

SAFETY IMPLICATIONS OF THE
PRESENCE OF NUCLEIC
ACIDS OF INACTIVATED
MICROORGANISMS IN FOODS

REPORT

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INTRODUCTION

Although microorganisms (including bacteria, viruses and microscopic parasites) are ubiquitous in the food chain, the vast majority are not hazardous. Indeed, they are integral to many food production processes (e.g. fermentation), as well as profoundly influencing the final appearance, taste and quality of many foods. They also play a significant role in food spoilage. Nucleic acids are a primary constituent of all living organisms. These macromolecules, including deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), are composed of nucleotides and store and transfer genetic information. Nucleic acids constitute 5–15% of the dry weight of all living cells. They can exist as either single- or double-stranded RNA or as single- or double-stranded DNA. The individual nucleotides are purines (guanine and adenine) and pyrimidines (cytosine and thymine in DNA or cytosine and uracil in RNA). The sequence in which these nucleotides occur forms the basis for storing and copying genetic information and provides a mechanism for the translation of information to functional proteins.

Some microorganisms (including selected bacteria, viruses, parasites and moulds) are capable of causing food poisoning – defined as any disease of an infectious or toxic nature caused by the consumption of contaminated food or water. These food-poisoning agents cause disease by a number of different mechanisms. In “intoxications” the microorganisms grow on the food prior to ingestion and produce metabolites (including toxins), which cause damage to the host (Table 1).

Table 1. Examples of food poisoning agents that cause disease via toxic metabolites produced prior to food consumption

Class	Food poisoning agent	Species involved
Bacterial	Toxic amines (histamine, tyramine)	Various species as a result of food spoilage or fermentation
	Toxic proteins and other peptides	
	Botulinum neurotoxin	<i>Clostridium botulinum</i>
	Cereulide	<i>Bacillus cereus</i>
	Staphylococcal enterotoxin	<i>Staphylococcus aureus</i>
Eukaryotic	Marine algal toxins	
	Diarrhoeic shellfish poisoning (okadaic acid, dinophysis toxins, yessotoxin and pectenotoxin)	<i>Prorocentrum</i> spp., <i>Dinophysis</i> spp., <i>Protoceratium reticulatum</i>
	Paralytic shellfish poisoning (saxitoxin, neo-saxitoxin and gonyautoxin)	<i>Alexandrium</i> spp., <i>Pyrodinium</i> spp., <i>Gonyaulax</i> spp., <i>Gymnodinium</i> spp.
	Amnesic shellfish poisoning (domoic acid)	<i>Nitzschia pungens</i>
	Ciguatera toxin	<i>Gambierdiscus</i> spp, <i>Procerentrum</i> spp.
	Mycotoxin	
	Aflatoxin	<i>Aspergillus</i> spp.
	Ochratoxin	<i>enicillium</i> spp., <i>Aspergillus</i> spp.
	Patulin	<i>Penicillium</i> spp., <i>Aspergillus</i> spp., <i>Byssoschlamys</i> spp.

The toxin may act in the gastrointestinal tract or at another body site (e.g. nervous system or kidneys). In intoxicative food poisoning, the bacterium may no longer be viable at the time of consumption. In "infectious" food poisoning, the food supports the growth or survival of the pathogenic microorganism, and it is the consumption of the live microorganism that leads to disease. The ingested microorganism may grow in, or invade through, the host gastrointestinal tissues, or it may produce toxins in the gastrointestinal tract (Table 2). Again, tissues outside of the gastrointestinal tract may be affected.

Table 2. Examples of food poisoning agents that cause disease through an infectious or toxico-infectious process and require consumption of viable organisms in food

Viral	Gastrointestinal	Calicivirus (Norovirus and Sapovirus), Rotavirus, Astrovirus, Adenovirus, Enterovirus
	Extra-gastrointestinal	Poliovirus, hepatitis A virus, hepatitis E virus
Bacterial	Gastrointestinal	<i>Salmonella enterica</i> , <i>Campylobacter</i> spp., <i>Clostridium perfringens</i> , <i>Bacillus cereus</i> , <i>Shigella</i> spp., <i>Escherichia coli</i> , <i>Yersinia</i> spp., <i>Vibrio</i> spp.
	Extra-gastrointestinal	<i>Listeria monocytogenes</i> , <i>Brucella</i> , <i>Salmonella</i> spp. (enteric fever), <i>Mycobacterium</i> spp.
Parasitic	Gastrointestinal	<i>Cryptosporidium</i> spp., <i>Giardia</i> , <i>Cyclospora</i> , <i>Entamoeba</i> , <i>Diphylobotrium</i> , <i>Taenia</i> , <i>Anisakiasis</i> , <i>Ascaris</i> , <i>Fasciola</i>
	Extra-gastrointestinal	<i>Toxoplasma</i> spp., <i>Entamoeba</i> , <i>Trichinella</i>

Over the past 25 years, there have been considerable advances in the development and use of molecular techniques for the detection of microorganisms in foodstuffs as a result of the increasing demand for rapid results. These are normally based on detecting specific DNA or RNA target sequences using amplification processes, in particular the polymerase chain reaction (PCR). The detection of pathogen-specific proteins as well as other biomolecules can also prove useful, particularly when these represent a metabolic toxic compound produced by the microorganisms (e.g. entero- or endotoxins, lipopolysaccharides). However, these methods will not be included in this report. Because nucleic acids are relatively stable and may be present in a food matrix, even after processing steps routinely used in food preparation that can kill viable food-poisoning agents, one must be careful in interpreting the public health significance of their presence in processed foods (Cenciarini-Borde *et al.*, 2009).

The purpose of this report is to provide guidance on the interpretation of results for the detection of nucleic acids from pathogenic microorganisms in raw materials and processed food products, including the detection of inactivated organisms. The effect of food processing, particularly thermal treatment, on bacterial and viral integrity and their nucleic acids are discussed. This report also considers the implications of pathogen-derived nucleic acid ingestion, particularly as related to horizontal gene transfer.

2. FOOD AND ENVIRONMENTAL RESERVOIRS OF PATHOGENS

Foods are heterogeneous and complex ecosystems in which interactions between chemical, physical and structural characteristics markedly influence microbial growth and survival. Understanding of the distribution and ecology of foodborne pathogens and their metabolites, together with the ways of transmission, is essential for the safe preparation of food. This understanding will increasingly include information obtained using nucleic-acid-based techniques. This knowledge should be used to reduce and, hopefully, prevent contamination of food by pathogens, thereby allowing a reduction in the number of food poisoning incidents.

