HLY A* AND *NUC* PRIMERS FOR THE SIMULTANEOUS DETECTION OF *S. AUREUS* AND *L. MONOCYTOGENES* Lanes 1, 15, Hyperladder II, 50–2,000 bp; lane 2, negative control (no DNA template in the PCR reaction); lanes 3, 4, 5, 6, *S. aureus* DNA samples only; lanes 7, 8, 9, 10, *L. monocytogenes* DNA samples only; lanes 11, 12, 13, 14, *S. aureus* and *L. monocytogenes* DNA in the same sample.

## Analysis of Food Samples and Assay Reproducibility

Sensitivities were first determined for each type of spiked food using the newly developed duplex SYBR Green-based, real-time PCR procedure. Secondly, in-house real-time PCR data were confirmed by a comparison with the commercial real-time PCR kits on the sample indicating the detection limit and the samples that were 10 times more or 10 times less contaminated. The foodproof® *Listeria monocytogenes* detection kit and the Hygiene Screening Detection kit provided the same sensitivity data as the duplex real-time PCR and by

**TABLE 5.** SENSITIVITIES* OF THE DUPLEX SYBR GREEN-BASED, REAL-TIME PCR IN cfu/MASS OF FOOD OBTAINED IN THE ANALYSIS OF NINE TYPES OF FOOD SAMPLES AFTER SPIKING OF *L. MONOCYTOGENES* OR *S. AUREUS* AND 18 H ENRICHMENT WITH BUFFERED PEPTONE WATER

| Food samples | *L. monocytogenes* | *S. aureus* |
|---|---|---|
| Tagliatelle and ham | 5 cfu/25 g | 6 cfu/25 g |
| Chicken supreme | 5 cfu/25 g | 6 cfu/25 g |
| Vegetable soup | 3 cfu/25 g | 5 cfu/25 g |
| Chicken soup | 3 cfu/25 g | 5 cfu/25 g |
| Cottage pie | 3 cfu/25 g | 5 cfu/25 g |
| Ice cream | 18 cfu/25 g | 6 cfu/25 g |
| Coleslaw | 7 cfu/g | 6 cfu/25 g |
| Cottage cheese | 6 cfu/25 g | 5 cfu/25 g |
| Minced meat | 6 cfu/25 g | 2 cfu/g |

* Sensitivities were defined according to qualitative duplex real-time polymerase chain reaction data (presence/absence) on enriched samples analyzed in triplicate and previously spiked with serial dilutions of an inoculum where the initial concentration was defined by plate counting on nutrient agar.

plating on selective media, as shown on Table 5. Sensitivities were defined by plate count data obtained after initial spiking with suspensions of *L. monocytogenes* or *S. aureus*, and computed by factoring in the dilution factor applied to each food sample.

In microbiological food analysis, an enrichment step of 18 to 24 h is generally required prior to PCR (Candrian 1995). This step appears to be essential for the detection of bacteria in foods at minimum concentration levels from 1 to 10 cfu per gram of food. Nevertheless, such enrichment media, notably a selective one, require hazardous supplements and additional costs in consumables (Duffy *et al.* 2001). Therefore, the possibility of eliminating or minimizing the enrichment step is of potential interest in order to simplify and even shorten the time of food analysis. In this study, the use of BPW allowed the recovery of low bacterial levels, depending on the food sample analyzed, the sensitivity of the method varied (Table 5). In this respect, the recovery of *L. monocytogenes* normally depends on enrichment to amplify low bacterial populations or injured cells in food products. However, the repair and detection of sub-lethally injured *Listeria* may be inhibited by the use of selective agents in such enrichment media (Duffy *et al.* 2001; Donnelly 2002). The ISO 11290-1 reference method (Anon 2004), uses half Fraser broth and Fraser broth for the detection of *L. monocytogenes* and is time consuming. Besse *et al.* (2005) and Oravcova *et al.* (2008) suggested shortening this two-step enrichment. Indeed, similar or slightly higher recoveries of *L. monocytogenes* were obtained in the presence of *L. innocua* after the first enrichment in half Fraser compared with the second enrichment with Fraser broth. Many publications report the limitations of such media in relation to the coexistence of other *Listeria* species (Duh 1993; Curiale and Lewus 1994; Macdonald and Sutherland 1994; Beumer *et al.* 1996). This may have an impact on the detection of *L. monocytogenes* based on the fact that *L. innocua* has a faster growth rate. In contrast, Besse *et al.* (2005) discussed the effect of enrichment, using the ISO 11290-1 reference method, on samples contaminated with *Listeria* species, where *L. innocua* was able to grow more easily in various foods than *L. monocytogenes*. The predominance of *L. innocua* over *L. monocytogenes* could also be explained by the higher initial contamination level of *L. innocua* (Besse *et al.* 2005). As an alternative to half Fraser and Fraser broths, Oravcova *et al.* (2008) obtained similar *L. monocytogenes* recoveries using a nonselective enrichment broth such as brain heart infusion. In the current study, it is reasonable to speculate that BPW, used for enrichment, may be sufficiently reliable for the recovery of *L. monocytogenes* or *S. aureus*. Studies comparing BPW with selective enrichment broth showed that similar bacterial recoveries were obtained for the detection of *L. monocytogenes* and other *Listeria* species in food samples as described by Walsh *et al.* (1998) where the selectivity for *L. monocytogenes* was increased. However, the recovery depends on the inherent buffering capacity. Dairy products are an example of foods with a low inherent buffering capacity and Walsh *et al.* (1998) advised instead the use of a highly buffered non selective medium. In contrast, duplex real-time PCR results indicated positive samples for *L. monocytogenes* in cottage cheese and ice cream samples enriched with BPW with a detection limit of 6 and 18 cfu/25 g of food. The lowest sensitivity was obtained in coleslaw with *L. monocytogenes* (7 cfu/g). However, this detection limit is still satisfactory for the analysis of such pathogens in foods. Duffy *et al.* (2001) compared the performances of BPW and University of Vermont Media for the recovery of *L. monocytogenes* in minced meat samples and obtained similar results after 24 h of enrichment. In the current study, enriched food samples could be analyzed after 18 h, which represents a gain of 6 h.

For the detection of *S. aureus*, the EN ISO 6888-3 (Anon 2003c) is the reference standard currently available and describes a selective enrichment step with Giolitti and Cantoni broth followed by isolation of colonies with characteristic morphology and subsequent identification tests. The use of BPW for enrichment of samples contaminated with *S. aureus* has not been reported in the literature. However, *S. aureus* is recognized as not having specific nutrient requirements and *S. aureus* appeared to grow well in BPW. Data for duplex real-time PCR in the tested food samples found the lowest detection limit of 2 cfu/g of minced meat (Table 5).

As regards reproducibility of the data obtained using the LightCycler® 1.2 (Table 6), the crossing point for both targeted gene was 0.20% for intra-assay CV and 0.63% for inter-assay CV in the case of DNA extracted from pure cultures. In comparison, for spiked foods an intra-assay CV of 0.32% and inter assay of 0.70% CV were obtained.

**TABLE 6.** REPRODUCIBILITY DATA OF THE DUPLEX SYBR GREEN-BASED, REAL-TIME POLYMERASE CHAIN REACTION (PCR)

| | | DNA samples§ | |
|---|---|---|---|
| | | Pure cultures | Spiked foods |
| CV (%)* Intra-assay† | *Ct* | 0.20 | 0.32 |
| | *Tm* | 0.04 | 0.08 |
| CV (%)* Inter-assay‡ | *Ct* | 0.63 | 0.70 |
| | *Tm* | 0.19 | 0.20 |

* The coefficient of variation (CV) was calculated by dividing standard deviation by the mean Ct and Tm values obtained by measurement.
† Mean CVs calculated from the replicates measured for both targeted genes.
‡ Mean CVs calculated from data obtained in three separate PCR runs. In each run, the mean of the triplicates corresponding to both targeted genes was used as the value for the Ct and Tm from each run.
§ For comparison purposes according to the nature of the sample, CVs were calculated in the case of DNA extracted directly from a pure culture (1 ng/2 µL) and DNA extracted from 1 mL of enriched food sample (cottage cheese).

Similar CV% for intra-assay and inter-assay crossing points were observed for both targeted genes. In contrast, the intra-assay CV obtained for Tm was lowest using pure cultures, which confirmed a satisfactory degree of precision within the same assay when defining Tm data. Moreover, the CV obtained for Tm was much lower than that obtained for inter-assay Ct for both types of samples. Because of the relatively small error between assays in the determination of Tm values the duplex real-time PCR system appeared to be a reproducible analytical method.

In the current study, an evaluation of two commercial kits from Biotecon's range showed advantages in using these systems for the detection of *L. monocytogenes* and *S. aureus*. While the kits are easy to use, rapid, reliable and highly specific the high cost per reaction may preclude their widespread use in the food industry. In this study combining optimal primer sets in a duplex real-time PCR platform was shown to allow simultaneous detection of *L. monocytogenes* and *S. aureus* with low sensitivities found in minimally enriched food products. SYBR Green I identified two different amplification products by melting curve analysis in spiked samples with detection limits of 2 cfu/g in minced beef spiked with *S. aureus* and 7 cfu/g in coleslaw spiked with *L. monocytogenes*. BPW recovered both pathogens in foods in 18 h and food analysis including DNA extraction and duplex real-time PCR took ~1 h 30 min to 2 h depending on the number of samples for analysis. This multiplex real-time PCR method provides a two-in-one screening solution to detect positive samples of *S. aureus* or *L. monocytogenes* in minimally enriched food samples. The main limitation is that it can exclude simultaneously native samples of *S. aureus* and *L. monocytogenes*. In the presence of both microorganisms in the same sample, only *S. aureus* will be detected using SYBR Green in duplex real-time PCR. However, while double contaminations of this type cannot be excluded, they remain rare in foods.

In conclusion, the newly developed method may need to be validated as an alternative analytical method to be widely accepted. On this aspect, inclusivity and exclusivity of the method may be tested over a wider range of strains among foodborne and non-foodborne pathogens, including *L. monocytogenes* and *S. aureus* subspecies. Interlaboratory studies may be conducted in the analysis of defined food samples comparing ISO methods versus alternative method and using different real-time PCR devices in order to confirm the application of this duplex method.

## ACKNOWLEDGMENT

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## REFERENCES

ALARCON, B., VICEDO, B. and AZNAR, R. 2006. PCR-based procedures for detection and quantification of *Staphylococcus aureus* and their application in food. J. Appl. Microbiol. *100*, 352–364.

ALDEA, C., ALVAREZ, C.P., FOLGUEIRA, L., DELGADO, R. and OTERO, J.R. 2002. Rapid detection of herpes simplex virus DNA in genital ulcers by real-time PCR using SYBR green I dye as the detection signal. J. Clin. Microbiol. *40*, 1060–1062.

AMAGLIANI, G., BRANDI, G., OMICCIOLI, E., CASIERE, A., BRUCE, I.J. and MAGNANI, M. 2004. Direct detection of *Listeria monocytogenes* from milk by magnetic based DNA isolation and PCR. Food Microbiol. *21*, 597–603.

ANONYMOUS. 1999. ISO 6887-1:1999 Microbiology of food and animal feeding stuffs – preparation of test samples, initial suspension and decimal dilutions for microbiological examination – Part 1: General rules for the preparation of the initial suspension and decimal dilutions.

ANONYMOUS. 2002. ISO 8261:2002 Milk and milk products – general guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiological examination, Geneva, Switzerland.

ANONYMOUS. 2003a. ISO 6887-2:2003 Microbiology of food and animal feeding stuffs – preparation of test samples, initial suspension and decimal dilutions for microbiological examination – Part 2: Specific rules for the preparation of meat and meat products.

ANONYMOUS. 2003b. ISO 6887-4:2003 Microbiology of food and animal feeding stuffs – preparation of test samples, initial suspension and decimal dilutions for microbiological examination – Part 4: Specific rules for the preparation of

products other than milk and milk products, meat and meat products, and fish and fishery products.

ANONYMOUS. 2003c. ISO 6888-3:2003 Microbiology of food and animal feeding stuffs – horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) – Part 3: Detection and MPN technique for low numbers, Geneva, Switzerland.

ANONYMOUS. 2003d. ISO 16140:2003 Microbiology of food and animal feeding stuffs – protocol for the validation of alternative methods, Geneva, Switzerland.

ANONYMOUS. 2004. ISO 11290-1:2004 Microbiology of food and animal feeding stuffs – horizontal method for the detection and enumeration of *Listeria monocytogenes*, Geneva, Switzerland.

ANONYMOUS. 2005. Commission Regulation (EC) No 2073/2005 of 15 November 2005 on Microbiological criteria for foodstuffs.

ANONYMOUS. 2007a. Foodproof® *Listeria monocytogenes* detection kit – hybridization probes (LC 1.x, 2.0). Instruction manual – Biotecon Diagnostic GmbH, Potsdam, Germany.

ANONYMOUS. 2007b. Hygiene screening system (*Staphylococcus, Micrococcus, Corynebacterium*) – hybridization probes (LC 2.0) – Biotecon Diagnostics GmbH, Potsdam, Germany.

ANONYMOUS. 2009. *Detecting X-Fold Differences in Template Concentration – Roche Diagnostics*, Vol. 2001 (R.D. Ltd ed.) Burgess Hill, U.K.

AZNAR, R. and ALARCÓN, B. 2002. On the specificity of PCR detection of Listeria monocytogenes in food: A comparison of published primers. Syst. Appl. Microbiol. *25*, 109–119.

BANSAL, N.S. 1996. Development of a polymerase chain reaction assay for the detection of Listeria monocytogenes in foods. Lett. Appl. Microbiol. *22*, 353–356.

BARKHAM, T. 2004. Internal amplification control for PCR should not be mandatory in the clinical medical environment. J. Clin. Microbiol. *42*, 3379–3379.

BESSE, N.G., AUDINET, N., KEROUANTON, A., COLIN, P. and KALMOKOFF, M. 2005. Evolution of *Listeria* populations in food samples undergoing enrichment culturing. Int. J. Food Microbiol. *104*, 123–134.

BEUMER, R.R., TEGIFFEL, M.C., ANTHONIE, S.V.R. and COX, L.J. 1996. The effect of acriflavine and nalidixic acid on the growth of *Listeria spp* in enrichment media. Food Microbiol. *13*, 137–148.

BRAKSTAD, O.G., AASBAKK, K. and MAELAND, J.A. 1992. Detection of *Staphylococcus aureus* by polymerase chain reaction, amplification of the *nuc* gene. J. Clin. Microbiol. *30*, 1654–1660.

CANDRIAN, U. 1995. Polymerase chain reaction in food microbiology. J. Microbiol. Methods *23*, 89–103.

CHIANG, Y.C., FAN, C.M., LIAO, W.W., LIN, C.K. and TSEN, H.Y. 2007. Real-time PCR detection of *Staphylococcus aureus* in milk and meat using new primers designed from the heat shock protein gene *htrA* sequence. J. Food Prot. *70*, 2855–2859.

CHOUDHURRY, R.S.R., MELLES, D.C., EADIE, K., VOS, M., WERTHEIM, H.F.L., VERBRUGH, H.A., VAN BELKUM, A. and VAN LEEUWEN, W.B. 2006. Direct detection of human *Staphylococcus aureus* carriage in the nose using the Lightcycler *Staphylococcus* kit. J. Microbiol. Methods *65*, 354–356.

CIGLENECKI, U.J., GROM, J., TOPLAK, I., JEMERSIC, L. and BARLIC-MAGANJA, D. 2008. Real-time RT-PCR assay for rapid and specific detection of classical swine fever virus: Comparison of SYBR Green and TaqMan MGB detection methods using novel MGB probes. J. Virol. Methods *147*, 257–264.

CURIALE, M.S. and LEWUS, C. 1994. Detection of *Listeria monocytogenes* in samples containing *Listeria innocua*. J. Food Prot. *57*, 1048–1051.

DOMANN, E., LEIMEISTERWACHTER, M., GOEBEL, W. and CHAKRABORTY, T. 1991. Molecular Cloning, Sequencing, and Identification of a Metalloprotease Gene from *Listeria monocytogenes* that is species specific and physically linked to the Listeriolysin gene. Infect. Immun. *59*, 65–72.

DONNELLY, C.W. 2002. Detection and isolation of *Listeria monocytogenes* from food samples: Implications of sublethal injury. J. AOAC Int. *85*, 495–500.

DUFFY, G., WALSH, D., SHERIDAN, J.J., LOGUE, C.M., HARRINGTON, D., BLAIR, I.S. and MCDOWELL, D.A. 2001. Comparison of selective and non-selective enrichment media in the detection of *Listeria monocytogenes* from meat containing *Listeria innocua*. J. Appl. Microbiol. *90*, 994–999.

DUH, Y.H. 1993. Modelling the effect of temperature on the growth rate and lag time of *Listeria innocua* and *Listeria monocytogenes*. J. Food Prot. *56*, 734–734.

ELIZAQUIVEL, P. and AZNAR, R. 2008. A multiplex RTi-PCR reaction for simultaneous detection of *Escherichia coli* O157: H7, *Salmonella spp.* and *Staphylococcus aureus* on fresh, minimally processed vegetables. Food Microbiol. *25*, 705–713.

