Chapter 14

Nucleic Acid-Based Methods to Identify, Detect and Type Pathogenic Bacteria Occurring in Milk and Dairy Products

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1. Introduction

Foodborne illnesses caused by pathogenic microorganisms, including bacteria, viruses and parasites are among the most serious public health concerns worldwide. A recent document of the Center for Disease Control and Prevention (CDC) estimates that each year 1 out of 6 American (or 48 million people) get sick, 128,000 are hospitalized and 3,000 die due to foodborne diseases, with *Norovirus*, nontyphoidal *Salmonella*, *Clostridium perfringens*, *Campylobacter* spp. and *Staphylococcus* (*S.*) *aureus* being the top five pathogens causing domestically acquired foodborne illnesses [1]. In the European Union, during 2009, 5,550 food-borne outbreaks occurred, mainly due to *Salmonella*, viruses and bacterial toxins, causing 48,964 human cases, 4,356 hospitalisations and 46 deaths [2]. Bacteria such as *Salmonella* spp., *Campylobacter* spp., *Listeria* (*L.*) *monocytogenes*, *Escherichia* (*E.*) *coli* O157:H7 and (*S.*) *aureus* have generally been identified as etiologic agents of most food-borne illnesses, with milk and its derivatives products among the most frequently involved food matrices. Moreover, although "less hazardous", these pathogens are a constant threat to the agro-food security, since they can be used to contaminate the environment, crops and animals, causing heavy damage to public health, agriculture and environment [3].

Traditionally, cultivation methods, ranging from plate counting to biochemical characterization, have been used to monitor pathogenic microorganisms in foods. However, these methodologies are labour-intensive and time-consuming, requiring from days to weeks to get results, with the consequence that products are often released for sale before the microbiological results become available. Moreover, these traditional methods as well as their advanced (such as cell wall composition analysis, whole-cell protein fingerprinting and fatty acid analysis) and automated (miniaturised kits or devices) applications often lead to uncertain identification or even misidentification, especially in cases of phenotypically closely related

species. Failure to detect pathogens can have adverse health effects as well as substantial economic losses and fatalities. New approaches based on the application of molecular methods have being developed in the last years, bringing new insights in the detection of pathogenic bacteria in milk and milk-based products. In this chapter, we will endeavour to touch upon several nucleic acid based methods (such as PCR and its derivatives, real time PCR, REA-PFGE, fAFLP, etc.) and their application in milk and dairy products.

2. Nucleic acid-based detection and identification of milk- and dairy-borne pathogens

Detection and identification methods to detect milk- and dairy- pathogens may be traced back to at least two basic techniques: direct hybridization and *in vitro* amplification. In the following paragraphs, due to their importance in the microbial safety of milk and its derivatives, and since most of the advanced molecular methods derive from these fundamental techniques, we will provide the basics of nucleic acid hybridization and polymerase chain reaction (PCR) as well as an excursus of the most used nucleic acid-based techniques to identify, quantitatively detect and type pathogenic microorganisms occurring in milk and dairy products.

2.1. Nucleic acid hybridization

2.1.1. Basics

DNA hybridization is mainly based on an intrinsic feature of the DNA molecule, such as the high specificity of base pairing (Figure 1) between homologous strands of single-stranded DNA. The deoxyribonucleic acid (DNA) structure consists in a double helix conformation of two polynucleotide strands held together by hydrogen bonds. DNA is composed of four repeating nucleotides: Adenine, Thymine, Cytosine, and Guanine (Figure 1). Each base is linked to a deoxyribose molecule, which is attached to a phosphate moiety. The various nucleotides are linked together via the 5′ carbon of the deoxyribose molecule and the phosphate group attached to the 3′ carbon (Figure 1). Each nucleotide base in the DNA strand will cross-link (via hydrogen bonds) with a nucleotide base in a second strand of DNA forming a structure that resembles a ladder (Figure 1). These bases cross-link in a very specific order: Adenine will only link with Thymine (and vice-versa), and Cytosine will only link with Guanine (and vice-versa) (Figure 1). Two single strands of DNA will bond together only if their base-pairs match up properly or complement one another [4].

The double stranded DNA may be broken by heat or high pH. The reannealing between single stranded DNAs from different sources is called hybridization (Figure 2).