Microorganisms occur ubiquitously in the environment and, although the vast majority of these are harmless, a small, but significant, number can either produce toxic products or can infect humans, animals or plants. Routes of transmission include:

- Person to person transmission
- Animal to animal transmission
- Animal to human transmission (zoonosis)
- Human to animal transmission
- Direct contact with the environment (including via contaminated water)

Any of the above routes can either directly or indirectly involve contaminated food, beverages or water. Contamination by food-poisoning agents may occur at various stages in the food chain. Contaminants can be present in raw products (animal or plant origin) prior to harvesting; they may gain access during slaughter or processing; may be introduced by the addition of contaminated food components or processing aids; may arise from the factory environment or by cross-contamination from other contaminated foods or from food handlers. The implementation of a hazard analysis and critical control point (HACCP) approach, complemented by Good Agricultural Practices (GAP), Good Manufacturing Practices (GMP) and Good Hygiene Practices (GHP) is pivotal to food safety management. The system includes defining control measure(s) to reduce the foodborne microbial hazard to an acceptable level. In many situations, heat inactivation may be defined as a critical control point (CCP) to ensure that foodborne pathogens are killed or substantially reduced. For example, high temperature short time (HTST) pasteurisation (71.2°C, 15 s) is a commonly applied validated process to ensure the microbial safety of milk.

To cause disease, many pathogens need to grow and, in some instances, produce toxin(s) during processing and storage. A clear understanding of the growth, survival and death of microbes in foods and beverages is essential for the production of safe and wholesome products. Apart from physical barriers that separate food from microorganisms in the environment (such as cans, bottles and other hermetically sealed packages), microbiological control is mostly achieved by exclusion of pathogens from the food chain and/or by physical factors, i.e. the application of specific treatments that destroy or inhibit microbes. The most common physical factors manipulated in the food industry are temperature (both heat and cold), water activity, acidity, gaseous environment and available nutrients. Some pathogens (viruses, parasites and some bacteria) are unable to multiply in food, but occur in sufficient numbers from their original point of contamination to present an unacceptable risk. Since viruses cannot multiply in food products, their numbers will never exceed the initial contamination level during processing or storage and they will never cause deterioration of the food product. In practice, this means that the viral load in food samples might be both low and heterogeneously distributed, which partly explains the diagnostic difficulties in detecting viral food contaminants (Rodríguez-Lázaro *et al.*, 2012).

Finally, toxins present in raw food components might not be inactivated during processing. Examples of microbial hazards present in raw foods of animal origin, seafood and the environment are listed in Tables 3, 4 and 5, respectively. Similar microbial hazards may be present in foods of plant origin and furthermore water, used in production and processing, can serve as a conduit of microbiological pathogens.

Table 3. Raw foods of vertebrate animal origin and selected zoonotic agents

Food type	Agents	Occurrence and route of contamination
Red meat	<i>Toxoplasma gondii</i> , <i>Trichinella spiralis</i>	Muscle
	<i>Hepatitis E virus</i> , <i>Clostridium perfringens</i> , <i>Listeria monocytogenes</i> , <i>Campylobacter</i> spp. (<i>C. jejuni</i> , <i>C. coli</i>)	Faeces and faecal contamination
	<i>E. coli</i> (STEC), <i>Salmonella enterica</i> , <i>Yersinia enterocolitica</i> , <i>Brucella</i> spp.	Faeces and faecal contamination
	<i>Cryptosporidium parvum</i>	Faeces and faecal contamination
	<i>Taenia solium</i> , <i>T. sagitana</i>	Faeces and faecal contamination
	<i>Fasciola hepatica</i>	Faeces and faecal contamination
Poultry meat	<i>Toxoplasma gondii</i>	Muscle
	<i>Clostridium perfringens</i> , <i>Listeria monocytogenes</i> , <i>Campylobacter</i> spp. (<i>C. jejuni</i> , <i>C. coli</i>)	Faeces and faecal contamination
	<i>Salmonella enteritidis</i>	Faeces and faecal contamination
Eggs	<i>Salmonella enteritidis</i>	Internal contents via trans-ovarian infection
	<i>Campylobacter</i> spp. (<i>C. jejuni</i> , <i>C. coli</i>), <i>Salmonella enteritidis</i> , <i>Clostridium perfringens</i> , <i>Listeria monocytogenes</i>	Surface contamination from faeces or the environment
Milk	<i>Bacillus cereus</i> , <i>Clostridium perfringens</i> , <i>Listeria monocytogenes</i> , <i>Staphylococcus aureus</i> , <i>Campylobacter</i> (<i>C. jejuni</i> , <i>C. coli</i>)	Contamination from faeces or the environment, or natural occurrence of mastitis
	<i>E. coli</i> (STEC), <i>Salmonella enterica</i> , <i>Brucella</i> spp. <i>Mycobacterium</i> spp., (<i>M. bovis</i> , <i>M. avium</i>) <i>Cryptosporidium parvum</i>	Faeces and faecal contamination

Table 4. Food-poisoning agents associated with seafood

Type of contamination	Agents	Notes
Pre-harvest contamination	Diarrhoeic shellfish poisoning (DSP) and paralytic shellfish poisoning (PSP)	Algal toxin most usually accumulated in filter-feeding shellfish. Toxin survives cooking.
	<i>Ciguatera</i> (mainly ciguatoxin-1, maitotoxin, scaritoxin, palitoxin, okadaic acid)	Algal toxin accumulated in tropical carnivorous reef fish. Toxin survives cooking.
	Hepatitis A, Norovirus	Viral contamination of filter-feeding shellfish via sewage. Especially associated with oysters, mussels, cockles and clams that are consumed raw or lightly cooked.
	<i>Giardia</i> spp., <i>Cryptosporidium</i> spp.	Protozoan parasite contamination of filter-feeding shellfish via sewage. As above, may be associated with consumption of raw or lightly cooked product.
	<i>Anisakiasis</i> spp., <i>Diphyllobothrium</i> spp.	Nematode worm and cestode, which naturally occur in marine fish. Will not survive cooking or freezing, consequently associated with raw fish consumption.
Pre-or post-harvest contamination that may require growth	Scombrototoxin	Toxin resulting from the conversion of histidine to histamine especially in scombrid fish (tuna, mackerel and bonito). Toxin can be produced at any time from catching to final preparation and will survive cooking.
	Botulism	Toxin resulting from growth of <i>Clostridium botulinum</i> during post-processing. Most usually type E, which is common in marine muds and sediments. Will not survive thorough cooking.
	<i>Vibrio cholerae</i> , <i>V. parahaemolyticus</i> , <i>V. vulnificus</i>	Contamination from marine and estuarine bacteria. May grow very rapidly on seafood. Will not survive thorough cooking.
	<i>Salmonella</i> spp., <i>Campylobacter</i> spp.	Human or animal faecal contamination. Will not survive thorough cooking.
	<i>Listeria monocytogenes</i> , <i>Staphylococcus aureus</i>	Bacterial contamination from factory sites (<i>L. monocytogenes</i>) and food handlers (<i>S. aureus</i>).
	<i>Clostridium perfringens</i>	Bacterial contamination from spores present in foods or kitchen areas. Spores will survive cooking. The bacterium can rapidly grow when temperature and time control is poor.