FAN, W., HAMILTON, T., WEBSTER-SESAY, S., NIKOLICH, M.P. and LINDLER, L.E. 2007. Multiplex real-time SYBR Green I PCR assay for detection of tetracycline efflux genes of Gram-negative bacteria. Mol. Cell. Probes *21*, 245–256.

FERNANDEZ, F., GUTIERREZ, J., SORLOZANO, A., ROMERO, J.M., SOTO, M.J. and RUIZ-CABELLO, F. 2006. Comparison of the SYBR green and the hybridization probe format for real-time PCR detection of HHV-6. Microbiol. Res. *161*, 158–163.

FUKUSHIMA, H., TSUNOMORI, Y. and SEKI, R. 2003. Duplex real-time SYBR green PCR assays for detection of 17 species of food- or waterborne pathogens in stools. J. Clin. Microbiol. *41*, 5134–5146.

GIGLIO, S., MONIS, P.T. and SAINT, C.P. 2003. Demonstration of preferential binding of SYBR Green I to specific DNA fragments in real-time multiplex PCR. Nucleic Acids Res. *31* (22), 6381–6386.

GLYNN, B., LAHIFF, S., WERNECKE, M., BARRY, T., SMITH, T.J. and MAHER, M. 2006. Current and emerging molecular diagnostic technologies applicable to bacterial food safety. Int. J. Dairy Technol. *59*, 126–139.

GUBALA, A.J. 2006. Multiplex real-time PCR detection of *Vibrio cholerae*. J. Microbiol. Methods *65*, 278–293.

GUILBAUD, M., DE COPPET, P., BOURION, F., RACHMAN, C., PREVOST, H. and DOUSSET, X. 2005. Quantitative detection of *Listeria monocytogenes* in biofilms by real-time PCR. Appl. Environ. Microbiol. *71*, 2190–2194.

GUNDRY, C.N., VANDERSTEEN, J.G., REED, G.H., PRYOR, R.J., CHEN, J. and WITTWER, C.T. 2003. Amplicon melting analysis with labelled primers: A closed-tube method for differentiating homozygotes and heterozygotes. Clin. Chem. *49*, 396–406.

HEIN, I., LEHNER, A., RIECK, P., KLEIN, K., BRANDL, E. and WAGNER, M. 2001. Comparison of different approaches to quantify *Staphylococcus aureus* cells by real-time quantitative PCR and application of this technique for examination of cheese. Appl. Environ. Microbiol. *67*, 3122–3126.

HOORFAR, J. 2004. Internal amplification control for PCR should not be mandatory in the clinical medical environment – reply. J. Clin. Microbiol. *42*, 3379–3380.

IKEDA, T., TAMATE, N., YAMAGUCHI, K. and MAKINO, S.I. 2005. Quantitative analysis of *Staphylococcus aureus* in skimmed milk powder by real-time PCR. J. Vet. Med. Sci. *67*, 1037–1041.

JASSON, V., JACXSENS, L., LUNING, P., RAJKOVIC, A. and UYTTENDAELE, M. 2010. Alternative microbial methods: An overview and selection criteria. Food Microbiol. *27*, 710–730.

JUNGE, B. and BERGHOF-JAGER, K. 2006. Roche/BIOTECON diagnostics LightCycler foodproof *L. monocytogenes* detection kit in combination with ShortPrep foodproof II kit – performance-tested method(SM) 070401. J. AOAC Int. *89*, 374–398.

KIM, C.H., KHAN, M., MORIN, D.E., HURLEY, W.L., TRIPATHY, D.N., KEHRLI, M., OLUOCH, A.O. and KAKOMA, I. 2001. Optimization of the PCR for detection of *Staphylococcus aureus nuc* gene in bovine milk. J. Dairy Sci. *84*, 74–83.

KIM, J.S., LEE, G.G., PARK, J.S., JUNG, Y.H., KWAK, H.S., KIM, S.B., NAM, Y.S. and KWON, S.T. 2007. A novel multiplex PCR assay for rapid and simultaneous detection of five pathogenic bacteria: *Escherichia coli* O157: H7, *Salmonella, Staphylococcus aureus, Listeria monocytogenes*, and *Vibrio parahaemolyticus*. J. Food Prot. *70*, 1656–1662.

KOBAYASHI, H., KUBOTA, J., FUJIHARA, K., HONJOH, K., ITO, M., FUJIKI, N., NAKABE, M., ODA, S., SATOYAMA, T., TAKASU, K. *ET AL*. 2009. Simultaneous Enrichment of *Salmonella spp, Escherichia coli* O157:H7, *Vibrio parahaemolyticus, Staphylococcus aureus, Bacillus cereus*, and *Listeria monocytogenes* by single broth and screening of the pathogens by multiplex real-time PCR. Food Sci. Technol. Res. *15*, 427–438.

KUGELMAN, G., TAPSALL, J.W., GOIRE, N., SYRMIS, M.W., LIMNIOS, A., LAMBERT, S.B., NISSEN, M.D., SLOOTS, T.P. and WHILEY, D.M. 2009. Simple, rapid, and inexpensive detection of *Neisseria gonorrhoeae* resistance mechanisms using heat-denatured isolates and SYBR green-based real-time PCR. Antimicrob. Agents Chemother. *53*, 4211–4216.

KUMAR, T.D.K., MURALI, H.S. and BATRA, H.V. 2009. Simultaneous detection of pathogenic *B. cereus, S. aureus* and *L. monocytogenes* by multiplex PCR. Indian J. Microbiol. *49*, 283–289.

LEE, S.H., JUNG, B.Y., RAYAMAHJI, N., LEE, H.S., JEON, W.J., CHOI, K.S., KWEON, C.H. and YOO, H.S. 2009. A multiplex real-time PCR for differential detection and quantification of *Salmonella spp., Salmonella enterica* serovar *Typhimurium* and *Enteritidis* in meats. J. Vet. Sci. *10*, 43–51.

MACDONALD, F. and SUTHERLAND, A.D. 1994. Important differences between the generation times of *Listeria monocytogenes* and *Listeria innocua* in two *Listeria* enrichment broths. J. Dairy Res. *61*, 433–436.

MALORNY, B., TASSIOS, P.T., RADSTROM, P., COOK, N., WAGNER, M. and HOORFAR, J. 2003. Standardization of diagnostic PCR for the detection of foodborne pathogens. Int. J. Food Microbiol. *83*, 39–48.

MLYNARCZYK, A., MLYNARCZYK, G. and JELJASZEWICZ, J. 1998. The genome of *Staphylococcus aureus*: A review. Zentralbl. Bakteriol. *287*, 277–314.

NAKKEN, S., AUSSEDAT, O., KRISTOFFERSEN, A.B., HOLST-JENSEN, A. and TENGS, T. 2009. UniquePrimer – a web utility for design of specific PCR primers and probes. Ann. Microbiol. *59*, 391–393.

NELSON, K.E., FOUTS, D.E., MONGODIN, E.F., RAVEL, J., DEBOY, R.T., KOLONAY, J.F., RASKO, D.A., ANGIUOLI, S.V., GILL, S.R., PAULSEN, I.T. *ET AL*. 2004. Whole genome comparisons of serotype 4b and 1/2a strains of the food-borne pathogen *Listeria monocytogenes* reveal new insights into the core genome components of this species. Nucleic Acids Res. *32*, 2386–2395.

NOGVA, H.K., RUDI, K., NATERSTAD, K., HOLCK, A. and LILLEHAUG, D. 2000. Application of 5′-nuclease PCR for quantitative detection of *Listeria monocytogenes* in pure cultures, water, skim milk, and unpasteurized whole milk. Appl. Environ. Microbiol. *66*, 4266–4271.

O'GRADY, J., RUTTLEDGE, M., SEDANO-BALBAS, S., SMITH, T.J., BARRY, T. and MAHER, M. 2009. Rapid detection of *Listeria monocytogenes* in food using culture enrichment combined with real-time PCR. Food Microbiol. *26*, 4–7.

OMICCIOLI, E., AMAGLIANI, G., BRANDI, G. and MAGNANI, M. 2009. A new platform for Real-Time PCR detection of *Salmonella spp., Listeria monocytogenes* and *Escherichia coli* O157 in milk. Food Microbiol. *26*, 615–622.

ORAVCOVA, K., TRNCIKOVA, T. and KACLIKOVA, E. 2007. Comparison of three real-time PCR-based methods for the detection of *Listeria monocytogenes* in food. J. Food Nutr. Res. *46*, 63–67.

ORAVCOVA, K., TRNCIKOVA, T., KUCHTA, T. and KACLIKOVA, E. 2008. Limitation in the detection of *Listeria monocytogenes* in food in the presence of competing *Listeria innocua*. J. Appl. Microbiol. *104*, 429–437.

PINTO, B., CHENOLL, E. and AZNAR, R. 2005. Identification and typing of food-borne *Staphylococcus aureus* by PCR-based techniqnes. Syst. Appl. Microbiol. *28*, 340–352.

RIP, D. and GOUWS, P.A. 2009. Development of an internal amplification control using multiplex PCR for the detection of *Listeria monocytogenes* in food products. Food Anal. Method *2*, 190–196.

RIRIE, K.M., RASMUSSEN, R.P. and WITTWER, C.T. 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Anal. Biochem. *245*, 154–160.

ROSSMANITH, P., KRASSNIG, M., WAGNER, M. and HEIN, I. 2006. Detection of *Listeria monocytogenes* in food using a combined enrichment/real-time PCR method targeting the prfA gene. Res. Microbiol. *157*, 763–771.

SCHEU, P.M., BERGHOF, K. and STAHL, U. 1998. Detection of pathogenic and spoilage micro-organisms in food with the polymerase chain reaction. Food Microbiol. *15*, 13–31.

SCHEU, P., GASCH, A. and BERGHOF, K. 1999. Rapid detection of *Listeria monocytogenes* by PCR-ELISA. Lett. Appl. Microbiol. *29*, 416–420.

SHI, X.M., LONG, F. and SUO, B. 2010. Molecular methods for the detection and characterization of foodborne pathogens. Pure Appl. Chem. *82*, 69–79.

TRNCIKOVA, T., HRUSKOVA, V., ORAVCOVA, K., PANGALLO, D. and KACLIKOVA, E. 2009. Rapid and sensitive detection of *Staphylococcus aureus* in food using selective enrichment and real-time PCR targeting a new gene marker. Food Anal. Method *2*, 241–250.

WALSH, D., DUFFY, G. and SHERIDAN, J.J. 1998. Comparison of selective and nonselective enrichment media for the isolation of *Listeria* species from retail foods. J. Food Safety *18*, 85–99.

WANG, X.W., JOTHIKUMAR, N. and GRIFFITHS, M.W. 2004. Enrichment and DNA extraction protocols for the simultaneous detection of *Salmonella* and *Listeria monocytogenes* in raw sausage meat with multiplex real-time PCR. J. Food Prot. *67*, 189–192.

WU, L.C., HORNG, J.T., HUANG, H.Y., LIN, F.M., HUANG, H.D. and TSAI, M.F. 2007. Primer design for multiplex PCR using a genetic algorithm. Soft Comput. *11*, 855–863.

ZHANG, D., ZHANG, H., YANG, L., GUO, J., LI, X. and FENG, Y. 2009. Simultaneous detection of *Listeria monocytogenes, Staphylococcus aureus, Salmonella enterica* and *Escherichia coli* O157:H7 in food samples using multiplex PCR method. J. Food Safety *29*, 348–363.

# APPENDIX TWO

Publication in “Food Analytical Methods”

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# Comparison of In-house and Commercial Real-time PCR Systems for the Detection of Enterobacteriaceae and their Evaluation Within an Interlaboratory Study Using Infant Formula Samples

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**Abstract** Traditional detection methods for *Enterobacteriaceae* in foods are time-consuming and laborious. The current study assessed the specificity of three real-time PCR primer sets. Set A (*IEC* primers) targeted the conserved flanking regions of the 16S rRNA, the 16S-ITS-23S gene region. Set B (*ENT* primers) annealed to *Escherichia coli* 16S ribosomal RNA gene. The third set (C) used a D-LUX™ (Light Upon eXtension) single FAM-labelled forward primer and a corresponding unlabeled primer. Set A was specific for *E. coli* and for some non-Enterobacteriaceae. SYBR Green-based real-time PCR confirmed the specificity of set B for the Enterobacteriaceae but also detected Vibrionaceae. In contrast, set C was poorly specific. However, set D including the forward LUX™ primer from set C and the reverse primer from set B had a specificity comparable to that of set B, but with higher sensitivity. This combined set was successfully applied to detect Enterobacteriaceae in infant milk formula and compared favourably with a commercial real-time PCR kit.

**Keywords** Detection · Enterobacteriaceae · Real-time PCR · LUX™ primers · Infant formula milk

## Introduction

The Enterobacteriaceae family is composed of widely studied microorganisms and includes several species such as *Escherichia coli*, *Klebsiella pneumoniae* or *Salmonella* Typhimurium which are responsible for food intoxications (Blood and Curtis 1995). Routine monitoring of Enterobacteriaceae serves as a hygiene indicator within food processing plants and their presence typically signifies poor cleaning procedures for process surfaces or post-processing contamination of heat-processed foods. To date, most quality assurance laboratories use agar-based ISO procedures (de Boer 1998) in order to detect and quantify Enterobacteriaceae in food products or swab samples. Combined with enrichment steps, Violet Red Bile Glucose Agar (VRBGA) has been among the most popular media for detecting Enterobacteriacaeae in foods. However, this medium is recognised as having some shortcomings (Baylis 2006) as other strains like *Aeromonas* spp. can also grow. Consequently, the colonies that appear on VRBGA are qualified as presumptive with further confirmatory tests required. Overall, this method can take 5 to 7 days for a definitive result which is not satisfactory for allowing rapid product release. Therefore, it is of commercial interest to accelerate this procedure by investigating alternative rapid DNA-based methods.

Detection of the Enterobacteriaceae by conventional PCR has been previously reported. Bayardelle and Zafarullah (2002) developed PCR protocols for detection of the most frequent species of the Enterobacteriaceae in blood, urine, and water samples using primer sets targeting the *wec* gene cluster involved in the synthesis of the enterobacterial common antigen. Real-time PCR has been widely accepted because of rapidity, sensitivity, reproducibility and reduced carry-over contamination (Mackay et al. 2007). Real-time PCR protocols have also been developed and applied in food samples for the detection of Enterobacteriaceae (Nakano et al. 2003; Qiu et al.

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2009). Nakano et al (2003) used specific primers and SYBR Green I as a detection format. Currently, the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit—5′Nuclease (Biotecon, Potsdam, Germany) uses *TaqMan*® probes for the qualitative detection of the Enterobacteriaceae and *Cronobacter sakazakii* particularly in infant formula.

The LUX™ (Light Upon eXtension) primer system is another commercially available tool for detection of pathogens by real-time PCR. Such primers are labelled with a single fluorophore near the 3′-end in a hairpin structure that intrinsically quenches the fluorescence. When incorporated into double-stranded DNA, the fluorophore is de-quenched, resulting in a significant increase of fluorescence signal. The LUX™ primers are software-designed by entering the targeted DNA sequence and subsequently a range of primers are proposed for use.

The LUX™-based real-time PCR has been recognised as a cost-effective alternative to other fluorescence-based techniques (Nazarenko et al. 2002). Such fluorogenic PCR has the potential to be routinely used in food industries because of rapidity, simplicity, and lower cost compared with real-time PCR systems using probes for instance. LUX™ primers have been designed for use in a number of studies mainly in virology (Aitichou et al. 2005; Antal et al. 2007; Chen et al. 2004; Nordgren et al. 2008; Slavov et al. 2008). However, some applications in bacteriology have been reported (Balcazar et al. 2007; Kunchev et al. 2007; McCrea et al. 2007; Mitchell et al. 2009; Xu et al. 2008).

The use of LUX™ primers for the detection of Enterobacteriaceae in food samples has not been assessed in detail and it was of interest to evaluate their potential as a new molecular tool of analysis.

In this study, the specificity of three separate PCR primer sets was evaluated for the detection of the Enterobacteriaceae. Set A (*IEC* primers) consisted of classic primers, previously tested by Maheux et al. (2009) and Khan et al. (2007), and evaluated in this study using conventional PCR. Set B (*ENT* primers) developed by Nakano et al. (2003) was evaluated in this study by PCR and real-time PCR, using SYBR Green I as a detection format. The LUX™ primers (set C) were designed online using the 16S ribosomal RNA gene of *E. coli*, a sequence previously used by Nakano et al for the design of *ENT* primers (2003). Set C was tested by real-time PCR. Finally, the forward primer from set C and the reverse primer from set B were combined to obtain set D and evaluated for their specificity for detection of the Enterobacteriaceae. All primer sets were tested in parallel using Primer Blast available on the National Centre for Biotechnology Information website. Subsequently, the combined primer set (primer set D) was evaluated on infant formula samples and compared with a commercial kit as part of an interlaboratory study.