Standard nucleic acid hybridization assays require the use of a labelled nucleic acid probe (a denaturated DNA fragment varying in size from ten basepairs to kilobasepairs) to identify the target homologous DNA or RNA molecules within a complex mixture of unlabeled nucleic acid molecules, with the stability of the hybrid depending on the extent of base pairing that occurs [5]. Experimentally, the probe is usually labelled and the denatured

target nucleic acid (DNA or RNA) is immobilized on a membrane or a polymer support or, if size information of the hybridization target is required, the target DNA is first run through agarose gel electrophoresis and then transferred to a membrane. The labelled probe is then added in a solution allowing the hybridization. After a suitable incubation, the membrane is washed in order to remove any non-specifically bound probe, leaving only the probe base-paired with the target DNA. By controlling the stringency of the washing conditions, DNA sequences 100% complementary to the probe or with lower degrees of similarity (i.e. with some mismatching) might be detected. In particular, having a sequence complementary to that of the target DNA, the probe might bind specifically to the target (previously denatured) and form with it a duplex DNA hybrid, recognizable by the labelled probe. The intensity of the spot is proportional to the amount of hybridized probe and therefore is proportional to the amount of target DNA in the sample. The intensity of the spot can be compared visually with the intensity of spots that correspond to a standard curve yielding semi-quantitative results (i.e., visual quantification), or the intensity can be determined using an instrument (e.g., densitometer) to create a quantitative value comparable with values obtained from the standard curve [6]. To overcome biohazards associated with the use of probes labelled with radioactive isotopes (usually P^{32} and S^{35}), biotin, digoxigenin and different fluorochromes have been used for labelling [7,8,9,10].

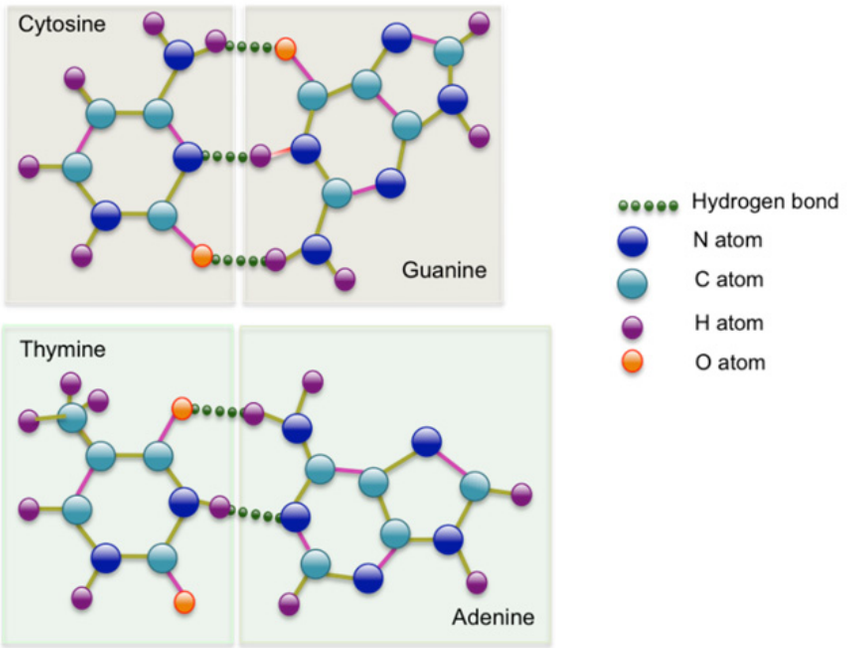


Figure 1. DNA base-pairing.

2.1.2. Application

Several technologies based on the nucleic acid hybridization, such as dot-blot [11], Southern- [12] and Northern-blot [13], colony hybridization [14], colorimetric DNA hybridization [15] etc. have been developed and successfully applied to the pathogen detection [16-41] in milk and dairy products.