Table 5. Food poisoning agents associated with soil, air and dust

Source of contaminant	Agents
Soil	<i>Bacillus cereus</i> , other <i>Bacillus</i> spp.
	<i>Clostridium perfringens</i> , <i>Clostridium botulinum</i>
	<i>Listeria monocytogenes</i>
	Fungi including <i>Aspergillus</i> spp., <i>Penicillium</i> spp., <i>Byssoschlamys</i> spp.
Air and dust	<i>Bacillus</i> spp.
	<i>Clostridium perfringens</i> , <i>C. botulinum</i>

Nucleic acids from microorganisms (including those from pathogens) may enter the food chain from multiple reservoirs: *inter alia* raw materials, process environments and personnel. Nucleic acids originating from microbial sources may also enter the food chain through processing aids, for example as adventitious contaminants of animal- or plant-derived enzymes. A further source can include virulent bacteriophages that have been used as decontaminants to reduce surface contamination of specific pathogens on foods (EFSA, 2009).

3. MICROBIAL NUCLEIC ACIDS IN FOOD

The bacterial membrane is one of the primary targets for inactivation treatments applied during food processing. The bacteriostatic and/or bactericidal effects of many inactivation and preservation processes are exerted through their impact on the microbial cell membrane potential and/or the structural integrity of the cell membrane. The secondary effect of such damage tends to be the loss of macromolecules (including nucleic acids) from the cell interior (Dock and Floros, 2000).

Microbial nucleic acids in foods, food ingredients and the environment may be present as a result of active secretion from living cells or by passive release from dead or injured cells. When cells are dying, autolysis generally occurs and the cytoplasmic content, including the DNA and RNA, is released into the surrounding environment. The integrity of the DNA and RNA that is released is dependent on the conditions preceding cell death. Once free in the environment, DNA is more susceptible to degradation. However, DNA can resist some environmental conditions and persist in various foods over a long time. DNA that remains stable over an extended time is probably protected from degradation in some way (e.g. it may be complexed with components of the food matrix). This means that it may not be available for uptake by "competent" bacteria (those able to take up naked DNA or RNA) and will probably not result in horizontal gene transfer (see Section 5). Free RNA is generally less stable than DNA, although viral RNA genomes have been shown to be stable in food and aquatic environments (Cook and Rzetuzka, 2004; De Roda Husman *et al.*, 2009).

DNA and RNA integrity can be altered as a consequence of almost all procedures used for food processing, including heat treatment, non-thermal processing and lytic agents such as phages. Thermal processing is the most commonly used food processing regime and, as a consequence, its effects on DNA integrity have been studied in some detail. High temperature treatments have been reported to exert the most dramatic effects on DNA stability, resulting in depurination or deamination of the DNA molecules (Gryson, 2010). At temperatures greater than 100°C, loss of secondary structure and strand scission occurs (Lindahl, 1993; Herman, 1997). It must be emphasised that heat treatments do not necessarily result in the complete degradation of DNA (in fact, the PCR process itself requires heating of DNA to 95°C to separate the two strands, albeit for relatively short times). Heating a DNA solution to 95°C for 60 min, for example, generates DNA fragments of less than 600 base pairs (bp) in length (Hupfer *et al.*, 2000). A more dramatic heat treatment of 99°C for 7 h resulted in DNA fragments of 400 bp (Debode *et al.*, 2007).

Several studies have examined the effects of heat treatments on DNA integrity in food; many such studies have been concerned with the detection of genetically modified DNA (Jonas *et al.* 2001). For example, boiling for 10 min did not completely degrade the genetically modified DNA from soybeans in a soymilk product, and 1339-bp length fragments were detected following this treatment (Bauer *et al.*, 2003).

The study also revealed that 714-bp DNA fragments could be detected in a highly processed commercial soy protein isolate. It can be expected that when heat is the sole treatment, only temperatures above boiling can substantially damage the coding regions and that complete gene coding fragments of >1 kb are likely to exist where the heat treatment is <100°C. Consequently, we can infer that dead cells of microorganisms with relatively intact genomic DNA will be present following normal processes applied to foods. This almost certainly explains the discrepancies that have been observed between the outcomes of quantitative PCR (qPCR) and culture-based methods for pathogen detection, where the former can predict up to ten times the number of viable cells detected (Wolffs *et al.*, 2005).

Sterilisation (autoclaving) combines pressure with high temperature and has been shown to degrade DNA to a greater extent than lower cooking temperatures. Fragments shorter than 295 bp were observed following an autoclave treatment of soybeans (Ogasawa *et al.*, 2003). In another study, cooking for 5 min or more, extrusion at a high temperature (170°C) and/or a high torque setting (36 nm) were shown to result in substantial degradation of maize DNA in most processed foods on retail sale. In this case, amplification of a 68-bp product by real-time qPCR was not possible following these treatments (Murray *et al.*, 2007). Spray-drying, which combines physical shearing with high temperature and sudden high pressure, was found to be the most effective process for degrading endogenous and exogenous genes of Roundup Ready soybean (Chen *et al.*, 2005). Irradiation treatments have also been shown to degrade DNA, with doses of up to 1000 Gray (Gy) resulting in damage (Villavicencio *et al.*, 2004).

In a study to differentiate viable from non-viable cells, McKillip *et al.*, (1998) determined the effects of various food processes on the viability of ribosomal RNA (rRNA) from the pathogens *Escherichia coli* O157:H7 and enterotoxigenic *Staphylococcus aureus*. rRNA could be detected up to 48 h after cells were killed by heat treatment at 80°C and UV irradiation at 254 nm. Interestingly, autoclave treatment of the cells degraded rRNA to the extent that it could not be detected by reverse transcription PCR (based on amplicon sizes of approximately 325 bp and 1400 bp) or by direct Northern blot analysis. In a study by Xiao *et al.*, (2010), RNA target sequences (86-152bp) of *Listeria monocytogenes* could be detected by reverse transcriptase PCR after heat treatments of 72.5°C and 98°C, although in general the signal was reduced with increased temperature and time of treatment.

In recent years, much attention has focussed on the application of bacteriophages (phages) to control susceptible pathogenic bacteria in foods. In this respect, phage infection of cells invariably leads to extensive leakage of host DNA into the environment. An inevitable consequence of lytic phage infection is the generation of high levels of progeny phages within the matrix, which contribute to the overall "load" of extraneous nucleic acids. Phage infection is also associated with degradation of the microbial chromosome, so that the resultant nucleic acids can provide the building blocks for generation of more phages (Wikner *et al.*, 1993). In this respect, phage infection can result in the degradation of the host genome within 6 min of infection (Powell *et al.*, 1992). However, the extent of the breakdown or fragmentation has not been characterised, although transfer of genes via lysogenic phages (incorporation of phage DNA into host genome) is well recognised.

Elevated temperature is a major cause of bacterial and virus inactivation in the environment, and food industries widely apply temperature as an intervention (Bertrand *et al.*, 2012, Mormann *et al.*, 2010). Thermal processes, such as pasteurisation substantially degrade viral proteins, which make up the phage capsid (head), resulting in the perturbation or rupture of the capsid. A recent study revealed that although the melting temperature of phage capsid is 87°C and the melting temperature of its DNA is 91°C, DNA escaped from the capsid at 68°C due to disruption of the phage tail at the lower temperature, which then triggered DNA release from the capsid (Qiu, 2012). Other virus inactivation methods such as UV light, high-pressure processing and chlorination also target the capsid and cause nucleic acid degradation.