## Materials and Methods

### Assessment of In-house and Commercial Real-Time PCR Methods Using Pure Cultures

#### *Bacterial Strains*

Type strains of Enterobacteriaceae, *E. coli* (ATCC 11775), *Enterobacter aerogenes* (ATCC 13048), *Erwinia persicina* (ATCC 1381), *K. pneumoniae* (ATCC 700603), *S.* Typhimurium (ATCC 13311), *Serratia marcescens* (ATCC 13880), *Shigella flexneri* (ATCC 9199), *Y. enterocolitica* (ATCC 9610); *A. hydrophila* (ATCC 7966), *Campylobacter jejuni* (ATCC 29428), *Listeria monocytogenes* (ATCC 19115) and *Vibrio parahaemolyticus* (ATCC 17802) were obtained from MicroBioLogics Inc, Saint Cloud, USA. *C. sakazakii*, *Enterobacter cloacae*, *Pseudomonas aeruginosa* and *Proteus mirabilis* isolates were obtained from the culture collection at the Department of Life Sciences, University of Limerick. *Staphylococcus aureus* (NCTC 8325) was obtained from the National Collection of Type Cultures (Health Protection Agency Culture Collections, Salisbury, UK). All strains were stored on Protect™ beads 109 (LangenBach Services Ltd, Dublin, Ireland) at −20 °C until cultivation.

#### *Culture Conditions*

All Enterobacteriaceae were grown overnight on nutrient agar (NA; Oxoid Ltd, Basingstoke, UK) at 37 °C, except *E. persicina* which was incubated at 30 °C. *L. monocytogenes* and *S. aureus* were incubated at 37 °C overnight on NA. *V. parahaemolyticus* was grown overnight at 35 °C on tryptic soya agar (TSA; Oxoid Ltd). *A. hydrophila* was grown overnight at 35 °C on Columbia blood agar (Oxoid Ltd). *P. aeruginosa* was incubated overnight on NA at 25 °C. *C. jejuni* was grown on Columbia blood agar in a microaerophilic environment (CampyGen 2.5l, Oxoid Ltd) for 72 h at 37 °C. Prior to DNA extraction, a preculture of each strain was made by inoculating one loopful of each culture into a flask of 30 ml of nutrient broth (NB; Oxoid Ltd) with overnight shaking at 37 °C for the Enterobacteriaceae and *S. aureus*. Tryptic Soya Broth (TSB; Oxoid Ltd) was used for growth of *L. monocytogenes* (37 °C), the *Vibrionaceae* (35 °C), and *P. aeruginosa* (25 °C). Following growth, 300 µl of the preculture was transferred into 30 ml of fresh broth. A culture of each strain was obtained under the conditions outlined above and grown to exponential growth phase. *C. jejuni* colonies grown on a Columbia agar plate

were suspended in 1 ml of 0.85% saline sterile water prior to DNA extraction.

*DNA Extractions*

DNA used for PCR and real-time PCR experiments was extracted following manufacturer's instructions for Gram-negative or -positive bacteria using the DNeasy Tissue Kit (Qiagen, Crawley, UK) based on DNA purification through chromatography columns. For each strain, DNA was extracted from pellets obtained from cultures in the exponential growth phase following centrifugation of 1 ml of a bacterial suspension at 5,000×*g* for 10 min. DNA quantifications were performed by spectrophotometry at 260 nm using a Nanodrop Spectrophotometer ND-1,000 (Thermo Scientific, Wilmington, USA). Finally, DNA concentrations were adjusted to 1 ng per 2 µl. The sensitivity of DNA detection was determined by diluting *E. coli* DNA, assuming that the targeted gene is present in 7 copies in the *E. coli* genome according to the Ribosomal RNA Operon Copy Number Database (Klappenbach et al. 2001), and using 4,990 kb as the size of the *E. coli* genome (Bergthorsson and Ochman 1995). Therefore 1 ng of DNA was calculated to equal $2.6\times10^4$ genomes.

*Commercial Real-time PCR Detection*

Enterobacteriaceae were detected using the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit–5′ Nuclease which allows rapid detection of Enterobacteriaceae and simultaneous identification of *C. sakazakii*. A 25-µl reaction included 18 µl of Master mix containing the primers and the Hydrolysis Probes, 1 µl of foodproof® Enzyme solution and 1 µl of foodproof® IC (Internal Control) and 5 µl of sample DNA. A positive control was used for each experiment where the DNA template was replaced by the DNA from the foodproof® Control Template. The reaction purity and cross-contamination were checked by adding a negative control consisting of PCR-grade water, in place of the sample. Experiments were carried out using an AB 7,900 HT (Applied BioSystems Inc, Foster City, USA) or a LightCycler® 480 II (Roche Diagnostics GmbH, Mannheim, Germany). The programme settings included: a single pre-incubation step: 37 °C for 4 min and 95 °C for 5 min, an amplification step of 40 cycles: 95 °C for 10 s, 65 °C for 70 s with a step down at each cycle by 0.1 °C. A Hydrolysis Probe binds specifically to the IC, allowing detection in the ROX/Texas Red channel with the AB 7,900 HT. *C. sakazakii* DNA is detected in the FAM channel and the *Enterobacteriaceae* DNA is detected in the VIC/HEX.

In-House Methods of PCR and Real-time PCR Detection

*Primer Selection*

Different primer sets were compared for their specificity for the detection of Enterobacteriaceae. All primers were purchased from MWG Eurofins Operon (Ebersberg, Germany). The specificity of the primer sets was tested using in silico PCR analysis against complete genome sequences of Enterobacteriaceae and non-Enterobacteriaceae. All sequences were provided by the National Centre for Biotechnology Information (NCBI) nucleotide database. The Primer BLAST (Basic Local Alignment Search Tool) programme, available on http://www.ncbi.nlm.nih.gov/tools/primer-blast/, was used for simulated PCR with the primer sets for the detection of the Enterobacteriaceae. This software allows searches for primer annealing sites on DNA sequences using the BLAST algorithm which is a segment-to-segment alignment principle.

Primer sets were named sets A, B, C, and D (Table 1). Set A or *IEC* primers were purported to target the conserved flanking regions of the 16S ribosomal RNA gene, the internal transcribed spacer region (ITS) and the 23 ribosomal RNA gene. Set B corresponded to *ENT* primers targeting the DNA sequence coding for *E. coli* 16S ribosomal RNA (accession number J01859). For set C, the LUX™ primers were provided by Invitrogen, Paisley, UK. These included a 6-carboxyfluorescein 3′-labelled forward primer. The online D-LUX Designer software was used for their design by specifying the *E. coli* 16S ribosomal RNA gene (accession number J01859) as the target sequence. For set D, the forward primer from set C and the reverse primer from set B were used in a new real-time PCR assay.

*PCR Conditions*

For conventional PCR, mixes were prepared with the ready-to-use kit FastStart Taq DNA Polymerase, dNTPack 5 U/µl purchased from Roche Diagnostics GmbH. On the basis of a final 50-µl reaction volume, the master mixture contained 48 µl of 4 mM $MgCl_2$ (2 mM from 10× PCR buffer and 2 mM from $MgCl_2$ solution), 500 nM of forward primer, 500 nM of reverse primer, dNTP mixture and the FastStart Taq DNA Polymerase. Two µl of DNA sample was added to each reaction. The PCR programmes were carried out as shown in Table 2. The specificity of the primer sets was tested against the bacterial strains described above.

PCR was performed on a G-Storm GS2 Thermal Cycler (Genetic Research Instrumentation Ltd, Braintree, UK). Each PCR product was subsequently run on a 2% agarose electrophoresis gel, stained using SYBR Safe™ (Molecular Probes, Eugene, USA) and visualised with a transilluminator G-BOX (Syngen, Frederick, USA) under UV light. The

**Table 1** Primers used in PCR and real-time PCR

| Genetic target | Set | Primer | Primer sequence | *a* | *b* | Reference |
|---|---|---|---|---|---|---|
| 16SrRNA–ITS–23SrRNA | A | IEC—F[a] | 5′-CAATTTTCGTGTCCCCTTCG-3′ | 57 | 450 | Khan et al. 2007 |
| | | IEC—R[b] | 5′-GTTAATGATAGTGTGTCGAAAC-3′ | | | Maheux et al. 2009 |
| 16S rRNA | B | ENT—F | 5′-GTTGTAAAGCACTTT CAGTGGTGAGGAAGG-3′ | 59 | 424 | Nakano et al. 2003 |
| | | ENT—R | 5′-GCCTCAAGGGCACAACCTCCAAG-3′ | | | |
| | C | LUX—F | 5′-CGGTGTACCCGCAG AAGAAGCAC[FAM]G-3′ | 55 | 69 | This study, generated by D-LUX™ software |
| | | LUX—R | 5′-GCTTGCACCCTCCGTATTACC-3′ | | | |
| | D | LUX—F | 5′-CGGTGTACCCGCAG AAGAAGCAC[FAM]G-3′ | 55 | 368 | This study, primer combinations |
| | | ENT—R | 5′-GCCTCAAGGGCACAACCTCCAAG-3′ | | | |

*a* Annealing temperature (°C), *b* Amplicon size (bp)

[a] Forward

[b] Reverse

Perfect DNA™ 100 bp ladder (Novagen, Madison, USA) was used as a molecular marker.

*Real-time PCR Conditions*

When testing the *ENT* primers, conventional PCR procedures were adapted to a real-time procedure on a LightCycler® 1.2 (Roche Diagnostics GmbH), using LightCycler® FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH). In each capillary, a 20 µl reaction mix contained 1× concentration LightCycler® FastStart Reaction Mix SYBR Green; 4 mM $MgCl_2$; 500 nM concentration of each primer; and 2 µl of the template and carried out under conditions outlined in Table 2. Real-time DNA amplifications were observed in Channel 1 at 530 nm of the LightCycler® 1.2. The *ENT* primers were also tested using a LightCycler® 480 with the same PCR mix in channel FAM/SYBR Green, except that the volume per reaction was 10 µl, using the same programme settings.

The detection of Enterobacteriaceae using LUX™ primers (sets C and D) was first assayed with a LightCycler® 1.2, using glass capillaries. In each capillary, the 20 µl reaction mix contained 10 µl of Platinum® qPCR SuperMix-UDG (Invitrogen), 5 mM $MgCl_2$ (final concentration); 500 nM concentration of each primer; 1 µl of Bovine Serum Albumin; 0.12 µl of FastStart Taq DNA Polymerase (Roche Diagnostics GmbH); and 2 µl of the template. Real-time PCR conditions are shown in Table 2.

The LUX™ primers (sets C and D) were also evaluated on the LightCycler® 480 in channel FAM/SYBR Green with 10 µl reaction of the same PCR mix.

Analysis of Enterobacteriaceae in Infant Formula Milk Samples

An interlaboratory study was performed across Europe as part of the MicroVal EN ISO 16140:2003 procedure for validation of an alternative method (Anonymous 2003a).

**Table 2** PCR and real-time PCR conditions using primer sets A, B, C, and D

| Programme | PCR | SYBR green-based real-time PCR | LUX™-based real-time PCR |
|---|---|---|---|
| Pre-incubation | 95 °C, 6 min | 95 °C, 10 min | 50 °C, 2 min |
| | | | 95 °C, 2 min |
| Amplification | 28 cycles | 35 cycles | 35 cycles |
| | 95 °C, 30 s | 95 °C, 5 s | 94 °C, 5 s |
| | °C, 15 s[a] | °C, 10 s[a] | 55 °C, 10 s (single) |
| | 72 °C, 30 s | 72 °C, 20 s (single) | 72 °C, 10 s |
| Melting | N/A[b] | 95 °C, 0 s | 95 °C, 0 s |
| | | 65 °C, 10 s | 55 °C, 15 s |
| | | 95 °C, 0 s | 95 °C, 0 s |
| | | 0.2 °C/s (continuous) | 0.1 °C/s (continuous) |
| Cooling | N/A[b] | 40 °C, 30 s | 30 °C, 30 s |

[a] Specific to each primer set used, similar to conventional PCR annealing temperatures

[b] Non applicable

This study evaluated specifically the foodproof® kit for the detection of *Enterobacteriaceae* and *C. sakazakii* in powdered infant formula samples. The 40 samples of 100 g powdered infant formula in sterile stomacher bags were provided by RIKILT—Institute of Food Safety, Wageningen, The Netherlands. A blind study was undertaken using whereby all samples labelled EBES 1 to 40 with no further information provided to the participants.

Each sample was enriched with 900 ml pre-warmed Buffered Peptone Water (BPW; Bio Trading, Mijdrecht, The Netherlands) at 37 °C and incubated for 18 h at 37 °C. One milliliter of BPW culture was added to a separate tube containing 10 ml Enterobacteriaceae Enrichment broth (EE; Bio Trading) and incubated for 24 h at 37 °C. Then, 50 µl of BPW culture were pipetted into 450 µl of fresh BPW and incubated for 3 h at 37 °C. DNA from dead cells (100 µl of each 3 h culture) was eliminated by adding 300 µl of Reagent D (Biotecon, Potsdam, Germany) to remove DNA from dead bacterial cells and avoid false-positive PCR results. DNA was prepared following the manufacturer's instructions using a Start Prep One kit (Biotecon) by heating a cell pellet resuspended in 200 µl of the provided lysis buffer at 95 °C.

In parallel, reference methods were used for comparison purposes with the suitable commercial and in-house real-time PCR systems.

For the detection of Enterobacteriaceae, the ISO 21528-1:2004 method was used (Anonymous 2004). A loopful of each EE broth tube was plated out on the selective isolation medium, VRBGA (Bio Trading). Characteristic colonies on VRBGA plates were pink to red or purple, with or without precipitation haloes. At least one representative colony from each VRBGA plate was subcultured onto NA plates incubated for 24 h at 37 °C. An oxidase test was performed on each subculture and a colony from each plate was stabbed into a glucose agar tube for incubation 24 h at 37 °C.

*C. sakazakii* was detected using the ISO/TS 22964:2006 (Anonymous 2006). After pre-enrichment of samples with BPW for 18 h at 37 °C, 100 µl of each BPW preculture was transferred into a 10 ml tube of modified Lauryl Sulphate Tryptose broth, (mLST/v; Bio Trading) containing 0.1% of vancomycin and incubated for 24 h at 44 °C. Each of the mLST/v broth tubes was plated out on a *C. sakazakii* Isolation Medium (CSIM; Bio Trading) and incubated for 24 h at 44 °C. Typical colonies on CSIM plates appeared small to medium in size (1–3 mm) and were green to blue-green colonies. Atypical colonies were slightly transparent and violet coloured. A presumptive colony from each plate was then subcultured on TSA (Bio Trading) and incubated for 48 h at 25 °C. A single yellow pigmented colony from each TSA plate was tested on an ID 32 E System (Biomerieux, Craponne, France) for the identification of Enterobacteriaceae and other non-fastidious Gram-negative rods according to the manufacturer's instructions.

The total viable count was obtained following the ISO 4833:2003 method (Anonymous 2003b). A 10-g reference powdered infant formula sample was dissolved into 90 ml of Peptone Physiological Salt (PFZ) (Bio Trading) and diluted to 1/100 dilution. One milliliter of each dilution (1/10, 1/100) was plated in duplicate with pre-melted Plate Count Agar (PCA; Bio Trading). The plates were incubated at 30 °C for 72 h.

## Results and Discussion

### Evaluation of the Commercial Detection Kit

The occurrence of Enterobacteriaceae including *Salmonella* spp. (Cahill et al. 2008), *Pantoea* spp., *E. hermanii*, *E. cloacae* (Estuningsih et al. 2006) or *Klebsiella* spp. (Gao et al. 2010) has been previously demonstrated in infant formula milks. The occurrence of *C. sakazakii* is of major concern to infant formula manufacturers (Chap et al. 2009; Drudy et al. 2006; Giovannini et al. 2008). In-house Real-time PCR systems have recently been developed for detection of *C. sakazakii* (Krascsenicsova et al. 2008; Lehmacher et al. 2007) or *K. pneumoniae* (Sun et al. 2010) in infant powdered milks. In addition, the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit–5′ Nuclease on the LightCycler® 480 is also commercially available for these applications. As manufacturers of the commercial kit, Biotecon purport to have tested a range of 121 *C. sakazakii* strains and 120 non-*C. sakazakii* strains including various species. All *C. sakazakii* gave a signal in the FAM and VIC/HEX channels while all the non-*C. sakazakii* Enterobacteriaceae produced a signal in VIC/HEX channel only. For the detection of Enterobacteriaceae, this exclusivity was confirmed by Biotecon with more than 60 non-Enterobacteriaceae including most of the closely related genera of *Aeromonas* and *Vibrio*. None of the non-Enterobacteriaceae was detected in any channel. After an enrichment step, 1 to 10 cells per 25 to 100 g of relevant type of food sample could be detected.

In the present study, the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit—5′Nuclease was assayed using DNA samples from Enterobacteriaceae and non-Enterobacteriaceae species. All the tested Enterobacteriaceae, including *P. mirabilis*, were detected in the VIC channel. *C. sakazakii* DNA had a Ct value equal to 18.77 whereas *K. pneumoniae* DNA was detected at a Ct equal to 20.73. In the FAM channel, the identification of *C. sakazakii* DNA was confirmed with a Ct equal to 19.90. Data obtained from real-time PCR on serial dilutions of copy numbers of *E. coli* DNA indicated a detection limit of

4 copies in a pure culture at a Ct equal to 30.08. This real-time PCR system was easy to use and appeared highly specific for the Enterobacteriaceae. To date, it is the only commercial kit available for the detection of Enterobacteriaceae by real-time PCR. In a single kit, 96 real-time PCR reactions can be performed; however the cost per reaction remains high. Therefore, if real-time PCR determinations are required to be performed in duplicate or triplicate to obtain statistical data it may not be economically feasible to use this kit but this obviously depends on the particular end user application. For this reason, modifications to these actual protocols and in-house methods were investigated and some general cost comparisons per real-time PCR reaction were subsequently made.