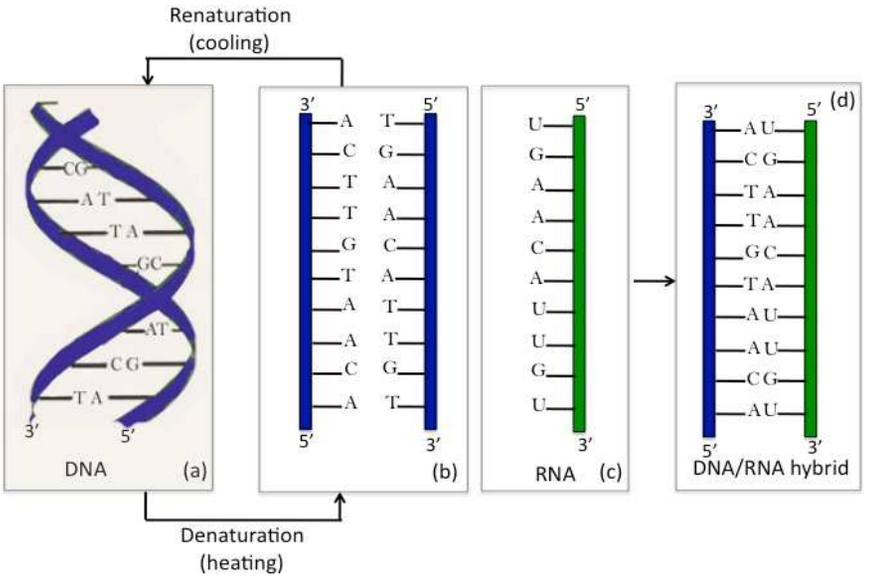


Figure 2. Nucleic acid hybridization.

However, a major drawback of the hybridization assays is their lack of sensitivity, which limits the use of these analyses to populations of cells or genes occurring in relatively high numbers in samples. For this reason, hybridization assays are currently mainly used for culture confirmation rather than direct detection and identification.

Among all the hybridization assays to date available, a particular focus should be given to the fluorescence *in situ* hybridization (FISH). FISH uses fluorescently labeled ribosomal RNA (rRNA) targeted probes and fluorescent microscopy to detect intact bacteria directly in food and clinical specimens, such as blood and tissue, or after enrichment culture [42,43]. Since this technique can visualize the precise location of a particular nucleic acid in the cytoplasm, organelli or nuclei of biological materials, it allows detecting metabollically active microorganisms directly in the environment without cultivation also providing useful information on the spatial distribution of the target organism in the colonised matrix. Experimentally, the procedure consists in preparing the samples, fixing it, preparing a smear or section on a microscope slide, permeabilizing the cells, hybridizing the probe to the DNA or RNA target in the sample and detecting the hybridization event by fluorescence microscopy. FISH assays have been developed and used to detect at family, genus and species level *Staphylococcus* spp., *Listeria* spp., *Campylobacter* spp., *Salmonella* spp. and *E. coli* [44,45,46,47,48].

As the other hybridization assays, FISH suffers from sensitivity. Moreover, FISH may be hindered by microorganism and substrate inherent autofluorescence, insufficient permeability of cell walls, non-specific binding of probes and low ribosome contents.

2.2. Polymerase chain reaction

2.2.1. Basics

The polymerase chain reaction (PCR) succeeded in revolutionizing the analysis of nucleic acids, so much that a Nobel Prize was conferred to Kary Mullis [49]. It is an *in vitro* three-

step amplification process first introduced by Saiki and co-workers [50]. In PCR reaction (Figure 3), mixtures of oligonucleotides (primers), properly designed to be complementary to the flanking regions of the target sequence to be amplified, are mixed in molar excess with the DNA template, free deoxyribonucleotides and a DNA polymerase enzyme in an appropriate buffer. Following heating to denature the original strands and cooling to promote primer annealing, the oligonucleotide primers bind to their complementary sequences in the target DNA. Then, the temperature is raised to the optimal temperature of a DNA polymerase, which begins polymerization, adding nucleotides to the 3′ end of each primer attached to a single DNA strand. After one complete cycle, there are two double stranded copies of the target DNA. This process of denaturation, annealing, and polymerase extension repeated cyclically, produces many copies. Theoretically, 30 cycles over a billion copies of the target sequence (2^{30} = 1.07 billion) could be provided.

The availability of both thermostable DNA polymerases, which resist to inactivation at the high temperatures used during the thermal cycling, and thermal cyclers, which could shift their temperatures up and down rapidly, automatically and in a programmed manner, have allowed the PCR to be automated. Amplicons, i.e. PCR products attended, can be visualized through several methods. Apart from DNA hybridization, one of the most used techniques to accomplish amplicon detection is the agarose gel electrophoresis using a buffer stained with a dye (ethidium bromide, SYBR Green etc.) that binds double stranded DNA and fluoresces upon excitation with UV light. By this way, it is possible to observe and photograph the gel by using an apparatus with a UV light source and an appropriate camera [51]. The presence of the target pathogen, regardless of its conditions (live or dead), can be ascertained by the presence in gel of the band relevant to the specific amplicon. Moreover, due to the inverse linear correlation recognised between the $\log_{10}$ size of the DNA fragment (basepair) and the distance migrated by the DNA fragment in the agarose gel during electrophoresis, it is possible to estimate the size of the amplicon from DNA standards loaded in the agarose gel. Other ways to accomplish the detection of PCR products include DNA hybridization and non-gel methods such as the enzyme-linked immunosorbent assay (ELISA) [52].