This disrupts the early phases of the virus life cycle, including attachment, penetration and uncoating (Dancho *et al.*, 2012). Where RNA and/or non-enveloped viruses are destroyed, the nucleic acid(s) are vulnerable to rapid environmental degradation, although smaller fragments may persist due to the protective effects of the food matrix.

Where such “free” RNA or DNA molecules persist, false-positive results in molecular diagnostics may occur even though no intact phages are present. In those instances, a pre-treatment with RNase or DNase is advocated. In addition, intact food and environmental viruses that have been rendered non-infectious as a consequence of capsid or other protein damage may still contain intact RNA or DNA (virus dependent) (Sano *et al.*, 2010; Diez-Valcarce *et al.*, 2011). Where molecular tools are used to measure the inactivation of viruses that cannot be propagated *in vitro*, it is considered that they largely underestimate the reduction effect and are poor indicators of treatment efficiency (Gassiloud *et al.*, 2003; Hewitt and Greening, 2006; Teunis *et al.*, 2009; Diez-Valcarce *et al.*, 2011; Knight *et al.*, 2012).

Attempts have been made using an RNA-exposure assay to correlate capsid integrity with infectivity after heat treatment (Topping *et al.*, 2009). Tulaadhar *et al.* (2012), Baert *et al.* (2008) and Kovac *et al.* (2012a, b) demonstrated the persistence and detection of amplifiable viral RNA and DNA by qPCR, despite complete loss of infectivity by thermal processing or high hydrostatic pressure. In addition, Tsujikawa *et al.* (2011) detected viral genome fragments (0.5–1.0 kb) in filtrates (15 and 19 nm filters) of parvovirus B19, thus underestimating the removal capacity of a manufacturing process in which degraded fragments of the viral genome may serve as targets in the qPCR assay.

On average, most bacterial genes are approximately 1000 bp in size. A comparison of potentially short coding sequences (<300 bp) from a range of bacterial genomes revealed that the majority are protein-coding regions, although many remain to be tested experimentally (Ochman, 2002). In terms of food processing, the resulting DNA fragments from microbial sources (including pathogens) have been shown to vary from <295 bp in size to >1000 bp. The question remains as to whether the resulting fragments still possess sufficient coding information and are stable enough to be incorporated into other bacterial genomes. Clearly, thermal processing, while resulting in the elimination or substantial reduction of viable/infective bacterial or viral pathogens, does not render complete degradation of the nucleic acid macromolecules within the food matrix. Consequently, amplifiable, pathogen-derived nucleic acids may persist in the food post processing.

4. POWER AND LIMITATIONS OF MOLECULAR DETECTION METHODS

4.1 Conventional microbiological techniques

Routine approaches for testing food for microbiological parameters based on culturing bacteria have largely been unchanged over the last 20 years and remain the gold standard for detection of bacterial pathogens and indicator organisms. In current EC legislation (EC Regulation 2073/2005, last amended by 1441/2007 – European Commission, 2005, 2007), culture-based approaches are identified as reference methods, although alternative techniques can be used provided that they are appropriately validated and produce equivalent results.

Culture-dependent methods may have limitations, including:

- Difficulties in the recovery of sub-lethally injured bacteria, which may not be able to grow in selective media, meaning that false-negative results might be obtained, e.g. *Campylobacter jejuni* (Melero *et al.*, 2011)
- Difficulty in the recovery of slow-growing food-associated microbes, e.g. *Mycobacterium tuberculosis* or *Mycobacterium avium* subsp. *paratuberculosis* (Botsaris *et al.*, 2010), and “unculturable” bacteria (which account for more than 99% of the existing microbial cosmos)
- Difficulties in growing microorganisms for which *in vitro* cultivation is complicated or impossible, e.g. norovirus and hepatitis E virus (Pan *et al.*, 2012; Le Blanc *et al.*, 2010)
- Difficulties in recognising microorganisms with atypical characteristics (e.g. sorbitol-fermenting Verocytotoxin-producing *Escherichia coli*) or where selective media are not available or function poorly (*Clostridium botulinum*)
- Culture may be time-consuming, labour-intensive, require specialised laboratories (unless automation is used), high levels of skill and specialised media specific for an individual target, although low-cost and satisfactory media are usually readily available for the routine detection and identification of most common pathogens.

4.2 Technological advances in nucleic acid amplification

As previously described, new techniques are now available for the detection and quantification of specific microorganisms in food by targeting sequences of their DNA and/or RNA. These techniques originated from considerable advances in the field of molecular biology, and the invention of new methods have revolutionised the ways in which microbiological detection can be performed. PCR (Mullis *et al.*, 1986) has augmented traditional microbiological analysis by allowing a culture-independent detection directly on the food, without the necessity for isolation and identification of the pathogenic microorganism. From its first introduction, PCR has become an essential analytical tool for research into foodborne pathogens, and a considerable commercial diagnostics market has developed around molecular assays.

PCR results in the amplification of a specific target nucleic acid region of DNA or RNA. PCR products are identical copies of the original target DNA sequence, which can be detected by a variety of methods including gel electrophoresis or by using further pieces of complementary DNA (known as reporter probes) within the amplified product. These probes are chemically labelled to fluoresce (emit light when excited by UV light) when binding occurs to the complementary pathogen-target sequence.

Because these labels allow the detection of PCR products during the reaction, these methods are known as real-time PCR. Quantitative PCR has the considerable advantage that the reaction and detection process is combined, which increases the analytical sensitivity, reduces the time needed for analysis and greatly reduces the possibility of spurious cross-contamination within the laboratory that can lead to false-positive reactions. When compared to classical PCR, qPCR reactions are more efficient where small fragment lengths (<100 bp) are targeted. This can potentially represent a drawback because fragments as small as this can persist even after nucleic acid degradation processes have occurred. RNA can be detected by PCR if it is first converted to DNA using the reverse transcriptase enzyme. Because of the considerable body of data now available on nucleic acid sequences, computer-assisted analysis (bioinformatics) can be used to assess the specificity of any individual assay. However, the specificity will only be as good as the genetic sequences available in the bioinformatics databases.

Because of the considerable advantages of PCR-based technologies, these are becoming not only an essential analytical tool for research into foodborne pathogens, but also allow for more routine diagnostic, epidemiological and surveillance activities. International standards are also available that allow these techniques to be incorporated into equivalent quality systems (Table 6). The advantages and disadvantages of the application of PCR-based approaches are summarised in Table 7.

Table 6. International standards for the application of PCR to food analysis

ISO 22174:2005	Microbiology of food and animal feeding stuffs. PCR for the detection of foodborne pathogens. General requirements and definitions.
ISO 20837:2006	Microbiology of food and animal feeding stuffs. PCR for the detection of foodborne pathogens. Requirements for sample preparation for qualitative detection.
ISO 20838:2006	Microbiology of food and animal feeding stuffs. PCR for the detection of foodborne pathogens. Requirements for amplification and detection for qualitative methods.
ISO 22118:2011	Microbiology of food and animal feeding stuffs. PCR for the detection and quantification of foodborne pathogens. Performance characteristics.
ISO 22119:2011	Microbiology of food and animal feeding stuffs. Real-time PCR for the detection of foodborne pathogens. General requirements and definitions.