## Comparison of Primer Sets

Figure 1 shows the PCR products run on a SYBR Safe™ pre-stained 2% agarose electrophoresis gel obtained after PCR using set A. Among the range of species tested, these primers amplified only DNA from *E. coli*, a finding not in agreement with that of Maheux et al. (2009). These workers reported that the *IEC* primers, tested by Khan et al., amplified DNA belonging to species from the Enterobacteriaceae family that are phylogenetically relatively close to *E. coli* and *Shigella* spp. According to Khan et al. (2007), the ITS region allows discrimination of bacterial species, and the flanking highly conserved 16S and 23S ribosomal RNA genes can be targeted by primers for specific amplification of *E. coli* strains. Data in the present study would support the findings of Khan et al. (2007) and would suggest that set A may not be suitable for detection of the entire Enterobacteriaceae family. In silico PCR analysis using Blast Primers confirmed this statement (Table 3). PCR procedures can be developed for the specific detection of *E. coli*, as described by Khan et al. (2007). However, other species like *S. aureus* or *P. fluorescens* may also be detected according to Primer Blast data. In the present study, when specificity was tested over a range of Enterobacteriaceae and non-Enterobacteriaceae, *S. aureus* was not detected using this primer set. Therefore, the specificity of the *IEC* primers for *E. coli* requires further analysis by testing of DNA extracted from other non-Enterobacteriaceae.

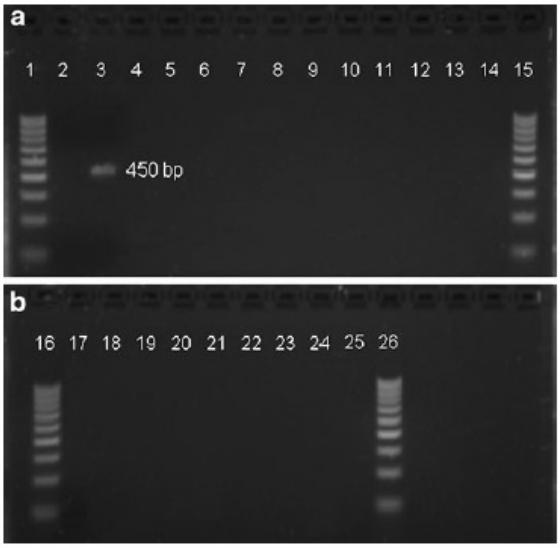


**Fig. 1** Amplification results obtained by PCR using the *IEC* primers (set A). a *Lanes 1*, *15*, *16*, *26*, Perfect DNA™ 100 bp ladder; *lane 2*, *17*, negative control (no DNA template in the PCR reaction); *lane 3*, *E. coli*; *lane 4*, *E. aerogenes*; *lane 5*, *E. cloacae*; *lane 6*, *C. sakazakii*; *lane 7*, *S.* Typhimurium; *lane 8*, *S. flexneri*; *lane 9*, *K. pneumoniae*; *lane 10*, *Y. enterocolitica*; *lane 11*, *E. percisina*; *lane 12*, *S. marcescens*; *lane 13*, *P. mirabilis*; *lane 14*, *V. parahaemolyticus*. b *Lanes 16*, *26*, Perfect DNA™ 100 bp ladder *lane 18*, *A. hydrophila*; *lane 19*, *P. aeruginosa*; *lane 20*, *L. monocytogenes*; *lane 21*, *S. aureus*; *lane 22*, *S. xylosus*; *lane 23*, *Micrococcus* spp.; *lane 24*, *B. cereus*; *lane 25*, *C. jejuni*

Set B (*ENT* primers) amplified DNA from all the Enterobacteriaceae tested except for *P. mirabilis*. However, DNA from *V. parahaemolyticus* (424-bp product) was also detected using this primer set by agarose gel electrophoresis (Fig. 2.). Nakano et al. (2003) evaluated the specificity of *ENT* primers with over 72 different bacterial species and noted amplifications for every tested strain belonging to the Vibrionaceae or Enterobacteriaceae family except *P. mirabilis*. For all other bacterial strains tested, results were negative. Data obtained in this study following PCR with the same set indicated similar results by conventional PCR, where the Enterobacteriaceae, *V. parahaemolyticus* and *A. hydrophila* were detected. Real-time PCR assays using mixes prepared with *ENT* primers were performed on both LightCycler® 1.2 and 480 instruments. The amplification curves obtained with the LightCycler® 480 confirmed that all the Enterobacteriaceae tested were detected as well as *P. mirabilis*, *V. parahaemolyticus*, and *A. hydrophila* were positive using the LightCycler® 1.2 (Table 4). Ct data were retrieved from real-time PCR using the LightCycler® 1.2 (Table 4). Primer set B appeared to be highly specific, as all the Enterobacteriaceae DNA samples tested would generate a PCR product based on Primer Blast analysis, except *Pantoea agglomerans* and *P. mirabilis*. However, the NCBI software showed detection of species from the *Vibrio* and *Aeromonas* genera, as confirmed by PCR and real-time PCR. Overall, this primer set would appear to be a good option for the detection of the Enterobacteriaceae, compared to the designed LUX™ primers (set C) which lacked specificity, as described below.

Initial assays using LUX™ primers targeting the 16S rRNA gene designed using the D-LUX™ software (set

**Table 3** In silico PCR analysis using primer sets A, B, C and D with Primer Blast software

| Bacterial species | Set | | | |
|---|---|---|---|---|
| | A | B | C | D |
| *Budvicia aquatica* | − | + | + | + |
| *Cedecea davisae* | − | + | + | + |
| *Citrobacter braakii* | − | + | + | + |
| *Citrobacter farmeri* | − | + | + | + |
| *Citrobacter freundii* | − | + | + | + |
| *Citrobacter koseri* | − | + | + | + |
| *Cronobacter sakazakii* | − | + | + | + |
| *Enterobacter aerogenes* | − | + | + | + |
| *Enterobacter cloacae* | − | + | + | + |
| *Erwinia persicina* | − | + | + | + |
| *Escherichia coli* | + | + | + | + |
| *Escherichia coli 0157:H7* | − | + | + | + |
| *Ewingella americana* | − | + | + | + |
| *Hafnia alvei* | − | + | + | + |
| *Klebsiella pneumoniae* | − | + | + | + |
| *Klebsiella oxytoca* | − | + | + | + |
| *Kluyvera ascorbata* | − | + | + | + |
| *Kluyvera intermedia* | − | + | + | + |
| *Pantoea agglomerans* | − | − | − | − |
| *Plesiomonas shigelloides* | − | + | + | + |
| *Proteus mirabilis* | − | − | − | − |
| *Proteus vulgaris* | − | + | + | + |
| *Salmonella* Typhimurium | − | + | + | + |
| *Salmonella enteritidis* | − | + | + | + |
| *Serratia liquefaciens* | − | + | + | + |
| *Serratia marcescens* | − | + | + | + |
| *Shigella flexneri* | − | + | + | + |
| *Shigella sonnei* | − | + | + | + |
| *Yersinia enterocolitica* | − | + | + | + |
| *Yersinia pseudotuberculosis* | − | + | + | + |
| *Yersinia rohdei* | − | + | + | + |
| *Acinetobacter baumanii* | − | − | + | − |
| *Aeromonas hydrophila* | − | + | + | − |
| *Aeromonas punctata* | − | + | + | + |
| *Aeromonas sobria* | − | + | + | + |
| *Alcaligenes faecalis* | − | − | + | − |
| *Bacillus cereus* | + | − | − | − |
| *Bacillus subtilis* | + | − | − | − |
| *Campylobacter coli* | − | − | + | − |
| *Campylobacter jejuni* | − | − | + | − |
| *Enterococcus faecalis* | + | − | − | − |
| *Listeria innocua* | − | − | − | − |
| *Listeria monocytogenes* | − | − | − | − |
| *Micrococcus* spp. | − | − | + | − |
| *Pseudomonas aeruginosa* | − | − | + | − |
| *Pseudomonas fluorescens* | + | − | + | − |
| *Pseudomonas putida* | − | − | + | − |
| *Staphylococcus aureus* | + | − | + | − |
| *Staphyloccoccus capitis* | − | − | − | − |
| *Staphyloccoccus lentus* | − | − | − | − |
| *Staphyloccoccus xylosus* | − | − | − | − |
| *Vibrio parahaemolyticus* | − | + | + | + |
| *Vibrio vulnificus* | − | + | + | + |

+ target templates found, − target templates not found

C) were performed using the LightCycler® 1.2 with Enterobacteriaceae and non-Enterobacteriaceae. All the non-Enterobacteriaceae were detected before 35 cycles (Table 4). As a consequence, the primers appeared to lack a satisfactory degree of specificity over the range of strains tested. For example, *P. aeruginosa* DNA could be detected at a Ct equal to 27.22, which was quite close to the value for *E. percisina* DNA (Ct=26.47). Moreover, fluorescence gains were observed for the negative control

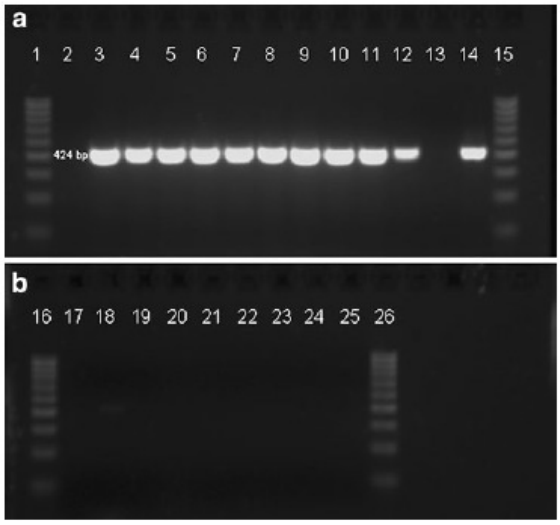


**Fig. 2** PCR products obtained with the *ENT* primers (set B). **a** *Lanes 1, 15, 16, 26*, Perfect DNA™ 100 bp ladder; *lane 2, 17*, negative control (no DNA template in the PCR reaction); *lane 3, E. coli*; *lane 4, E. aerogenes*; *lane 5, E. cloacae*; *lane 6, C. sakazakii*; *lane 7, S.* Typhimurium; *lane 8, S. flexneri*; *lane 9, K. pneumoniae*; *lane 10, Y. enterocolitica*; *lane 11, E. percisina*; *lane 12, S. marcescens*; *lane 13, P. mirabilis*. **b** *Lanes 16, 26*, Perfect DNA™ 100 bp ladder; *lane 14, V. parahaemolyticus*; *lane 18, A. hydrophila*; *lane 19, P. aeruginosa*; *lane 20, L. monocytogenes*; *lane 21, S. aureus*; *lane 22, S. xylosus*; *lane 23, Micrococcus* spp; *lane 24, B. cereus*; *lane 25, C. jejuni*

**Table 4** Sensitivity of primer sets B, C and D against test strains as determined by mean Ct (Cycle threshold) values determined using the automated method provided by the LightCycler® 4.1 software

| Tested strains | Primer set used | | |
|---|---|---|---|
| | B | C | D |
| *Escherichia coli* | 23.14 | 25.51 | 24.30 |
| *Enterobacter aerogenes* | 27.47 | 25.64 | 23.43 |
| *Enterobacter cloacae* | 28.67 | 25.79 | 26.08 |
| *Serratia marcescens* | 28.26 | 25.00 | 23.50 |
| *Erwinia percisina* | 26.85 | 26.46 | 25.68 |
| *Klebsiella pneumoniae* | 27.27 | 25.63 | 22.80 |
| *Shigella flexneri* | 22.74 | 24.83 | 24.29 |
| *Yersinia enterocolitica* | 24.71 | 25.01 | 22.95 |
| *Salmonella* Typhimurium | 29.00 | 25.32 | 25.19 |
| *Cronobacter sakazakii* | 23.52 | 25.17 | 24.81 |
| *Listeria monocytogenes* | 34.49 | 31.48 | 37.01 |
| *Staphylococcus aureus* | 31.34 | 32.28 | 37.84 |
| *Staphylococcus lentus* | 36.82 | 31.39 | >41.00 |
| *Staphylococcus xylosus* | 35.70 | 31.80 | >41.00 |
| *Staphylococcus capitis* | 33.71 | 31.89 | 37.92 |
| *Micrococcus* spp. | – | 29.86 | 38.04 |
| *Campylobacter jejuni* | 38.09 | 31.96 | 36.96 |
| *Bacillus cereus* | 33.02 | 32.68 | >41.00 |
| *Pseudomonas aeruginosa* | 34.14 | 27.21 | 36.79 |
| *Vibrio parahaemolyticus* | 22.73 | 22.79 | 21.18 |
| *Aeromonas hydrophila* | 29.44 | 25.33 | 34.99 |

and the other non-Enterobacteriaceae, suggesting that forward and reverse primers were possibly forming primer-dimers beyond 30 cycles. It is reasonable to speculate that the design aspects of the LUX™ primers can impact on the specificity even if a particular DNA sequence recognised to be highly conserved among species is entered into the software.

Set D appeared to give higher specificity for the Enterobacteriaceae, as all the Enterobacteriaceae tested were detected except *P. mirabilis*. All tested non-Enterobacteriaceae, including *A. hydrophila*, were detected at or beyond 35 cycles. Therefore, cycle number should not exceed 35 in order to avoid generation of false-positive data. However, Enterobacteriaceae and *V. parahaemolyticus* were detected within similar Ct values, between 21.18 and 26.08. Therefore, the specificity of primer set D appeared limited by the detection of *V. parahaemolyticus*, as confirmed by Primer Blast (Table 3). Using the LightCycler® 1.2, this combined primer set showed greater sensitivity as Ct values for the Enterobacteriaceae were lower than those obtained using the *ENT* primer set (Table 4). The detection limit was four cells per PCR reaction at a mean Ct equal to 32.32 for a pure culture. Non-Enterobacteriaceae species had Ct values greater than 30, except for *V. parahaemolyticus* (Ct= 21.18), as shown in Table 4.

Comparative studies between LUX™ primers and other detection formats have been reported recently. Xu et al. (2008) developed a LUX™-based real-time PCR for the detection of *V. parahaemolyticus* in seafood and obtained comparable results for rapidity, specificity and sensitivity to a *TaqMan*® probe-based real-time PCR procedure. However, Mitchell et al. (2009) found that the application of LUX™ primers used in the detection of *Chlamydophila pneumoniae* in clinical specimens displayed a log less sensitivity than their designed *TaqMan*-based assay. In their comparative analysis using SYBR Green I, *TaqMan*® probe and LUX™ primers as the detection format, McCrea et al. (2007) confirmed that the hairpin structure of the LUX™ primers may improve the specificity of PCR by reducing mispriming and primer–dimer formation. The primers used by Castillo et al. (2006) were reported as being suitable for real-time PCR in the detection of Enterobacteriaceae using primers targeting the 16S ribosomal RNA gene. However, these workers used SYBR Green I technology which is purported to be less specific than LUX™ primers (Anonymous 2010). Moreover, the LUX™ detection

format could not be adapted on pre-designed sequences such as those of Castillo et al. (2006).

## Limitations of the Assays Using Primer Sets B and D

Generally, with a LUX™-based real-time PCR, PCR products can be identified based to their melting temperature, Tm, on condition that the amplified region of the targeted gene is sufficiently variable from one species to another. In their study, Mitchell et al. (2009) confirmed their positive samples using melting curve analysis to ensure the specificity of the LUX™ primers. However, when using primer set D in a real-time PCR run, DNA from a range of Enterobacteriaceae, *V. parahaemolyticus* and *A. hydrophila* were amplified and melting curve analysis showed a very low variability of Tm from one species to another. The Tm values obtained were between 89 °C and 90 °C and indicated that the amplified region of the 16S ribosomal RNA gene may be highly conserved among the Enterobacteriaceae and the Vibrionaceae. Therefore, a melting curve analysis would not allow species identification and discrimination using this primer set. The main issue in the use of primer sets B and D is that the Vibrionaceae family was also detected. The 16S ribosomal RNA gene has been used for phylogenetic analyses (Olivier et al. 2005; Wertz et al. 2003). Some sequences of the gene include hypervariable regions and conserved regions. Sequence alignment using Blastn (Basic Local Alignment Search Tool nucleotide) of 16S ribosomal RNA genes of Enterobacteriaceae and Vibrionaceae showed sequences with homologies, which explains the detection of these families by the primer sets B and D.

According to Drake et al. (2007), all *Vibrio* species are ubiquitous in the marine environment and all species except *V. cholerae* and *V. mimicus* require sodium chloride supplementation of the media for growth. Therefore, PCR assays using primer sets B and D may not be applied for the analysis of certain samples including seafood. However, no outbreaks related to contamination with Vibrionaceae have, to date, been reported in infant formula milks. The growth of the Vibrionaceae may be prevented using Brain Heart Infusion (BHI), as noted by Nakano et al. (2003). However, Wong et al. (2004) reported that another low salt medium such as Morita Mineral Salt (MMS)-0.5% NaCl allowed resuscitation of *V. parahaemolyticus* which was present in a viable but non-culturable state. Hence, this may provide false-positive data with real-time PCR using primer sets B and D. Therefore, the use of BHI should be further tested in order to confirm the statement by Nakano et al. (2003).