2.2.2. Application

PCR, together with culture and counting methods is been indicated as the most popular method used in pathogen detection [53]. Sequencing analysis of rRNA genes intergenic spacer regions as well as other phylogenetically important genes such as *rpoB*, *hsp60* etc. and species-specific PCR of DNA fragments that are unique for a given microbial species, have been used to identify, detect and characterize pathogenic microorganisms. In Table 1, a list of PCR based methods developed and used to detect in milk and dairy products the five most concerned pathogens (*Campylobacter* spp., *Salmonella* spp., *E. coli* O157:H7, *L. monocytogenes* and *S. aureus*) is reported.

Among the different PCR variants to date available, multiplex PCR is very useful as it allows the simultaneous detection of several organisms by introducing different primers to amplify DNA regions coding for specific genes of each bacterial strain targeted. Apart from 16S and

23S rRNA and intergenic spacer regions, several genes have been targeted to allow multiplex milk- and dairy-borne pathogens identification, detection and characterization [54-66].

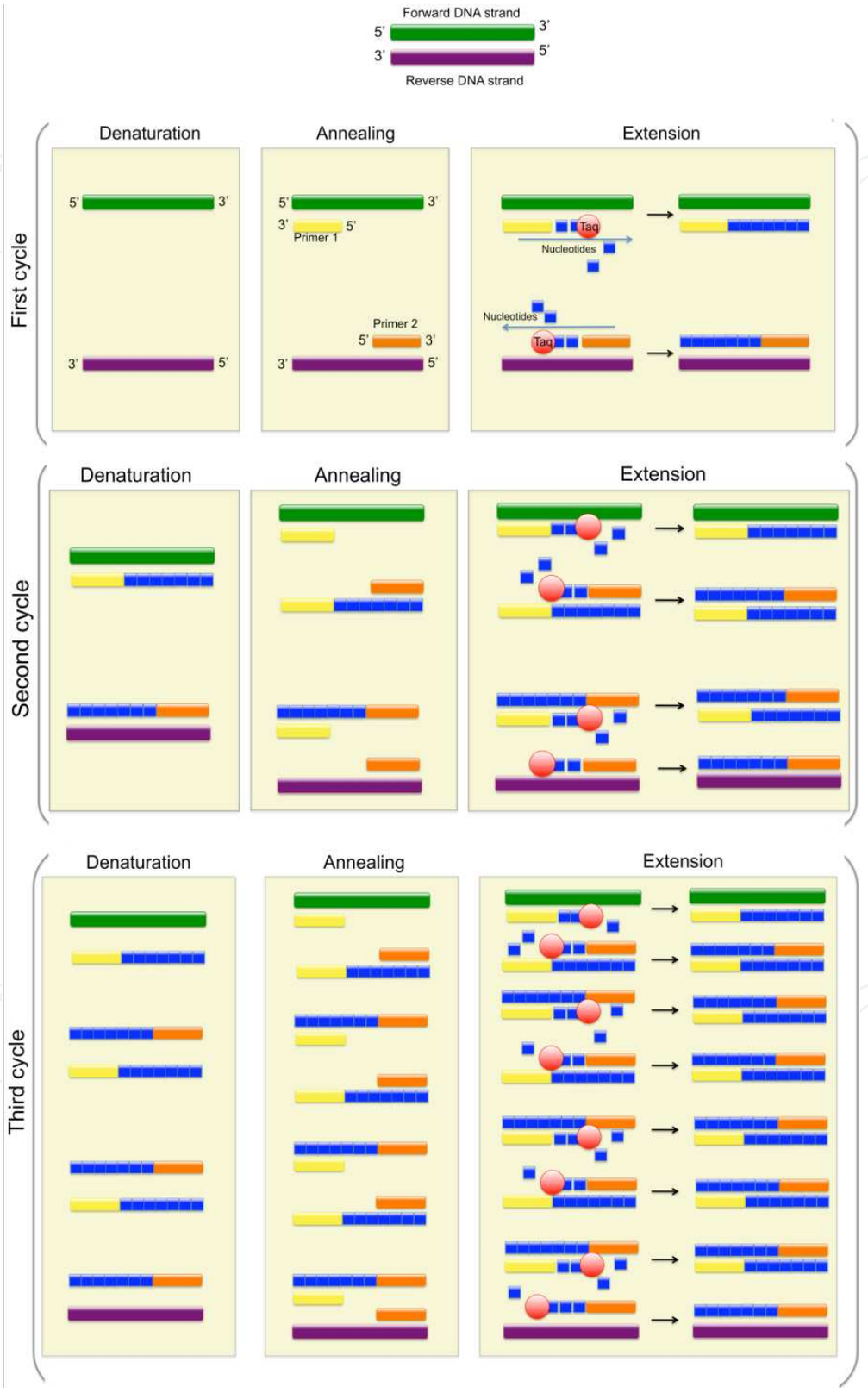


Figure 3. Polymerase chain reaction.