Table 7. Advantages and disadvantages of nucleic acid sequence-based amplification methods for detection of foodborne pathogens

Advantages	Disadvantages
Generic techniques and methodologies applicable to all food poisoning agents (viruses, bacteria and both unicellular and multicellular eukaryotic organisms)	It does not unambiguously distinguish between viable or infective and non-viable or non-infective organisms
Sensitive, specific, rapid	Does not detect biological activities (e.g. active proteins)
Detects non-viable organisms	Requires specialist equipment, infrastructure and trained staff
Can detect differences not available or technically difficult to detect using other techniques (e.g. identification under species taxon)	Less common in the portfolio of food microbiology laboratory testing methodologies
Large and ever-expanding database of sequences	Can be expensive
Can provide surrogates for expensive and unethical tests such as the use of experimental animals	Difficult to quantify targets (hazards) in all food matrices
Capable of a high level of automation	Difficult to test large volumes of food without extensive purification
Can detect organisms independent of the ability to grow them <i>in vitro</i>	Does not provide an equivalent result to conventional tests, including those in legislation
Can detect broad families of hazards	Destructive of food sample
Capable of providing information of pathogen detection and characterisation	Susceptible to interference (including nuclease activities) in food sample
Will provide additional information on biological hazards (e.g. likely survival in food processes, pathogenicity, toxicity, etc.)	

PCR allows the detection of target microorganisms independently of their physiological state. Consequently, stressed or injured cells can be detected by these methods, avoiding the false-negative results described above for the traditional microbiological methods. In addition, the time required for analysis is much shorter than for culture methods. A determination of the presence of pathogens in food can be obtained in under 3 h, including the extraction of the nucleic acids and the amplification process, to a maximum of 24 h if an enrichment step is necessary for the detection of low numbers of pathogenic microorganisms (1-100 colony forming units per 10 g). Despite the reduced costs of reagents and equipment, there is still a need for significant investment by an analytical laboratory in order to conduct state of the art PCR-based methodologies.

Currently PCR is the most common technique used for nucleic acid amplification. Nevertheless, other techniques able to amplify specific nucleic acid fragments have been developed. The nucleic acid sequence-based amplification (NASBA) and the loop-mediated isothermal amplification (LAMP) are examples of such techniques. Both methods have the advantage of being carried out at a constant temperature, thereby avoiding the purchase of expensive equipment for the thermal cycling in PCR amplification. NASBA uses three enzymes: a reverse transcriptase, RNaseH and T7 RNA polymerase, which act together to amplify sequences coming from an original single-stranded RNA molecule.

This method specifically allows detection of viable cells, since the target for amplification is the RNA. The method has been used to detect a range of pathogens in foods including *Campylobacter* spp., *Listeria monocytogenes*, *Salmonella enterica*, *Mycobacterium avium* subsp. *paratuberculosis* and others (Cook, 2003; Rodríguez-Lázaro *et al.*, 2006). However, it is reported that, in some circumstances, DNA can act also as a template and therefore is not a good tool for detection of viable bacterial cells (Rodríguez-Lázaro *et al.*, 2004). In the LAMP method, four primers recognising six regions of the target DNA are used, so the specificity is high. The method uses a polymerase to amplify target DNA (or RNA, after a reverse transcription step) following an autocycling strand-displacement mechanism to produce detectable products in approximately 1 h (Tomita *et al.*, 2008). LAMP has the potential for detection of foodborne pathogens (Sibley *et al.*, 2012).

For all tests applied to food microbiology, it must be emphasised that it is essential that all staff members are appropriately trained, that equipment performance is monitored and that analytical controls are included to assure that the performance of the test is within the required specification. The application of PCR is not an exception, and appropriate validation of sensitivity, specificity and predictive values of positive and negative test results (which account for any effects due to the food matrix) is required to allow proper interpretation. Therefore, it may be necessary to include process controls (cells inoculated in the food item/suspension taken up in parallel during the extraction of the food item under investigation) with each extraction. Incorporating process controls provides information on the efficiency of the nucleic acid extraction, and the PCR procedure and strategies to do this have been described (Murphy *et al.*, 2007). In this way, it is possible to estimate the influence of the food matrices, as well as the extraction and analytical processes, on the uncertainty of measurement of the original microbial target concentration.

Initially, PCR was used for presence and absence tests, but the method also allows for quantification of the original target. Following advancements in the late 1990s, instruments became available that greatly facilitated quantitative analysis by PCR. Not only it is now possible to quantify one specific microorganism in food, but it is also possible to study its behaviour as a consequence of the influence of the environment (food composition, temperature, pH, oxygen, etc.). Moreover, since the process is monitored in real-time, there is no need for post-amplification treatment of the samples, such as gel electrophoresis, thus reducing the analysis time (Cocolin *et al.*, 2011). For qPCR, calibration curves must be created to allow assessment of the effects of sample preparation and amplification for a specific food matrix. Validation to generate standard curves usually requires quantification of the target microorganism in a specific food because the testing of extracted DNA alone is not usually sufficient to allow an understanding of the relationship between target DNA and concentration of PCR product. qPCR requires the generation of standard curves by inoculating cell dilutions in each specific food matrix, which allows accurate quantification of the microorganism under investigation and compliance with quantitative targets set by legislation. Alternatively, DNA extracted from *in vitro* cultures can be used to construct the calibration curves; however, the efficiency of amplification should be corrected by a factor that takes into consideration the influence of the food matrix.

Currently, PCR is the method of choice for detection of nucleic acids in food, although, due to its high specificity, this technique can only detect well-defined targets, the specificities of which can be predicted on the basis of the sequences that are used for primers and reporter probes. This limitation may be alleviated in the future because new methodologies are emerging that allow high-throughput sequencing of entire ecosystems. These include the 454 Roche pyrosequencer (producing about a million sequences of 400-500 base length) or Illumina or Solid sequencing technologies (producing over a billion sequences of 50-100 base length). The power of these methods is that they can sequence the entire DNA content of an ecosystem (for example, a sample of food). These molecules could be a mixture of bacterial 16S rRNA genes, of which tens to hundreds of thousands of copies are sequenced,

or random DNA fragments corresponding to microbial DNA or retro-transcripts of RNA molecules, of which millions of short sequences are determined. The sequences generated can be analysed using bioinformatics to permit taxonomical assignment of the bacterial content of the original sample. From this data, viable and non-viable microorganisms present in the food sample can be ascertained (Josephson *et al.*, 1993). The limitations of these techniques are: (i) similar limitations to those explained previously that are inherent to the use of nucleic acids for diagnostic tests; (ii) lower sensitivity than PCR, so that detection of only the most abundant microorganisms is achieved; and (iii) the cost of the technology. These emerging high-throughput sequencing methods are increasingly used in the study of complex microbial ecosystems such as those linked to human microbiomes (oral, intestinal, etc.) and food ecosystems. Their cost has rapidly decreased (and will probably continue to do so), both for sequence acquisition and for facilities for automated analysis. However, these sequencing strategies are likely to remain more expensive than PCR against a small number of targets, and will therefore be restricted to investigative approaches for microbial source tracking, pathogen discovery and food history.