The ISO method 21528-1:2004 for the detection of Enterobacteriaceae in foods requires an enrichment step in EE broth, which was used in the interlaboratory study. Gurtler and Beuchat (2005) and Iversen and Forsythe (2007) have reported that some *Cronobacter* strains do not grow in EE broth, which can lead to false negative results. Joosten et al. (2008) advised an enrichment of the food samples with BPW only. However, Gram-positive flora may interfere with the recovery of Enterobacteriaceae. Weber et al. (2009) supplemented the BPW with 40 μM 8-hydroxyquinoline, 0.5 g/L ammonium iron (III) citrate, 0.1 g/L sodium deoxycholate and 0.1 g/L sodium pyruvate in order to optimise the enrichment and the selection of the Enterobacteriaceae.

## Comparative Analysis of Milk Samples

The results of the analysis of milk samples are shown in Table 5, and provide comparative data between the LUX™-based method (set D), the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit 5′-nuclease and ISO methods. In the interlaboratory study, five spiking modes

**Table 5** Comparison of percentage (%) of positive results obtained in the analysis of 40 samples of 100 g powdered infant formula after spiking and detected using ISO and alternative real-time PCR methods

| Spiking mode | Detection enterobacteriaceae | | | Detection *C. sakazakii* | |
|---|---|---|---|---|---|
| | Set D | foodproof® kit | ISO 21528-1:2004 | foodproof® kit | ISO/TS 22964:2006 |
| EBES0[a] | 0 | 0 | 12.5 | 0 | 0 |
| EB1[b] | 100 | 100 | 100 | 0 | 37.5 |
| EB2[c] | 100 | 100 | 100 | 0 | 0 |
| CS1[d] | 37.5 | 37.5 | 37.5 | 37.5 | 37.5 |
| CS2[e] | 100 | 100 | 100 | 100 | 100 |

[a] Non-spiked blank samples

[b] Samples spiked with low level of Enterobacteriaceae

[c] Samples spiked with higher level of Enterobacteriaceae

[d] Samples spiked with very low levels of *C. sakazakii*

[e] Samples spiked with higher levels of *C. sakazakii*

were decided by RIKILT who gave the identification of the samples after the pooling of results: blank samples or no spiking (EBES0), samples spiked with low levels (EB1) and higher level of Enterobacteriaceae (EB2), and samples spiked with low levels (CS1) and higher levels of *C. sakazakii* (CS2). For each spiking mode, eight samples were tested. Twenty seven out of 40 samples tested positive for the Enterobacteriaceae and 11 out of these 27 samples tested positive for *C. sakazakii* using the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit 5′-nuclease. The samples were analysed with the LightCycler® 480 (Fig. 3). Overall, the data obtained was in good agreement among all participants in the interlaboratory study. However, three false negative results were obtained for some CS1 samples among the three methods tested (also found by other participating laboratories). The occurrence of false negative results could be explained by a failure to detect *C. sakazakii*. As described by Edson et al. (2009) the bacteria may have failed to grow because of issues with media or incubation conditions.

One false-positive result for Enterobacteriaceae was obtained among the EBES 0 samples using the ISO 21528-1:2004 method only, while both alternative methods provided the expected results. Similarly, one false-positive result for *C.*

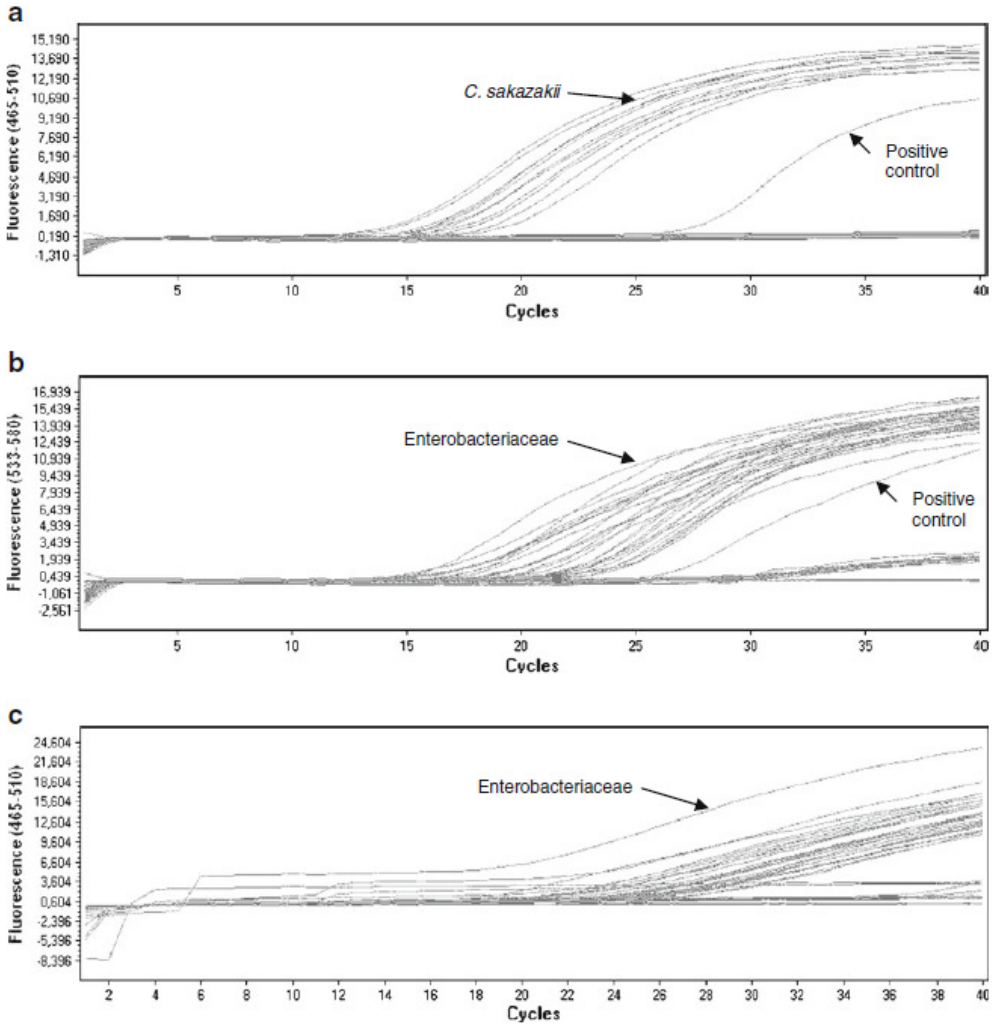


**Fig. 3** Amplification curves for the detection of Enterobacteriaceae using the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit 5′-nuclease on FAM channel for the detection of *C. sakazakii* on FAM channel (**a**) and for the Enterobacteriaceae on VIC channel (**b**), and using primer set D on FAM channel (**c**)

*sakazakii* was retrieved among the EB1 samples using the ISO/TS 22964:2006. As a hypothesis, both false-positive results may have arisen from cross-contamination in the laboratory while performing the ISO procedures, more than likely after the pre-enrichment step with BPW. This indicates that the real-time PCR methods used may reduce the occurrence of such false-positive results.

In summary, Enterobacteriaceae could be detected simultaneously using primer set D or the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit. The real-time PCR with set D appeared to be as rapid and sensitive as the commercial detection kit. However, the specificity of set D was limited by the detection of the Vibrionaceae family and the non-detection of *P. mirabilis*. As shown with real-time PCR and in silico PCR data, primer set D cannot be used for the detection of *P. mirabilis*. However, the presence of this species in food samples remains very rare and *P. mirabilis* has not been reported to date in infant formula. The identification of *C. sakazakii* was not possible using primer set D by melting curve analysis. The main commercial interest in the use of this primer set arises from its' potential lower cost based on current retail prices for the commercial kit and the costs of the individual components of the method developed in this study. Therefore, the choice is given to the user: either a lower specificity at low cost or higher specificity at higher cost with the guarantee to identify *C. sakazakii* from other Enterobacteriaceae. It should be stated that no other in-house methods or other commercial real-time PCR systems are available with the specificity of the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit for safety control in milk samples. Despite the lower specificity compared with the commercial real-time PCR kit, the LUX™-based real-time PCR system with set D may be considered as a low-cost screening method for the detection of Enterobacteriaceae. This primer set could directly discriminate negative samples, while presumptive positive samples could be further analysed using the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit for Enterobacteriaceae confirmation, or even identification of *C. sakazakii*.

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## References

Aitichou M, Javorschi S, Ibrahim MS (2005) Two-color multiplex assay for the identification of orthopox viruses with real-time LUX-PCR. Mol Cell Probes 19(5):323–328

Anonymous (2003a) ISO 16140:2003: microbiology of food and animal feeding stuffs—protocol for the validation of alternative methods. ISO, Geneva

Anonymous (2003b) ISO 4833:2003: microbiology of food and animal feeding stuffs—horizontal method for the enumeration of microorganisms—colony-count technique at 30 degrees C. ISO, Geneva

Anonymous (2004) ISO 21528-1:2004: microbiology of food and animal feeding stuffs—horizontal methods for the detection and enumeration of Enterobacteriaceae—Part 1: detection and enumeration by MPN technique with pre-enrichment. ISO, Geneva

Anonymous (2006) ISO/TS 22964:2006: milk and milk products—detection of *Enterobacter sakazakii*. ISO, Geneva

Anonymous. (2010). D-LUX™ Gene Expression Assays—www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Nucleic-Acid-Amplification-and-Expression-Profiling/qRT-PCR/qPCR/D-LUX-Assays.html?CID=fl-lux. In.

Antal M, Farkas T, German P, Belak S, Kiss I (2007) Real-time reverse transcription-polymerase chain reaction detection of Newcastle disease virus using light upon extension fluorogenic primers. J Vet Diagn Investig 19(4):400–404

Balcazar JL, Vendrell D, de Blas I, Ruiz-Zarzuela I, Girones O, Muzquiz JL (2007) Quantitative detection of *Aeromonas salmonicida* in fish tissue by real-time PCR using self-quenched, fluorogenic primers. Journal of Medical Microbiology 56(3):323–328

Bayardelle P, Zafarullah M (2002) Development of oligonucleotide primers for the specific PCR-based detection of the most frequent Enterobacteriaceae species DNA using wec gene templates. Can J Microbiol 48(2):113–122

Baylis CL (2006) Enterobacteriaceae. In: CdW Blackburn (ed) Food spoilage microorganisms (Campden and Chlorleywood Food Research Association). Woodhead, UK, pp 624–667

Bergthorsson U, Ochman H (1995) Heterogeneity of genome sizes among natural isolates of *Escherichia coli*. J Bacteriol 177 (20):5784–5789

Blood RM, Curtis GDW (1995) Media for total Enterobacteriaceae, coliforms and *Escherichia coli*. Int J Food Microbiol 26(1):93–115

Cahill SM, Wachsmuth IK, Costarrica MD, Ben Embarek PK (2008) Powdered infant formula as a source of *Salmonella* infection in infants. Clin Infect Dis 46(2):268–273

Castillo M, Martin-Orue SM, Manzanilla EG, Badiola I, Martin M, Gasa J (2006) Quantification of total bacteria, enterobacteria and lactobacilli populations in pig digesta by real-time PCR. Vet Microbiol 114(1–2):165–170

Chap J, Jackson P, Siqueira R, Gaspar N, Quintas C, Park J, Osaili T, Shaker R, Jaradat Z, Hartantyo SHP, Sani NA, Estuningsih S, Forsythe SJ (2009) International survey of *Cronobacter sakazakii* and other *Cronobacter* spp. in follow up formulas and infant foods. Int J Food Microbiol 136(2):185–188

Chen R, Huang WM, Lin ZX, Zhou ZF, Yu HQ, Zhu DZ (2004) Development of a novel real-time RT-PCR assay with LUX primer for the detection of swine transmissible gastroenteritis virus. J Virol Meth 122(1):57–61

de Boer E (1998) Update on media for isolation of Enterobacteriaceae from foods. Int J Food Microbiol 45(1):43–53

Drake SL, DePaola A, Jaykus LA (2007) An overview of *Vibrio vulnificus* and *Vibrio parahaemolyticus*. Compr Rev Food Sci Food Saf 6(4):120–144

Drudy D, Mullane NR, Quinn T, Wall PG, Fanning S (2006) *Enterobacter sakazakii*: an emerging pathogen in powdered infant formula. Clin Infect Dis 42(7):996–1002

Edson DC, Empson S, Massey LD (2009) Pathogen detection in food microbiology laboratories: an analysis of qualitative proficiency test data, 199–2007. J Food Saf 29(4):521–530

Estuningsih S, Kress C, Hassan AA, Akineden O, Schneider E, Usleber E (2006) Enterobacteriaceae in dehydrated powdered infant formula manufactured in Indonesia and Malaysia. J Food Prot 69(12):3013–3017

Gao H, Gao QL, Zhang X, Guan C, Luo MH, Zhang HB, Liu P, Zhang HY, Li J (2010) Improved medium for detection of *Klebsiella* in powdered milk. J. Food Saf. 30(1):12–23

Giovannini M, Verduci E, Ghisleni D, Salvatici E, Riva E, Agostoni C (2008) *Enterobacter sakazakii*: an emerging problem in paediatric nutrition. J Int Med Res 36(3):394–399

Gurtler JB, Beuchat LR (2005) Performance of media for recovering stressed cells of *Enterobacter sakazakii* as determined using spiral plating and ecometric techniques. Appl Environ Microbiol 71(12):7661–7669

Iversen C, Forsythe SJ (2007) Comparison of media for the isolation of *Enterobacter sakazakii*. Appl Environ Microbiol 73(1):48–52

Joosten H, Marugg J, Stephan R, Klijn A, Jackson T, Iversen C (2008) A rapid and reliable alternative to ISO 21528-1: 2004 for detection of Enterobacteriaceae. Int J Food Microbiol 125 (3):344–346

Khan IUH, Gannon V, Kent R, Koning W, Lapen DR, Miller J, Neumann N, Phillips R, Robertson W, Topp E, Bochove Ev, Edge TA (2007) Development of a rapid quantitative PCR assay for direct detection and quantification of culturable and non-culturable *Escherichia coli* from agriculture watersheds. J. Microbiol. Methods 69(3):480–488

Klappenbach JA, Saxman PR, Cole JR, Schmidt TM (2001) rrndb: the ribosomal RNA operon copy number database. Nucleic Acids Res 29(1):181–184

Krascsenicsova K, Trncikova T, Kaclikova E (2008) Detection and Quantification of *Enterobacter sakazakii* by Real-time 5'-Nuclease Polymerase Chain Reaction Targeting the *palE* Gene. Food Anal. Meth. 1(2):85–94

Kunchev M, Alexandrov E, Kamarinchev B, Marinov K, Gergova I, Mekouchinov K (2007) Effective LUX (Light upon eXtension) primer system for early and rapid detection of *Coxiella burnetii*. Biotechnology & Biotechnological Equipment 21(3):338–340

Lehmacher A, Fiegen M, Hansen B (2007) Real-time PCR of *Enterobacter sakazakii* in infant formula. Journal Fur Verbraucherschutz Und Lebensmittelsicherheit-Journal of Consumer Protection and Food Safety 2(2):218–221

Mackay IM, Mackay JF, Nissen MD, Sloots TP (2007) Real-time PCR: history and fluorogenic chemistries. In real-time PCR in microbiology, from diagnosis to characterization. Caister, Norfolk, pp 1–39

Maheux AF, Picard FJ, Boissinot M, Bissonnette L, Paradis S, Bergeron MG (2009) Analytical comparison of nine PCR primer sets designed to detect the presence of *Escherichia coli/Shigella* in water samples. Water Res (Oxf) 43(12):3019–3028

McCrea JK, Liu CY, Ng LK, Wang GH (2007) Detection of the *Escherichia coli* pathogenic gene *eae* with three real-time polymerase chain reaction methods. Can J Microbiol 53 (3):398–403

Mitchell SL, Budhiraja S, Thurman KA, Thacker WL, Winchell JM (2009) Evaluation of two real-time PCR chemistries for the detection of *Chlamydophila pneumoniae* in clinical specimens. Mol Cell Probes 23(6):309–311

Nakano S, Kobayashi T, Funabiki K, Matsumura A, Nagao Y, Yamada T (2003) Development of a PCR assay for detection of Enterobacteriaceae in foods. J Food Prot 66(10):1798–1804

Nazarenko I, Lowe B, Darfler M, Ikonomi P, Schuster D, Rashtchian A (2002) Multiplex quantitative PCR using self-quenched primers labeled with a single fluorophore. Nucleic Acids Research 30(9)

Nordgren J, Bucardo F, Dienus O, Svensson L, Lindgren PE (2008) Novel light-upon-extension real-time PCR assays for detection and quantification of genogroup I and II noroviruses in clinical specimens. J Clin Microbiol 46(1):164–170

Olivier A, Lee HY, Cote JC (2005) Study of the heterogeneity of 16S rRNA genes in gamma-proteobacteria: implications for phylogenetic analysis. J Gen Appl Microbiol 51(6):395–405

Qiu Y, Hu Q, Wang W (2009) Evaluation of real-time PCR in detecting enteric pathogens. China Tropical Medicine 9(1):12–13

Slavov S, Kalvatchev Z, Tsekov I, Simeonov P, Hristova L, Kotsev J, Mladenov D, Tsvetkov M (2008) Novel light-upon-extension (LUX) real-time PCR primer system for rapid detection of *Polyomavirus hominis* 1 (BKV) in clinical samples. Biotechnol Biotechnol Equip 22(2):759–761