4.3 Interpreting DNA and/or RNA amplification outcomes

The remarkable stability of DNA in the environment is illustrated by the recovery of DNA and successful amplification by PCR from archaeological and paleontological samples, which can be thousands of years old (Landweber, 1999). Although this technology is very useful for the detection, identification and quantification of genetically modified (GM) ingredients in processed foods, in the field of food microbiology the detection of DNA must be carefully interpreted. Amplification by PCR of DNA from a pathogenic microorganism does not infer the presence of live populations of the pathogen in the tested food and thus does not of itself constitute a food safety risk. A number of approaches have been developed to overcome detection of dead cells, such as the detection of RNA by reverse-transcription PCR (RT-PCR), as well as the exclusion of dead cells on the basis of their membrane permeability to intercalating dyes such as ethidium bromide monoazide (EMA) or propidiummono azide (PMA). In the latter approach, the dye is able to permeate into dead cells (because of the loss of functionality or damage of the cell wall and membrane) and binds to DNA preventing its amplification, but does not enter functional bacterial cells. In this way, only DNA from cells with uncompromised membrane integrity is amplified (Nogva *et al.*, 2003). Some drawbacks have been encountered with this EMA/PMA-PCR technique, particularly where inactivation methods do not target the cell membrane. These are discussed by Jofre and Blanch (2010).

RNA has been proposed as a more representative target for assessing bacteria viability (Bej *et al.*, 1991) because messenger RNA (mRNA) persists for short periods of time in actively growing bacteria cells, with an average half-life measured in minutes (Arraiano, *et al.*, 1988). Despite this potential advantage, mRNA-based approaches for routine diagnostic tests have proved difficult to exploit because of experimental complexity, the practical problems of extracting detectable levels of intact mRNA from small numbers of bacteria, difficulties in distinguishing mRNA from corresponding homologous DNA sequences and a lack of basic information about the significance of detecting mRNA in stressed cells (Sheridan *et al.*, 1998). Because the number of intact ribosomes approximately reflects the rate of protein synthesis, rRNA can be used as a marker for general metabolic activity (Gosalbes *et al.*, 2011), although it must be accepted that these molecules are characterised by a much higher level of protection than mRNA (up to weeks). Therefore, the longer half-life of rRNA and its persistence following potential stress treatments make this molecule a less reliable target than the more reliable mRNA for viable pathogen detection post processing. The most common method used to detect RNA is RT-PCR, in which the RNA molecule is first transcribed to complementary DNA (cDNA) by a reverse transcriptase and then amplified by PCR. If targeting constitutively and stably expressed gene(s) is coupled with a quantitative assay, such as RT-qPCR, the method is able to determine the correct number of viable cells present in a defined sample. In terms of food safety, the detection of bacterial RNA in a processed food should indicate the presence of a viable population of a specific foodborne pathogen.

4.4 The VBNC (viable but not culturable) state and its significance in the food industry

Foods are complex ecosystems of great microbial diversity in terms of possible genera and species present, their growth requirements and physiological states. Particularly relevant for the food industry is the viable but not culturable (VBNC) state of a bacterium, which was defined by Oliver (1993) as “a cell which is metabolically active, while being incapable of undergoing the cellular division required for growth in or on a medium normally supporting growth of that cell”. Rowan (2004) described the VBNC state as a survival strategy and response to adverse environmental conditions (e.g. starvation or acid stress). With the implementation of milder food processing (Leistner, 1995) as a response to the consumer demand for foods that avoid the extreme use of a single preservation technique and that maintain characteristics resembling freshness, there is a greater likelihood for the emergence and establishment of VBNC states. The VBNC state has been described for several foodborne pathogens, including *E. coli* O157:H7, *L. monocytogenes*, *C. jejuni* and *Salmonella* Typhimurium (Rowan, 2004), and may represent a risk for the safety of processed foods.

Although there is a general lack of knowledge about the risks from VBNC cells, it cannot be assumed that such cells will not emerge from this state after entering the human body and cause disease. VBNC cells often have reduced gene expression and protein synthesis compared to cells growing under optimal conditions. Nevertheless, VBNC cells exhibit normal mechanisms of transcription of DNA into RNA. As described above, the detection of DNA by PCR will not allow discrimination between bacteria in normal live states from those that are dead or in the VBNC state. Although RNA will give additional information about the presence of normally live cells of the target organism, it will not be possible to distinguish these from cells in the VBNC state. If analytical results, however, are integrated with culture-dependent approaches, such as cultivation in liquid or solid media, the presence of bacteria in a VBNC state will be identified.

4.5 Viral nucleic acid detection and infective status

For virus detection in foodstuffs, many laboratories use different RT-PCR strategies involving multistep processes (viral extraction, concentration, detection and confirmation). The harmonisation and standardisation of these methods are considered as part of a current draft International Organization for Standardization (ISO) method, under the auspices of an ISO-CEN (European Committee for Standardization) ad hoc group (Lees, 2010). Caution in the evaluation of results (false-positive and false-negative interpretations) and verification of method reliability has been urged (Richards, 1999; Rodríguez-Lázaro *et al.*, 2007). In the framework of the European Network for Environmental and Food Virology (ENVIRONET, <http://www.cost929-environet.org/>) and an EU-funded project (VITAL, <http://www.eurovital.org>) and based on general consensus agreement and experimental evidence, D’Agostino *et al.* (2011) have defined appropriate controls to be included in nucleic acid amplification-based methods for virus detection to avoid analytical misinterpretation.

Furthermore, as discussed earlier, amplification and detection of the nucleic acid target may not indicate infectivity. For example, in a meta-analysis of methods used and their detection rates, norovirus (NoV) genome copies were frequently detected in fresh produce in Belgium, Canada and France. However, infection or outbreaks rarely occurred (Baert *et al.*, 2011). Stals *et al.* (2011) showed that 18 of 72 fruit products tested positive for NoV by RT-qPCR; however, it was not possible to confirm these results by sequencing genotyping regions in the NoV genome, nor could methods differentiate between infectious or non-infectious particles. Recent methods have explored the receptor binding capacity of viruses to target cells in an attempt to resolve this difficulty (infectious versus non-infectious) for interpretation (Li *et al.*, 2011; Dancho *et al.*, 2012). The need for confirmation of virus detection and the technical issues involved in sequencing after RT-qPCR is also a debated issue.

For example, in addition to the above study, successful sequencing of only a small number (10%) of RT-qPCR-positive samples was achieved (Mattison *et al.*, 2010). Some of the concerns regarding virus detection and correlation with infectivity are discussed further in the review by Knight *et al.* (2012).

4.6 Conclusions on molecular detection method interpretation

The target for PCR amplification is DNA and in some cases RNA. Because the former molecule is characterised by high stability even after cell death, a positive result by PCR does not necessarily indicate the presence of live pathogenic microorganisms in foods and thus whether there is a food safety risk. This is extremely important for processed foods where production includes a step in which foodborne pathogens can be killed. In this context, PCR can be considered as a primary diagnostic tool, which can be used as a screening step. In the case of positive amplification results, a confirmation by culture methods should be performed. The lack of correspondence between positive signals in the PCR and live populations in foods has been addressed in different ways and, for example, if positive results are obtained after an enrichment step this implies the initial presence of viable populations (Rossen *et al.*, 1991). Nonetheless, even the presence of dead cells indicates that at some point in the food production line there has been contamination of raw materials or food ingredients and the potential for a breach in food safety. Messenger RNA is potentially a more suitable target for assessing microbial viability.

5. CONSEQUENCES OF INGESTION OF NUCLEIC ACIDS

Nucleic acids, including DNA and RNA, are not themselves harmful molecules, irrespective of their animal, vegetable or microbiological origin. Considerable amounts of these molecules are ingested daily with meat, vegetables and fermented foods that have been part of a normal healthy diet since the dawn of time.

Upon ingestion, most cells present in food are degraded and their nucleic acids released in the gut. This applies to the DNA from both eukaryotic and prokaryotic organisms. Factors affecting the release and persistence of DNA in the environment have been studied extensively. Limitations to these studies are reviewed elsewhere (Nielsen *et al.*, 2007). Netherwood *et al.* in 2004 reported the fate of plant DNA in the digestive tract. In this study, the survival of a transgenic DNA from genetically modified soya was followed in the small intestine and in the faeces. The amount of transgenic DNA that survived passage through the human small bowel varied between individuals, but did not survive passage through the full gastrointestinal tract. This study therefore suggests that although DNA could be available in the upper part of the digestive tract, it is then completely degraded in the large intestine.

During passage through the gastrointestinal tract, the majority of nucleic acids are cleaved, digested and ingested as metabolites. Any remaining nucleic acids are largely degraded and unable to express the functions they encode because the environment differs considerably from the cellular cytoplasmic or nuclear environments of bacteria or eukaryotic organisms. Nevertheless, foreign DNA uptake in the gastrointestinal tract may be a natural process, similar to the absorption of metabolites (Palka-Santini *et al.*, 2003). DNA released from plants in food can be detected in animal tissue (Mazza *et al.*, 2005). The exact mechanism that leads to this detection is not known, but data obtained in cellular cultures indicate that viral genes can be transmitted from apoptotic bodies to somatic cells, potentially allowing their spread (Holmgren, 2010).

A possible consequence of nucleic acid ingestion, as a risk factor, is linked to their transfer into a microbial cellular environment that allows expression of the information carried by the nucleic acids. This issue has been widely discussed for transgenes present in genetically modified organisms (Nielsen *et al.*, 2010; van den Eede *et al.*, 2004; Keese, 2008). In this respect, the transfer, expression and dissemination of DNA from food to the microbiota present in the alimentary tract is highly likely (Marchesi, 2011).

Gene transfer has been demonstrated between microorganisms living in communities. Most studies described the potential transfer of antimicrobial drug resistance or virulence genes between living microorganisms, such as between commensals or between food microorganisms and commensals (van Reenen and Dicks, 2011; Tuohy *et al.*, 2002). These studies showed that, in general, the frequencies of gene transfer were low but significant ($\sim 10^{-4}$) when the experiments were designed with living bacteria carrying mobile elements with specific mechanisms for gene transfer.

Another factor that influences whether the exogenous DNA in the environment can be taken up and have an effect on the host is the level of homology between the donor DNA and the recipient microorganism. In this respect, the probability of transfer of recombinant DNA to bacteria is not determined only by the presence of DNA of gene-coding length in food, but rather by the size and length of any homologous sequences. This is particularly important for short and non-conjugative fragments of DNA. In *E. coli*, 20-40 bp of homologous DNA is sufficient for DNA transformation to

occur (Watt *et al.*, 1985); 70 bp has been recorded as sufficient in *Bacillus subtilis* (Khasanov *et al.*, 1992); and 280 bp in *Campylobacter coli* (Richardson and Park, 1997). There is a high probability that DNA fragments of sufficient length to contain coding regions and which have the potential to transfer by horizontal gene transfer to other bacteria will remain intact at the end of some food processes.

In addition to these exchange mechanisms between living bacteria, DNA can also be captured from the environment and integrated into a bacterial genome. These mechanisms are termed "natural competence" (Griffith, 1928; Avery *et al.*, 1944). Similar events probably occur *in vivo* in humans and animals and between different species of foodborne and enteric pathogens. Different mechanisms for natural competence have been described for several groups of bacteria in which DNA acquisition is considered to be an important driver for both short-term survival and long-term evolution (Johnsborg *et al.*, 2007; Heuerand Smalla, 2007). Although there is evidence that many bacterial species develop specific states allowing for DNA acquisition, there are factors limiting the extent of such acquisition for transfer and expression in the environment (Lorenz and Wackernagel, 1994; Brigulla and Wackernagel, 2010). The first factor is that the quality of DNA may not be sufficient to ensure its functionality for transformation, as suggested by the lack of detectable DNA uptake by isolated gut bacteria grown *in vitro*, and by *Acinetobacter baylyi* in the rat model (Nordgård *et al.*, 2007).

A second factor is the dependency on particular environmental conditions. This limitation is believed to be the reason for the failure of transfer of recombinant DNA to *Streptococcus gordonii* in a model system in foods and in gnotobiotic rats (Kharazmi *et al.*, 2003). Another investigation also led to the conclusion that gut microbiota were not prone to acquire markers added in the diet, even when selective pressure was added. This restriction was influenced by DNA homologies between donor DNA and potential docking sites in recipient DNA, thus allowing integration in the genome and expression (Nordgard *et al.*, 2012). Finally, it was shown that the extent of DNA acquisition from naked DNA in the food is minimal. However, these experiments might not have been performed at a relevant scale to correctly assess the potential for gene transfer in the gut, in the same way as underlined more generally for DNA transfer in the environment (Nielsen and Townsend, 2004).

In summary, the mechanistic issues related to the uptake of DNA and its integration into a microbiological genome, including the integrity of the genetic information, acquisition mechanisms and limits associated with integration in the genome, all suggest that it is not likely to occur. Before having an impact on the environment (including humans), a number of events are subsequently required for a newly incorporated gene to express a new function at a significant level. The recipient has to develop and occupy a niche within its environment that will allow survival, multiplication and maintenance of the specific trait in subsequent progeny. Furthermore, maintenance of transferred genetic information is only favoured if it confers beneficial traits (or is at least neutral) for the recipient microorganism, e.g. the transfer of antibiotic resistance genes is selected in environments where the corresponding antibiotics are in use (Cantón and Morosini, 2011).