Sun FL, Wu DC, Qiu ZG, Jin M, Wang XW, Li JW (2010) Development of realtime PCR systems based on SYBR Green for the specific detection and quantification of *Klebsiella pneumoniae* in infant formula. Food Control 21(4):487–491

Weber C, Stephan R, Druggan P, Joosten H, Iversen C (2009) Improving the enrichment procedure for Enterobacteriaceae detection. Food Microbiol 26(6):565–572

Wertz JE, Goldstone C, Gordon DM, Riley MA (2003) A molecular phylogeny of enteric bacteria and implications for a bacterial species concept. J Evol Biol 16(6):1236–1248

Wong HC, Wang PL, Chen SY, Chiu SW (2004) Resuscitation of viable but nonculturable *Vibrio parahaemolyticus* in a minimum salt medium. Fems Microbiology Letters 233(2):269–275

Xu R-s, Chen R, Lin C-h, Yang Z-j, Chen G-w (2008) Development of a LUX™ PCR assay for rapid detection of *Vibrio parahaemolyticus*. Chin Vet Sci 38(2):1075–1079

# APPENDIX THREE

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RESEARCH

# Development of Defined Microbial Population Standards Using Fluorescence Activated Cell Sorting for the Absolute Quantification of *S. aureus* Using Real-Time PCR

**Alice Martinon · Ultan P. Cronin · Martin G. Wilkinson**



**Abstract** In this article, four types of standards were assessed in a SYBR Green-based real-time PCR procedure for the quantification of *Staphylococcus aureus* (*S. aureus*) in DNA samples. The standards were purified *S. aureus* genomic DNA (type A), circular plasmid DNA containing a thermonuclease (*nuc*) gene fragment (type B), DNA extracted from defined populations of *S. aureus* cells generated by Fluorescence Activated Cell Sorting (FACS) technology with (type C) or without purification of DNA by boiling (type D). The optimal efficiency of 2.016 was obtained on Roche LightCycler® 4.1. software for type C standards, whereas the lowest efficiency (1.682) corresponded to type D standards. Type C standards appeared to be more suitable for quantitative real-time PCR because of the use of defined populations for construction of standard curves. Overall, Fieller Confidence Interval algorithm may be improved for replicates having a low standard deviation in Cycle Threshold values such as found for type B and C standards. Stabilities of diluted PCR standards stored at −20°C were compared after 0, 7, 14 and 30 days and were lower for type A or C standards compared with type B standards. However, FACS generated standards may be useful for bacterial quantification in real-time PCR assays once optimal storage and temperature conditions are defined.

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**Keywords** Quantification · Real-time PCR · Fluorescence activated cell sorting · DNA standards · *Staphylococcus aureus*

## Introduction

Quantitative real-time PCR is extensively used for the simultaneous amplification, detection and quantification of nucleic acids in many research and diagnostic applications. Most of the real-time PCR procedures in microbiology generate qualitative data by giving a simple presence or absence result. Absolute quantification of target DNA by real-time PCR can be used if it is assumed a direct relationship exists between the concentration of nucleic acid and the number of microorganisms in a sample. The Cycle Threshold (Ct) corresponding to the analysed sample is compared with the other Ct data by the amplification of a dilution series of standards to generate a standard curve, which subsequently allows determination of the initial concentration of target DNA in a sample. Mackay et al. [1] noted the lack of commercial applications of quantitative PCR (qPCR) in routine microbial analysis due to difficulties in the optimization, standardization and normalization of qPCR procedures. In parallel, research laboratories have developed their own methods in real-time PCR for the absolute quantification of microorganisms in samples. However, the reliability of a real-time PCR quantification method depends on providing accurate and precise titres of microbial standards.

To date, standards for absolute quantification of microorganisms using real-time PCR are expressed as 'copy number' or Colony Forming Unit (CFU). Copy number unit corresponds to the quantity of target molecules

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initially present in the PCR reaction. Standards in 'copy number' are typically prepared using genomic DNA [2–5], plasmids containing the target sequence [6–9], or PCR products [10].

For the quantification of bacteria as CFU per unit volume of sample, DNA standards are prepared by serial dilution of a pure bacterial culture followed by DNA extraction [11, 12]. Alternatively, standard curves can be achieved from several dilutions of a spiked food sample which generates crossing points (Ct) which are plotted against the logarithmic concentration of the serial dilutions [13–15].

An alternative novel possibility for bacterial quantification by real-time PCR is to prepare standards with exact known quantities of microorganisms generated using single-cell isolation by Fluorescence Activated Cell Sorting (FACS). In flow cytometry, physical and/or chemical characteristics of single cells or particles are measured in a fluid stream where pressure and orifice sizes are controlled such that a single cell passes through once into an illumination zone [16]. Cell sorting then uses electrical and/or mechanical means to separate, isolate and collect the cells of interest according to the parameters entered by the user [17]. Among its advantages, FACS allows the rapid concentration and enrichment of cell populations present in low abundance [18] or sorting of defined cell numbers into tubes or microplates [19]. However, very few cell sorting applications have been reported in the literature where cell sorting has been combined with molecular analysis techniques such as PCR. Gillebault et al. [19] combined cell sorting with PCR to identify sorted marine bacterial subpopulations. Bowers et al. [20] used real-time PCR combined with cell sorting to confirm the presence of a specific algal prey inside the vacuoles of sorted algal bloom species. Hoffemann et al. [21] studied gene expression levels in sorted eukaryote cell populations which provided a relative quantification methodology. To date, the use of FACS has not been assessed as a potential methodology for the generation of bacterial standards for use in real-time PCR assays to improve the quantification of microorganisms of interest to the food industry such as *Staphylococcus aureus*.

*Staphylococcus aureus* is an important indicator of food quality and the efficiency of industrial cleaning procedures. This microorganism is routinely monitored by the food industry using selective agar based methods which can take up to 48 h for a definitive result. Therefore, the development of a rapid and quantitative real-time PCR detection system for this pathogen is of both industrial and public health interest. A number of studies have described real-time PCR assays for quantification of *S. aureus* in various foods using genomic or plasmid DNA standards [22–26]. Moreover, real-time PCR systems for the quantitative detection of *S. aureus* can be applied to clinical diagnosis, as described by Peters et al. [27], for the determination of bacteremia. In this study, the thermonuclease (*nuc*) gene of *S. aureus* was selected as the target DNA for real-time PCR assays as Brakstad et al. [28] demonstrated that this gene had sequences common to all tested *S. aureus* isolates and was unique to the species. Mackay et al. [1] stated that quantitative real-time PCR technology may be improved by the introduction of additional reference materials and calibrators and the release of more commercial real-time quantitative PCR kits. This lack of commercially available kits for quantification of microorganisms is itself problematic, but is further compounded by the absence of standardization among existing in-house PCR assays that enable valid comparison of methodologies and data [29]. Some commercial real-time PCR systems currently available on the market do not include quantitative features such as a quantification calibrator or a protocol to allow absolute quantification, whereas others use calibrated plasmid solutions to create a standard curve. Therefore, the novel strategy of using standards generated by FACS in real-time PCR was assessed to address some of the issues outlined above using *S. aureus* as the target microorganism for development of a quantification model.

In this study, the potential of DNA standards generated by cell sorting was investigated for use in real-time PCR quantification. Four DNA standards were compared for the absolute quantification of the *nuc* gene of *S. aureus* using a SYBR Green-based real-time PCR: (a) Pure genomic DNA, (b) circular plasmid DNA, (c) and (d) FACS-generated DNA standards. The resulting standard curves were compared on the basis of PCR efficiency using real-time PCR software, and the stability of the diluted standards was tested over a range of storage times.

## Materials and Methods

### Bacterial Strain

The type strain of *S. aureus* (NCTC 8325) was obtained from the National Collection of Type Cultures (Health Protection Agency Culture Collections, Salisbury, UK) and was stored on Protect beads 109 (LangenBach Services Ltd, Dublin, Ireland) at −20°C until cultivation. *S. aureus* was revived by smearing of an inoculated bead onto nutrient agar (NA; Oxoid Ltd, Basingstoke, UK). After overnight incubation at 37°C, isolated colonies were obtained and maintained at 4°C on NA plates.

### Type A Standards (Genomic DNA)

Before DNA extraction, a preculture of *S. aureus* was made by inoculating one loopful of each culture into a flask of

30 ml of nutrient broth (NB; Oxoid Ltd) with overnight shaking at 37°C. Following this, 300 µl of preculture was transferred into 30 ml of fresh broth. A culture of *S. aureus* was obtained under the conditions outlined above and grown to exponential growth phase. DNA used for specificity evaluation was extracted according to the manufacturer's instructions for Gram negative or Gram positive bacteria using a DNeasy Blood and Tissue Kit (Qiagen, Crawley, UK) based on DNA purification through chromatography columns. DNA was extracted from pellets obtained from cultures in the exponential growth phase following centrifugation of 1 ml of a bacterial suspension at 5000×*g* for 10 min in a Sigma 1–15 centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) and eluted in 200 µl of AE buffer (Qiagen). DNA quantifications were automatically determined by spectrophotometry at 260 nm using a Nanodrop Spectrophotometer ND-1000 (Thermo Scientific, Wilmington, USA) which systematically computes the 260/280 nm absorbance ratio.

Standard curves were prepared by amplifying DNA samples containing 300,000 copies or $9.27 \times 10^{-10}$ g *S. aureus* DNA to three copies or $9.27 \times 10^{-15}$ g of *S. aureus* DNA per 2 µl or PCR reaction over a 10-fold dilution series in six dilution points in duplicate in DNA grade water (Fisher Scientific, Loughborough, UK). A standard curve was generated from genomic DNA according to Applied Biosystems' instructions [30] based on a 2,821,361 bp sized genome of *S. aureus* NCTC 8325 [31] assuming that the *nuc* gene was present as a single copy. The copy numbers of the *nuc* gene were calculated based on the following formula, in which $n$ is the number of base pairs, $m$ is the DNA mass, $N_A$ is Avogadro's number ($6.02 \times 10^{23}$ bp/mol) and $M$ is the average molecular weight of a base pair (610 g/mol).

$$n = (m \times N_A)/M$$

$$m = n \times 1.096 \times 10^{-21} \text{g/bp}$$

Type B Standards (Plasmid DNA)

First, a 279-bp PCR product corresponding to the insert was produced by amplifying a *S. aureus*-specific fragment of the *nuc* gene. The Genbank number of *S. aureus* NCTC 8325 is NC_007795 with SAOUHSC_00818 as the locus tag for the thermonuclease. The primers were purchased from MWG Eurofins Operon (Ebersberg, Germany). The forward primer was 5'-GCGATTGATGGTGATACGGT-3' and the reverse primer was 5'-AGCCAA GCCTTGACGAACTAAAGC-3' [28].

The PCR mix was prepared with the FastStart Taq DNA Polymerase, dNTPack 5 U/µl kit (Roche Diagnostics GmbH, Mannheim, Germany). On the basis of a final 50 µl reaction volume, the master mixture contained 48 µl of 4 mM $MgCl_2$ (2 mM from 10× PCR buffer and 2 mM from $MgCl_2$ solution), 500 nM of forward primer, 500 nM of reverse primer, dNTP mixture and the FastStart *Taq* DNA Polymerase to which 2 µl of *S. aureus* DNA sample (1 ng) was added to each reaction. The PCR was performed on a G-Storm GS2 Thermal Cycler (Genetic Research Instrumentation, Braintree, UK). The PCR programmes were carried out as shown in Table 1.

**Table 1** PCR and real-time PCR conditions using *nuc* primer set

| Program | PCR | SYBR Green-based real-time PCR |
|---|---|---|
| Pre-incubation | 95°C, 6 min | 95°C, 10 min |
| Amplification | 35 cycles | 35 cycles |
| | 95°C, 30 s | 95°C, 5 s |
| | 62°C, 15 s | 62°C, 10 s |
| | 72°C, 30 s | 72°C, 20 s (Single) |
| Melting | N/A[a] | 95°C, 0 s |
| | | 65°C, 10 s |
| | | 95°C, 0 s |
| | | 0.2°C/s (Continuous) |
| Cooling | N/A[a] | 40°C, 30 s |

[a] Not applicable

Plasmid DNA standards were constructed by purifying the PCR product using a Qiaquick PCR Purification Kit (Qiagen) according to the manual's instructions. The DNA was quantified using a Nanodrop Spectrophotometer ND-1000. Purified PCR products were cloned using the TOPO TA pCR® 4 Cloning® Kit for Sequencing (Invitrogen Corporation, Carlsbad, USA) as per manufacturer's instructions. The plasmid was transformed into Mach1™-T1® chemically competent *Escherichia coli* cells (Invitrogen Corporation). 50 µl of transformed inoculum were spread onto Luria–Bertani (LB; Sigma-Aldrich, Saint Louis, USA) agar supplemented with 50 µg/ml of ampicillin (Sigma-Aldrich). Only positive recombinants obtained after the transformation of the chemically competent cells grow in this medium. Characteristic white colonies were picked and cultured overnight in LB broth (Sigma-Aldrich) containing 50 µg/ml of kanamycin (Sigma-Aldrich). Plasmids were prepared using the HiSpeed Plasmid Midi kit (Qiagen), according to manufacturer's instructions. The presence of the insert was confirmed by a restriction digestion using Eco RI (Sigma-Aldrich). The circular plasmid was quantified using a Nanodrop Spectrophotometer ND-1000.

Standard curves were prepared by amplifying plasmid samples containing 300,000 copies or $1.39 \times 10^{-12}$ g of plasmid DNA to three copies or $1.39 \times 10^{-7}$ g of plasmid DNA per 2 µl or per PCR reaction over a 10-fold dilution series in six dilution points in duplicate in DNA grade

water, according to Applied Biosystems instructions for creating a standard curve using plasmid DNA [30], based on a 4235-bp sized of plasmid including the insert.

### Type C and D Standards (FACS Generated)

A single colony of *S. aureus* NCTC 8325 was inoculated onto 5 ml of Nutrient Broth (NB; Oxoid Ltd, Basingstoke, UK) and grown overnight at 37°C with shaking at 125 rpm. The next day, 1 ml of the preculture was transferred to 49 ml of fresh NB, and the culture was left shaking at 125 rpm at 37°C for 2 h. From the culture, 1 ml aliquots were pipetted into microfuge tubes and centrifuged for 10 min at 4000×*g* using a Sigma centrifuge. The supernatant was discarded, and the pellet was resuspended in 5 ml of sterile Phosphate Buffer Saline (PBS; Oxoid). This suspension was subsequently analysed and sorted using a standard MoFlo cell sorter (Beckman Coulter Inc, Fullerton, USA) with a 488 nm Ar laser and a 635 nm laser diode and the only modification of which was the inclusion of a photomultiplier tube for detecting forward scattered (FSC) light. For bacterial analysis, the instrument's 488 nm laser was set at 100 mW, with triggering on side scatter (SSC). Bacteria were separated from noise and cellular debris using combinations of FSC pulse height versus SSC pulse height and FSC pulse area versus SSC pulse area. Thereafter, single cells were discriminated from doublets and chains using FSC pulse width. Using Summit v4.0. Software, a gate was drawn around single cells and $1.0 \times 10^6$ single cells were sorted into Eppendorf tubes, using the instrument's single cell/3 drop sort mode. Sorting was performed with a 70 µm nozzle at typical drop drive frequencies of ~95,000 Hz and drop drive amplitudes of ~15 V. Charge phase and defanning were adjusted to maintain side stream integrity. If necessary, drop drive amplitude was adjusted to maintain the physical position of the last attached drop (using the stream camera) and drop delay was calculated immediately before and after each sort. The acquisition rates of 3,000–9,000 events/s (eps), sort rates of 2,500–8,000 eps and abort rates of 180–200 eps were typical. Plate counts were carried out in triplicate on the day of sorting to confirm that $1.0 \times 10^6$ cells per tube were sorted. Post sorting, the tubes were centrifuged at 4000×*g* using an Allegra X22R centrifuge (Beckman Coulter, Brea, USA). Pellets were stored at −20°C until DNA extraction.

Type C standards were prepared by isolating DNA from sorted cells resuspended in 180 µl of lysing buffer using DNeasy Blood and Tissue Kit according to manual instructions. After DNA purification, tubes contained $1.0 \times 10^6$ equivalent cells or $1.0 \times 10^6$ Signal Generating Unit (SGU)/200 µl of elution buffer. Wang and Spadoro [32] introduced the SGU to measure the concentration of template available for PCR amplification. A SGU corresponds to the smallest unit that generates a positive signal by PCR amplification or one particle containing at least one amplifiable molecule. This unit was employed in the present study when dealing with FACS generated standards.

Then, serial dilutions were performed in DNA grade water over six points: $10^4$, $5 \times 10^3$, $10^3$, 300, 100, and 10 SGU per 2 µl or per PCR reaction.

Type D standards were prepared by resuspending pellets obtained after centrifugation of sorted cells in 200 µl of DNA grade water and the inclusion of a lysing step by boiling of the cells, according to Queipo-Ortuno et al. [33]. Serial dilutions were performed as for the preparation of type C FACS standards.