Once all these steps have occurred, it is also necessary to determine whether efficient gene transfer will lead to the expression of a harmful trait. The transfer of a gene does not necessarily imply that the recipient microorganism is more dangerous than its ancestor, even if the new gene is related to so-called "virulence factors". A recent review discussed the ways in which genomes of pathogenic bacteria are shaped (Pallen and Wren, 2007) and shows increasing evidence that this is the result of a long history of co-evolution. Pathogenicity is a multifactorial trait, including proper expression and regulation of numerous factors. For example, the production of a single pathogenicity factor, the cysteine protease in Group A streptococci, requires several processes that are tightly regulated by several genes (Carroll and Musser, 2011). Consequently, acquisition of a single gene usually has no effect on pathogenicity

of a non-pathogenic species, and most transfers inducing change in pathogenicity are related to the acquisition of sets of genes, such as those carried by genomic pathogenicity islands or prophages (Juhas *et al.*, 2009; Muniesa *et al.*, 2012). These islands are usually large, which would hamper their acquisition through DNA uptake in the gut. As such, these considerations suggest that the food safety risk associated with the uptake and expression of food-derived microbial DNA by the gut microbiota is minimal.

6. SUMMARY AND CONCLUDING REMARKS

- Microorganisms are ubiquitous in the food chain. The vast majority are not hazardous. Indeed, they are required for many production processes such as fermentation, where they profoundly influence the final appearance, taste and flavour of the food. A primary constituent of microorganisms (as well as all other living organisms) is nucleic acids.
- Microbial nucleic acids (including those from pathogens) may enter into the food chain from multiple reservoirs. Nucleic acids may also enter the food chain through processing aids, including virulent bacteriophages that have been used as decontaminants to reduce surface contamination of specific pathogens on foods.
- The nature and mechanism of microbial cell damage or death during processing may be multifactorial, in some cases resulting in dead (intact) cells and/or released nucleic acids from such cells. The released nucleic acids may be fragmented but most probably include relatively large segments (detectable by nucleic acid amplification methods) in the food product.
- Over the past 25 years, there have been considerable advances in the use of molecular biological techniques for detection of microorganisms in foodstuffs. The targets most often selected are DNA and RNA, which are detected using sequence amplification processes such as PCR.
- Because nucleic acids are relatively stable and may be present in a food matrix even after normal processing (including in the absence of viable food-poisoning agents), the probability that they will be detected in food is high.
- DNA and RNA integrity can be dramatically altered as a consequence of most food processing regimes, including heat treatment, high hydrostatic pressure and lytic agents such as phage. High-temperature treatments have been reported to have the most dramatic effects on DNA stability, resulting in depurination or deamination of the DNA molecules. RNA is a less stable molecule and is degraded rapidly once the microorganisms die, although rRNA possesses some level of protection.
- When the target is DNA, a molecule characterised by high stability even after cell death, a positive result by PCR does not infer the presence of live pathogenic microorganisms in foods. Thus, proper interpretation of such an analytical result is extremely important for processed foods where the production includes a critical control point (CCP) in which foodborne pathogens are killed or substantially reduced. In the majority of cases, CCPs are validated to achieve an appropriate reduction in the perceived microbiological hazard. Nonetheless, the presence of dead cells may indicate that somewhere along the food production line there had been contamination of raw materials or food ingredients or abuse of the product allowing the growth of bacteria.
- Routine approaches for testing food for microbiological parameters using *in vitro* culture of bacteria have largely remained unchanged over the last 20 years and remain the gold standard for detection of bacterial pathogens and indicator organisms.
- In current EU legislation, culture-based approaches are identified as reference methods, although alternative techniques (including immunoassays and PCR-based technologies) can be used, provided that they are appropriately validated and produce equivalent results.
- Currently, PCR is the method of choice for detecting nucleic acids in food although, due to its high specificity, this technique can only detect well-defined targets, the specificities of which can be predicted on the basis of the sequences that are used for primers and reporter probes. This limitation may be alleviated in the future because new methodologies are emerging that allow global studies of ecosystems by high-throughput sequencing.
- The nucleic acid sequence-based amplification (NASBA) and the loop-mediated isothermal amplification (LAMP) methodologies are examples of alternative isothermal nucleic-acid based amplification techniques.

- mRNA (transcribed to cDNA) is potentially a more suitable target for assessing microbial viability, although technical issues can be prohibitive for routine analysis. When this approach is used to determine inactivation or survival of non-cultivable viruses, an underestimation of the inactivation can occur.
- The use of molecular diagnostic methods and application of high-throughput sequencing (or metagenomics or massive ecological studies) provides an effective means to assess the presence of pathogen-derived and/or adventitious nucleic acids in processed foods and food-related environments. Their detection is not necessarily a reflection of unsafe products. They can, however, provide information on raw material and processing aid quality, hygiene and manufacturing practices.
- Viable but non-culturable (VBNC) states have been described for several foodborne pathogens, including *E. coli*, *L. monocytogenes*, *C. jejuni* and *S. Typhimurium* and may represent a risk for the safety of processed foods. Although there is a general lack of knowledge of the risks from VBNC cells, it cannot be assumed that such cells will not emerge from this state and cause disease.
- Nucleic acids, including DNA and RNA, are not harmful molecules themselves, irrespective of their origin. Indeed, considerable amounts of these molecules are ingested daily with meat, vegetables and fermented foods that are part of a normal healthy diet.
- DNA present in the digestive tract may be taken up by bacteria from the microbiota, but the high degradation state of these molecules due to the digestion process makes it extremely unlikely that an intact gene is integrated .

7. DATA GAPS AND RECOMMENDATIONS

In the last 30 years, significant advances in the field of molecular biology have allowed new diagnostic tools to be developed. The best example is PCR. From initial applications in clinical microbiology, in the 1990s PCR started to be applied in food microbiology, mainly for the detection of pathogenic microorganisms. Since then, a huge amount of data has been generated and nowadays the literature describes many PCR applications for foodborne pathogenic bacteria, viruses and parasites, spoilage microorganisms and technologically important bacteria and yeasts. In the last decade, PCR has been used by food processing companies as a diagnostic tool, mainly for the detection of pathogenic microorganisms. However, PCR can also detect DNA from dead microorganisms, and so a positive result does not necessarily infer the presence of live populations of target organisms. This is an important issue for food companies. Besides heat treatments, alternative processes exist to enhance food shelf-life and improve safety, including high-pressure treatments, enzymatic treatments, alterations in pH, irradiation, etc. In many cases, the effects of these treatments on pathogen survival have been evaluated. However, the consequence of such treatments on the integrity of pathogenic DNA remains to be elucidated.

A clearer understanding of the influence of food matrices on the stability of (free) nucleic acids during food processing and post-processing needs further investigation. For example, intrinsic food factors may influence the degree of nucleic acid fragmentation and, consequently, their accessibility for detection. It is well known that nucleases play a dominant role in the degradation of nucleic acids, and this may also be studied further in relation to food processing and food safety.

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