### SYBR Green I Real-Time PCR Assay for Absolute Quantification

Real-time PCR procedures were performed on a LightCycler® 1.2 (Roche Diagnostics GmbH). The primers used were the same as those used in the production of PCR products for the cloning procedure. In each capillary, the 20-µl reaction mix contained 1× concentration LightCycler-FastStart DNA Master SYBR Green (Roche Diagnostics GmbH); 4 mM $MgCl_2$; 500 nM concentration of each primer and 2 µl of the template. SYBR Green I was included as detection format for its simplicity, low cost, and as binding is not affected by mutation of the target gene [5], to avoid increased handling and possible loss of detection found for probes such as TaqMan® or HybProbes [34, 35]. Indeed, data obtained in the study of Martinon and Wilkinson [26] confirmed that the primer set to target the *nuc* gene was highly specific for *S. aureus* and that SYBR Green I was a suitable detection format. Times and temperatures applied are displayed on Table 1, and DNA amplification was followed in real-time in channel F1 or at 530 nm. A no template control was included in each PCR assay.

The Roche Diagnostics LightCycler® 4.1 Software was utilized to measure the increase in fluorescence emitted by SYBR Green I bound to double-stranded DNA. The crossing point (Cp) values were calculated to prepare the standard curves. Data were shown as amplification plots with fluorescence values in abscissa and the cycle number in intercept. Standard curves were displayed on a graph displaying the Cp versus the logarithm of defined copy numbers or SGU, according to the standard type. Cp values were measured in triplicate within the same PCR run.

### Absolute Quantification and Statistical Analysis

Standard curves were created under Excel 2003 software from Cp data retrieved from the LightCycler® 4.1. Software. Each standard curve was assessed by the calculation of a correlation coefficient ($R^2$) that monitors the reproducibility

of pipetting [36] and the slope. Amplification efficiencies ($E$) were automatically generated by the LightCycler® 4.1 Software to give a quantitative expression of the quality of PCR. Absolute quantification analysis was performed using the automated method provided by the software, where the Cp was identified as that where the fluorescence curve turns sharply upwards which was the first maximum of the second derivative of the curve. An efficiency of 2 indicated that the number of target molecules was doubling at each PCR cycle [37]; $E$ is theoretically calculated from the calibration curve slope, according to the equation:

$$E = 10^{[-1/\text{slope}]}.$$

To obtain the efficiency value in percentage, the following formula was used [38]:

$$E = (10^{[-1/\text{slope}]} - 1) \times 100.$$

The quality of each quantification process was evaluated using Fieller's Confidence Interval (FCI) software, a R-based algorithm software [39]. Cp data collected from LightCycler® 4.1 Software were imported into FCI using Microsoft Excel as Comma Separated Values (csv). Data were fitted to the linear regression and ANOVA (ANalysis Of Variance) models detailed by Verderio et al. [39]. A lack of fit test was assayed with a 95% confidence interval for the regression coefficients to assess the efficiency of each standard curve. A plot was generated to allow the assessment of the PCR assay quality and a visual representation of the FCI graphical derivation [39].

Finally, to assess the stability of the real-time PCR assays over time using the different standards, experiments were performed using standards stored after 0, 7, 14 and 30 days at −20°C, repeated three times for each of the conditions. Viviskis et al. [40] studied DNA conservation at 4°C and did not detect any modification in DNA quantity, quality or suitability for PCR over 1 month of storage, confirmed also by Farkas et al. [41]. However, they did not exclude the likelihood of a constant DNA degradation in samples stored at this temperature. Consequently, in the present study, it was decided not to store the DNA standards at 4°C and to evaluate the uniformity of Cp values in a sample according to duration of storage at −20°C. Cp variations were estimated by calculating the intra-assay coefficient of variation (CV) per dilution point tested in triplicates. The CV was calculated by dividing the standard deviation by means of the measured Cp values.

## Results and Discussion

### Generation of Accurate Populations by FACS

The present study has introduced a new concept in the preparation of quantification standards using FACS, the analytical aspect of which allows the identification and selection of cell populations, and the sorting aspect of which allows rapid and precise deposition of known numbers of cells into tubes or onto plates. Scatter signals emitted by bacteria were used for discriminating the bacterial population from background noise and debris. Scatter pulse width was employed to separate single cells from doublets and chains on the principle that particles with larger diameters or cross sections produce wider pulses of scattered light [42]. In order to reduce the numbers of doublets and aggregates, mild ultrasonic treatments may be used before FACS; however, energy levels applied must be controllable and reproducible [43, 44]. An inherent background error of 1.9–2.5% was associated with FACS. The sorting process did not affect the viability or cellular integrity of the vast majority of sorted cells; microbial viability as assessed by plate counting was ~95% with typical yields of at least 98–99%. Furthermore, given that the sort mode employed to deflect single cells into tubes would have eliminated the co-sorting of any cellular debris, including chromosomes that produced a FSC signal above threshold, it seems unlikely that significant free DNA was sorted alongside intact cells. Several concerns have been raised regarding the use of standards expressed as 'copy number' units which are seen as theoretical rather than experimentally derived. The accuracy of external standard quantification depends on the correct determination of standard DNA concentration that is usually measured by UV spectrophotometry giving an approximate concentration value [36, 45]. However, the determination of the DNA concentration is sometimes overestimated and the major drawback is that the target gene cannot be quantified in the final dilution of the standard [32]. In microbiology, copy number of genomic DNA standards is calculated according to the molecular weight of the target genome. However, such data are not systematically available for a given species or strain of microorganism. In the preparation of plasmid standards, the insert is required to be purified and cloned into a vector. These operations appear to be expensive and laborious, and the efficiencies of cloning and transformation vary depending on the nature of the insert (size, toxicity, vector-to-insert ratio, freshness of the PCR products), the plasmid size and the competent state of the cells [46–48]. Also, the issue of whether to linearise the plasmid or not continues to be a matter for discussion [49]. The use of PCR products as standards [50] may be not advised since errors may be generated during PCR when PCR products are used as templates for amplification [51]. Secondary structures such as stem-loops may be involved in the generation of mistakes and bias in template detection when using probes for instance. The quantification by real-time PCR in CFU per mass or volume unit [11–15] is also disadvantageous as the serial dilution method employed

may refer to absorbance values or enumeration in a counting chamber, and standard values must be confirmed by plate counting. Moreover, these standards can only be said to contain an approximate amount of cells as one CFU may correspond to one cell or one cluster of cells. Furthermore, the bacterial cell may have a variety of replicons—chromosomes [52]. Therefore, FACS-generated standards may be a potential alternative to allow accurate quantitative real-time PCR assays.

## Comparison of Amplification Efficiencies Using LightCycler® 4.1 Software

Standard curves were constructed using type A, B, C and D standards. The melting curve analysis of the PCR products obtained after the SYBR Green-based real-time PCR showed the primers to be specific for all of the extracted DNAs irrespective of the type of standard (Table 2A). Slight differences in melting temperatures may be explained by the variation of salt concentrations from one reaction vessel to another which can influence the stability of the DNA helix [53]. In this study, the melting curve profiles were considered to be similar to each other.

The accuracy of real-time PCR is highly dependant on the PCR efficiency. The Roche LightCycler® 4.1. and FCI softwares were also utilized to define the efficiency of the real-time PCRs. As shown on Table 2B, efficiency values were relatively similar between both the software packages.

The reaction efficiency and linearity data provided by the LightCycler® 4.1 Software were of relatively good quality, as shown in (Table 2B and Fig. 1), except in the case of the standard curve type D, where outliers were observed.

**Table 2** Melting temperatures (*Tm*) obtained in relation to the specificity of the primers according to the calibration DNA standard amplified (A) and comparison of PCR efficiencies calculated using LightCycler® 4.1. Software and FCI Software (B)

| | Standards types | Mean value | SD |
|---|---|---|---|
| A | A | 80.61 | 0.08 |
| | B | 80.72 | 0.09 |
| | C | 80.14 | 0.11 |
| | D | 80.51 | 0.12 |
| | Standard type | Roche LightCycler 4.1. Software | FCI Software |
| B | A | 1.992 | 1.992 |
| | B | 2.013 | 2.011 |
| | C | 2.016 | 2.007 |
| | D | 1.682 | 1.857 |

Type B standard had an efficiency of 2.013 with the LightCycler® 4.1. software. Using the type A-generated standards, the resultant standard curve may possibly result in an overestimation of the DNA content especially when DNA quantities are low, such as in the range of 3–10 copies. To date, microbiological research and analysis by real-time PCR still have not reached a definitive preference for use of either genomic or plasmid DNA as quantitative standards. Type A, C and D standards were prepared using genomic DNA and, therefore, contained a large excess of non-target sequences, as opposed to type B where plasmid DNA was used and may have contained a relatively small amount of non-target DNA. Yun et al. [38] noted that besides the inherent properties of the primer pair, the complexity and nature of the DNA sample containing the amplicon has itself an influence on the amplification efficiency. However, the efficiency values defined by the LightCycler® 4.1. Software for type A and C standard curves were satisfactory, which may suggest that the DNA complexity was not an issue in these two quantification standard types. According to Pfaffl [54], real-time PCR efficiency varies with high linearity from 1.60 or 60% to maximal values up to 2.10 or 110%. The best efficiency was obtained for type C standards (101.6%), whereas satisfactory efficiencies were associated with plasmid DNA standards (101.3%) and genomic DNA standards (99.2%). Satisfactory coefficients of determination ($R^2$) for standard curves type A, B and C ranged from 0.991 to 0.998. However, the efficiency of 1.682 obtained using type D standards showed a poor amplification of the target DNA. Hodek et al. [55] explained that the efficiency of real-time PCR procedures have an essential impact on DNA quantification. In the case of poor efficiency values such as those obtained using the type D standards, an unsatisfactory slope was observed with a coefficient of determination ($R^2$) of 0.951 for the calibration curve and, correspondingly, the interpolation of DNA quantity may be misinterpreted. The presence of PCR inhibitors in type D standards may be the cause of low efficiency, as DNA was not purified for this standard type. The most significant inhibitors of PCR are endogenous contaminants present in insufficiently purified target DNA samples [1, 56]. Matrices containing high amounts of lipids such as those that constitute the bacterial membrane are inclined to be inhibitory.

## Evaluation Using FCI Software

FCI software uses an algorithm that follows a linear regression model according to an ANOVA that includes regression, error, lack of fit and pure error values. Efficiency is calculated based on the regression coefficient of the standard curve, whereas the precision is given by the

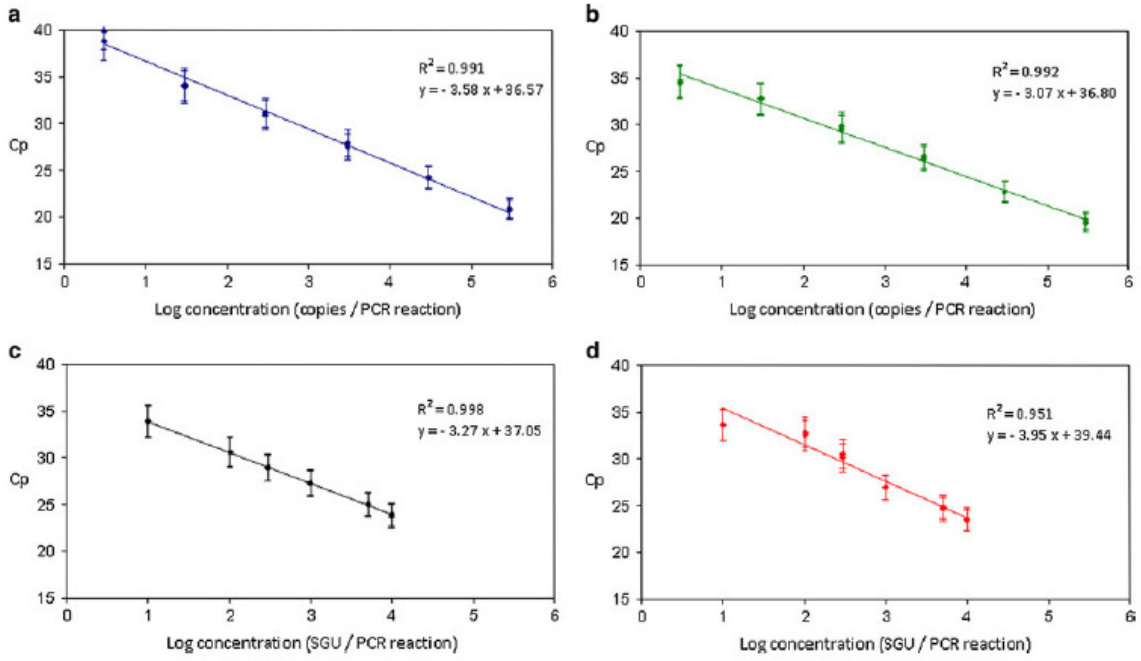


**Fig. 1** Standard curves showing Log of concentration (300,000–3 copies or 10,000–10 SGU per PCR reaction) versus crossing point (*Cp*) constructed using **a** type A, **b** type B, **c** type C and **d** type D standards

pure error as calculated by FCI software. Efficiency and pure error are two distinct features, and a standard curve with a good efficiency can be imprecise and vice versa. When the lack of fit test was applied under FCI software to standard curve data, only standard type A curves were reported as having optimal efficiency; a lack of fit was reported for types B, C and D standards. According to Verderio et al. [39] the 'lack of fit' message is purported to be a warning message that should be considered for DNA quantification, whereas, a lack of fit was clearly observed for type D standard curves. This was not obvious for type B or C generated standard curves, as seen on Fig. 1, especially given that both the standard curves had efficiencies close to 2 (Table 2B).

Commercial software such as Roche LightCycler® 4.1. generally do not investigate the fitting of the data to the linear regression as well as the FCI algorithm. As regards to a 'lack of fit' message appearing in the FCI output, communication with the manufacturer on this issue elicited a response which outlined that a standard curve should not be systematically discarded if the pure error is small because the 'lack of fit' warning may be due to the presence of near identical Cp values within each standard or a low standard deviation, as it was in the case for type B and C standards. Therefore, it is reasonable to consider that an improvement of the FCI algorithm may be required when formulating the 'lack of fit' message in the context of PCR data that actually fit the linear regression.

## Stability of the DNA Standards

The stability of the diluted standards depended on the storage duration at −20°C as shown in Fig. 2. Type D standards were excluded from the study on the basis of previous data regarding efficiency.

Overall, the CV did not exceed 9% on day 0 for each dilution standard. Type B standards were the most stable over time when stored over 30 days at −20°C. Despite the slight increase of variance noticed at 30 days, circular plasmid DNA standards appeared to be more stable over 1 month at −20°C. These results were in agreement in Dhanasekaran et al. [50] who noted that diluted circular plasmid standards were more stable than linear plasmid DNA or PCR products when stored at −20°C. However, the use of circular plasmid DNA is still controversial. In contrast, type A and C diluted standards had similar profiles to each other, but with higher CVs compared with type B diluted standards. At lower dilutions, it appeared that the CVs were increased by 2-fold from 14 days of storage at −20°C; the maximum CVs recorded for type A and C standards were ≥10 and ≤18%, respectively. Dhanasekaran et al. [50] considered it as acceptable diluted standards that showed a CV ≤ 10%.

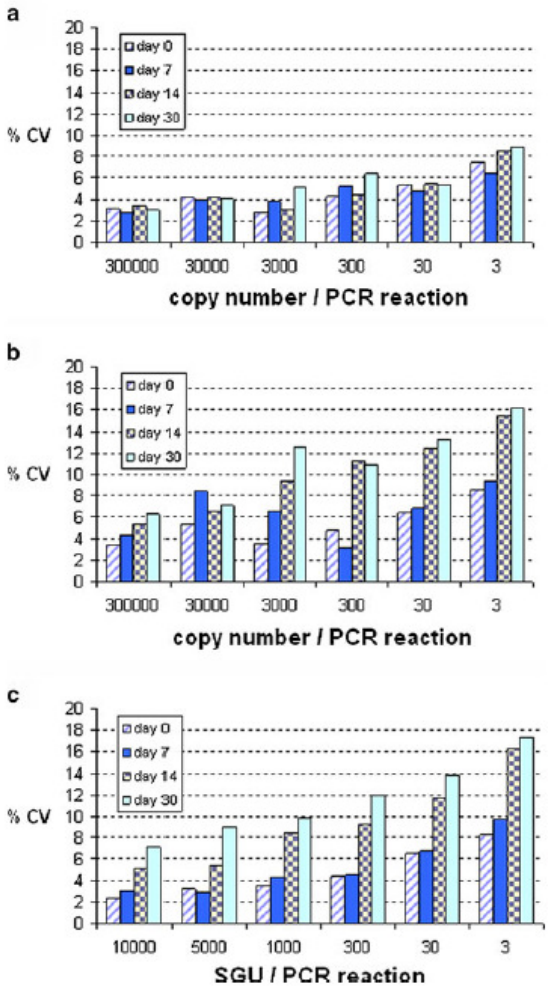


**Fig. 2** Coefficients of variation (*CVs*) of Cp values for real-time PCR quantification of the *nuc* gene using type A, B or C standards stored for 0, 7, 14 and 30 days at −20°C

The handling of DNA standards with multiple freezing and thawing procedures exposes DNA to conditions that may affect stability and integrity [57]. Consequently, we would recommend keeping such diluted standards for up to 7 days for re-use. Otherwise, diluted standards should be freshly prepared from an aliquoted DNA stock solution kept at −20°C. As an alternative to the inclusion of diluted standards for absolute quantification analysis, a previously saved standard curve may be imported under the LightCycler® 4.1. software. However, one dilution point must be included into the PCR run with the same analysis method, channel settings and concentration units. This dilution point has to fit to the external standard so that it can be applied for quantification. This option offered by the LightCycler® device allows the user to spare reagents, consumables and time. Nevertheless, it should not replace the preference for construction of a new standard curve for quantification purposes. However, aliquots corresponding to one dilution point may be prepared in advance and conserved at −20°C.

To improve the stability of types A and C standards, it is intended to further optimise storage conditions. In general, DNA is conserved in solution in distilled water, such as in the current study, or in Tris:$Na_2$EDTA. Tris has a buffering capacity, whereas the sodium salt stabilizes the DNA double helix and EDTA inhibits possible action of nucleases [41]. In contrast, DNA samples that contain EDTA can chelate divalent cations like $Mg^{2+}$ and act as a PCR inhibitor [58]. Consequently, storage conditions in the presence of EDTA should be further evaluated. Shaudien et al. [57] preserved DNA standards in 50% glycerol and found that 16 cycles of freezing and thawing did not affect DNA quantification in cDNA samples in contrast to DNA standards conserved in distilled water. Therefore, this method may be a good option to conserve type B and C standards at −20°C for up to 14 days. As an alternative, Visvikis et al. [40] were in favour of DNA lyophilisation provided that better methods of DNA hydration were found. However, it is not clear whether DNA degradation of lyophilised DNA is due to disruption during storage or to difficulties in rehydration. In this context, further experiments are needed to optimize DNA lyophilisation and rehydration procedures.

## Conclusion

In this study, the use of FACS-generated standards with optimal temperature and storage stability was shown to significantly improve the quantification of *S. aureus* using real-time PCR. This novel molecular tool provides a valuable alternative to quantification standards expressed in 'copy number' or 'CFU' as defined quantities of microorganisms are generated. Subsequent PCR assays using such standards had the best reaction efficiency. It was demonstrated that FACS-generated standards may be maintained for up to 7 days for re-use or alternatively fresh diluted standards from an aliquoted DNA stock solution kept at −20°C may be prepared. Storage conditions may be further optimised to improve stability over time. Using FCI software, a 'lack of fit' warning message was generated for plasmid and FACS-generated standard curves. This may be due to close or identical Cp values within each standard or a low standard deviation for plasmid and FACS-generated standard curves, which does not question the quality of both quantification processes. The FCI algorithm may need to be modified for such cases. In order to optimise

similarities with the analysed sample, accurate quantities of viable bacteria may also be sorted and resuspended into diluent containing the sample matrix or a synthetic formulation to mimic the biochemical background. Overall, the main advantages of this study may be to allow in-house real-time PCR methods to become more compatible and enable progress towards data standardization among laboratories worldwide. Despite the high cost of purchase of a cell sorter and, therefore, a limited access to such equipment for many microbiological laboratories, it is conceivable in the future to optimise and/or dedicate external FACS-equipped laboratories to the unique production of precise microbiology standards. Moreover, it is possible to include ready-to-amplify FACS generated standards into commercial real-time PCR detection kits or to provide them as separate components for use in real-time PCR systems.

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## References

1. Mackay, I. M., Bustin, S. A., Andrade, J. M., Kubista, M., & Sloots, T. P. (2007). Quantification of microorganisms: Not human, not simple, not quick. In I. M. Mackay (Ed.), *Real-time PCR in microbiology* (pp. 133–182). Norfolk: Caister Academic Press.
2. Rossmanith, P., Krassnig, M., Wagner, M., & Hein, I. (2006). Detection of *Listeria monocytogenes* in food using a combined enrichment/real-time PCR method targeting the *prfA* gene. *Research in Microbiology, 157*, 763–771.
3. Alarcon, B., Vicedo, B., & Aznar, R. (2006). PCR-based procedures for detection and quantification of *Staphylococcus aureus* and their application in food. *Journal of Applied Microbiology, 100*, 352–364.
4. Hein, I., Lehner, A., Rieck, P., Klein, K., Brandl, E., & Wagner, M. (2001). Comparison of different approaches to quantify *Staphylococcus aureus* cells by real-time quantitative PCR and application of this technique for examination of cheese. *Applied and Environmental Microbiology, 67*, 3122–3126.
5. Guilbaud, M., de Coppet, P., Bourion, F., Rachman, C., Prevost, H., & Dousset, X. (2005). Quantitative detection of *Listeria monocytogenes* in biofilms by real-time PCR. *Applied and Environmental Microbiology, 71*, 2190–2194.
6. Chen, X., Wang, J., & Liu, J. (2008). Development of real-time PCR approach for cellulolytic bacteria quantification in the rumen. *Chinese Journal of Animal Science, 44*, 36–40.
7. Xu, W., Li, S.-f., Liu, J., & Hu, D. (2008). The development of duplex real-time PCR for detection of *Listeria monocytogenes* and *Shigella*. *Zhonghua Weishengwuxue He Mianyixue Zazhi, 28*, 946–950.
8. Loddenkotter, B., Becker, K., Hohoff, C., Brinkmann, B., & Bajanowski, T. (2005). Real-time quantitative PCR assay for the detection of *Helicobacter pylori*: No association with sudden infant death syndrome. *International Journal of Legal Medicine, 119*, 202–206.
9. Sails, A. D., Fox, A. J., Bolton, F. J., Wareing, D. R. A., & Greenway, D. L. A. (2003). A real-time PCR assay for the detection of *Campylobacter jejuni* in foods after enrichment culture. *Applied and Environmental Microbiology, 69*, 1383–1390.
10. Furet, J.-P., Quénée, P., & Tailliez, P. (2004). Molecular quantification of lactic acid bacteria in fermented milk products using real-time quantitative PCR. *International Journal of Food Microbiology, 97*, 197–207.
11. Yang, H., Qu, L. W., Wimbrow, A. N., Jiang, X. P., & Sun, Y. P. (2007). Rapid detection of *Listeria monocytogenes* by nanoparticle-based immunomagnetic separation and real-time PCR. *International Journal of Food Microbiology, 118*, 132–138.
12. Ott, S. J., Musfeldt, M., Ullmann, U., Hampe, J., & Schreiber, S. (2004). Quantification of intestinal bacterial populations by real-time PCR with a universal primer set and minor groove binder probes: A global approach to the enteric flora. *Journal of Clinical Microbiology, 42*, 2566–2572.
13. Berrada, H., Soriano, J. M., Manes, J., & Pico, Y. (2006). Real-time quantitative PCR of *Staphylococcus aureus* and application in restaurant meals. *Journal of Food Protection, 69*, 106–111.
14. Berrada, H., Soriano, J. M., Pico, Y., & Manes, J. (2006). Quantification of *Listeria monocytogenes* in salads by real time quantitative PCR. *International Journal of Food Microbiology, 107*, 202–206.
15. Takahashi, H., Iwade, Y., Konuma, H., & Hara-Kudo, Y. (2005). Development of a quantitative real-time PCR method for estimation of the total number of *Vibrio parahaemolyticus* in contaminated shellfish and seawater. *Journal of Food Protection, 68*, 1083–1088.
16. Ishii, S., Tago, K., & Senoo, K. (2010). Single-cell analysis and isolation for microbiology and biotechnology: Methods and applications. *Applied Microbiology and Biotechnology, 86*, 1281–1292.
17. Shapiro, H. M. (2003). Overture. In *Practical flow cytometry* (4th ed., pp. 1–60). Hoboken, New Jersey: Wiley.
18. Wallner, G., Fuchs, B., Spring, S., Beisker, W., & Amann, R. (1997). Flow sorting of microorganisms for molecular analysis. *Applied and Environmental Microbiology, 63*, 4223–4231.
19. Guillebault, D., Laghdass, M., Catala, P., Obernosterer, I., & Lebaron, P. (2010). An improved method of bacterial cell capture after flow cytometry cell sorting. *Applied and Environmental Microbiology, 76*, 7352–7355. doi:10.1128/AEM.00621-10.
20. Bowers, H. A., Brutemark, A., Carvalho, W. F., & Graneli, E. (2010). Combining flow cytometry and real-time PCR methodology to demonstrate consumption by *Prymnesium parvum*. *Journal of the American Water Resources Association, 46*, 133–143.
21. Hoffmann, P. R., Gurary, A., Hoffmann, F. W., Saux, C. J. L., Teeters, K., Hashimoto, A. C., et al. (2007). A new approach for analyzing cellular infiltration during allergic airway inflammation. *Journal of Immunological Methods, 328*, 21–33.
22. Hein, I., Jorgensen, H. J., Loncarevic, S., & Wagner, M. (2005). Quantification of *Staphylococcus aureus* in unpasteurised bovine and caprine milk by real-time PCR. *Research in Microbiology, 156*, 554–563.
23. Ikeda, T., Tamate, N., Yamaguchi, K., & Makino, S. I. (2005). Quantitative analysis of *Staphylococcus aureus* in skimmed milk powder by real-time PCR. *Journal of Veterinary Medical Science, 67*, 1037–1041.
24. Kim, C. H., Khan, M., Morin, D. E., Hurley, W. L., Tripathy, D. N., Kehrli, M., et al. (2001). Optimization of the PCR for detection of *Staphylococcus aureus nuc* gene in bovine milk. *Journal of Dairy Science, 84*, 74–83.
25. Pinto, B., Chenoll, E., & Aznar, R. (2005). Identification and typing of food-borne *Staphylococcus aureus* by PCR-based techniques. *Systematic and Applied Microbiology, 28*, 340–352.
26. Martinon, A., & Wilkinson, M. G. (2011). Selection of optimal primer sets for use in a duplex SYBR Green-based real time PCR

protocol for the detection of *L. monocytogenes* and *S. aureus* in foods. *Journal of Food Safety*. doi:10.1111/j.1745-4565.2011.00301.x.

27. Peters, R. P. H., van Agtmael, M. A., Gierveld, S., Danner, S. A., Groeneveld, A. B. J., Vandenbroucke-Grauls, C., et al. (2007). Quantitative detection of *Staphylococcus aureus* and *Enterococcus faecalis* DNA in blood to diagnose bacteremia in patients in the intensive care unit. *Journal of Clinical Microbiology, 45*, 3641–3646.
28. Brakstad, O. G., Aasbakk, K., & Maeland, J. A. (1992). Detection of *Staphylococcus aureus* by polymerase chain reaction, amplification of the *nuc* gene. *Journal of Clinical Microbiology, 30*, 1654–1660.
29. Kao, C. L., King, C. C., Chao, D. Y., Wu, H. L., & Chang, G. J. (2005). Laboratory diagnosis of dengue virus infection: Current and future perspectives in clinical diagnosis and public health. *Journal of Microbiology Immunology and Infection, 38*, 5–16.
30. Anonymous. (2003). Applied biosystems—creating standard curves with genomic DNA or plasmid DNA templates for use in quantitative PCR. www6.appliedbiosystems.com/support/tutorials/pdf/quant_pcr.pdf.
31. Mlynarczyk, A., Mlynarczyk, G., & Jeljaszewicz, J. (1998). The genome of *Staphylococcus aureus*: A review. *Zentralblatt Fur Bakteriologie-International Journal of Medical Microbiology Virology Parasitology and Infectious Diseases, 287*, 277–314.
32. Wang, Z., & Spadoro, J. (1998). Determination of target copy number of quantitative standards used in PCR-based diagnostic assays. In F. Ferré (Ed.), *Gene quantification* (pp. 31–44). Carlsbad: Birkhäuser.
33. Queipo-Ortuno, M. I., Colmenero, J. D. D., Macias, M., Bravo, M. J., & Morata, P. (2008). Preparation of bacterial DNA template by boiling and effect of immunoglobulin G as an inhibitor in real-time PCR for serum samples from patients with brucellosis. *Clinical and Vaccine Immunology, 15*, 293–296.
34. Aldea, C., Alvarez, C. P., Folgueira, L., Delgado, R., & Otero, J. R. (2002). Rapid detection of herpes simplex virus DNA in genital ulcers by real-time PCR using SYBR green I dye as the detection signal. *Journal of Clinical Microbiology, 40*, 1060–1062.
35. Fernandez, F., Gutierrez, J., Sorlozano, A., Romero, J. M., Soto, M. J., & Ruiz-Cabello, F. (2006). Comparison of the SYBR Green and the hybridization probe format for real-time PCR detection of HHV-6. *Microbiological Research, 161*, 158–163.
36. Tse, C., & Capeau, J. (2002). Quantification des acides nucléiques par PCR quantitative en temps réel. *Annales de Biologie Clinique, 61*, 279–293.
37. Anonymous. (2007). *LightCycler® 2.0 instrument operators' manual—software version 4.1—manual B: For general laboratory use—Roche diagnostics GmbH*. Mannheim, Germany.
38. Yun, J. J., Heisler, L. E., Hwang, I. I. L., Wilkins, O., Lau, S. K., Hyrcza, M., et al. (2006). Genomic DNA functions as a universal external standard in quantitative real time PCR. *Nucleic Acids Research, 34*, e85.
39. Verderio, P., Pizzamiglio, S., Gallo, F., & Ramsden, S. C. (2008). FCI: An R-based algorithm for evaluating uncertainty of absolute real-time PCR quantification. *Bmc Bioinformatics, 9*, 7.
40. Visvikis, S., Schlenck, A., & Maurice, M. (1998). DNA extraction and stability for epidemiological studies. *Clinical Chemistry and Laboratory Medicine, 36*, 551–555.
41. Farkas, D. H., Kaul, K. L., Wiedbrauk, D. L., & Kiechle, F. L. (1996). Specimen collection and storage for diagnostic molecular pathology investigation. *Archives of Pathology and Laboratory Medicine, 120*, 591–596.
42. Shapiro, H. M. (2003). Parameters and probes. In *Practical flow cytometry* (4th edn., pp. 273–410). Hoboken, New Jersey: Wiley.
43. Braga, P. C., Bovio, C., Culici, M., & Dal Sasso, M. (2003). Flow cytometric assessment of susceptibilities of *Streptococcus pyogenes* to erythromycin and rokitamycin. *Antimicrobial Agents and Chemotherapy, 47*, 408–412.
44. Nebe-von-Caron, G., Stephens, P. J., Hewitt, C. J., Powell, J. R., & Badley, R. A. (2000). Analysis of bacterial function by multicolour fluorescence flow cytometry and single cell sorting. *Journal of Microbiological Methods, 42*, 97–114.
45. Vaerman, J. L., Saussoy, P., & Ingargiola, I. (2004). Evaluation of real-time PCR data. *Journal of Biological Regulators and Homeostatic Agents, 18*, 212–214.
46. Anonymous. (2006). *TOPO TA Cloning® kit for sequencing—five-minute cloning of Taq polymerase-amplified PCR products for sequencing*. Carlsbad: Invitrogen Corporation.
47. Siguret, V., Ribba, A. S., Cherel, G., Meyer, D., & Pietu, G. (1994). Effect of plasmid size on transformation efficiency by electroporation of *Escherichia coli* DH5 alpha. *Biotechniques, 16*, 422–426.
48. Szostkova, M., & Horakova, D. (1998). The effect of plasmid DNA sizes and other factors on electrotransformation of *Escherichia coli* JM109. *Bioelectrochemistry and Bioenergetics, 47*, 319–323.
49. Beld, M. G. H. M., Birch, C., Cane, P. A., Carman, W., Claas, E. C. J., Clewley, J. P., et al. (2007). Experts' roundtable: Real-time PCR and microbiology. In I. M. Mackay (Ed.), *Real-time PCR in microbiology from diagnosis to characterization* (pp. 357–443). Norwich: Caister Academic Press.
50. Dhanasekaran, S., Doherty, T. M., Kenneth, J., & TB Trials Study Group. (2010). Comparison of different standards for real-time PCR-based absolute quantification. *Journal of Immunological Methods, 354*, 34–39.
51. Loewen, P. C., & Switala, J. (1995). Template secondary structure can increase the error frequency of the DNA polymerase from *Thermus aquaticus*. *Gene, 164*, 59–63.
52. Krishnapillai, V. (1995). Comparative genome architecture and dynamics in bacteria. *Journal of Genetics, 74*, 61–76.
53. Tan, Z. J., & Chen, S. J. (2006). Nucleic acid helix stability: Effects of salt concentration, cation valence and size, and chain length. *Biophysical Journal, 90*, 1175–1190.
54. Pfaffl, M. W. (2004). Quantification strategies in real-time PCR. In S. A. Bustin (Ed.), *PCR A–Z of quantitative* (pp. 87–112). La Jolla, CA: International University Line.
55. Hodek, J., Ovesna, J., & Kucera, L. (2009). Interferences of PCR effectivity: Importance for quantitative analyses. *Czech Journal of Food Sciences, 27*, 42–49.
56. Wilson, I. G. (1997). Inhibition and facilitation of nucleic acid amplification. *Applied and Environmental Microbiology, 63*, 3741–3751.
57. Schaudien, D., Baumgartner, W., & Herden, C. (2007). High preservation of DNA standards diluted in 50% glycerol. *Diagnostic Molecular Pathology, 16*, 153–157.
58. Wiedbrauk, D., Werner, J., & Drevon, A. (1995). Inhibition of PCR by aqueous and vitreous fluids. *Journal of Clinical Microbiology, 33*, 2643–2646.