Several targets have been used and several researchers have used the same target to develop different primer pairs to detect the same bacterial species. However, individual assessments of the specificity and sensitivity of a given assay may differ markedly and care should be taken in designing a PCR [67,68]. Moreover, taxonomy of several pathogenic bacteria is being continuously changed in the recent years, thus calling for a periodic re-validation of PCR assays. As an example, the taxonomy of *Campylobacter* has evolved every year since 1988 and to date it contains over 100 *taxa*. Within the EC 6th Framework "MoniQA" Network of Excellence project on harmonisation and standardisation of detection methods for foodborne hazards, we have recently participated, with other 7 laboratories of other 7 countries, in a revalidation study of 31 PCR assays for the identification of *Campylobacter* species, with particular reference to taxa described since 2004, which are closely related to the most concerned pathogenic species *C. jejuni* and *C. coli* [69]. It resulted that i) the sensitivity and specificity of these PCR assays varied considerably, ii) PCR assays recently developed to identify and detect a novel *Campylobacter* species (namely *C. lari*) were no successful in detecting all strains of this species, probably reflecting its complex taxonomy; and iii) several PCR assays gave false positive results for *Campylobacter* species described since PCR tests were reported (including *C. cuniculorum, C. subantarticus, C. peloridis* and *C. volucris*), thus highligthing the need for attention to detail in the design and evaluation of a PCR assay and also for ongoing revalidation of previously already validated PCR assays [69].

Species	Target gene or fragment	Encoded product	Reference	
			a	b
Listeria monocytogenes	16S	16S rRNA	[70]	
	*hly*A	Listeriolysin	[71,72]	[73,74]
Campylobacter spp.	*act*A	Actin assembly-inducing protein	[75]	
	16S	16S rRNA	[76]	
Escherichia coli O157:H7	*eae*	Intimin	[77]	
	stx1 (or VT1)	Shiga-like toxin 1		[78]
	*stx*2 (or VT2)	Shiga-like toxin 2		[74,79,80]
	hlyA	Haemolysin		[62]
	*rfb*E	Lipopolysaccharide O side chain of *E. coli* O157		[79]
Salmonella spp.	*inv*A	Invasion protein		[81,82]
	stn	Enterotoxin determinant		[74]
Staphylococcus aureus	*nuc*	Thermostable nuclease of *S. aureus*	[83]	[74,84-88]
	sea	Staphylococcal enterotoxin A	[89]	[84,90-96]
	seb	Staphylococcal enterotoxin B	[89,97]	[84,90-96]
	sec	Staphylococcal enterotoxin C	[89]	[84,90-96,98]
	sec1	Staphylococcal enterotoxin C1	[97]	[99]

Species	Target gene or fragment	Encoded product	Reference	
			a	b
	sed	Staphylococcal enterotoxin D	[89]	[84,90-96]
	see	Staphylococcal enterotoxin E	[89]	[84,90-96]
	seg	Staphylococcal enterotoxin G	[89]	[84,91-93, 96,100,101]
	seh	Staphylococcal enterotoxin H	[89]	[91,92,94,96]
	sei	Staphylococcal enterotoxin I	[89]	[91,92,94,96,100,101]
	selj	Staphylococcal enterotoxin-like J	[89]	[84,91,94,96]
	selk	Staphylococcal enterotoxin-like K		[94]
	sell	Staphylococcal enterotoxin-like L	[89]	[91,94]
	selm	Staphylococcal enterotoxin-like M	[84,102]	[94,100,101]
	seln	Staphylococcal enterotoxin-like N	[84,102]	[94,100,101]
	selo	Staphylococcal enterotoxin-like O	[84,102]	[94,100,101]
	ser	Staphylococcal enterotoxin R		[92]
	ses	Staphylococcal enterotoxin S		[92]
	set	Staphylococcal enterotoxin T		[92]
	selu	Staphylococcal enterotoxin-like U		[101]
	selv	Staphylococcal enterotoxin-like A		[101]
	ϕent1	Pseudogene *ϕent1*		[100,101]
	ϕent2	Pseudogene *ϕent2*		[100,101]
	tsst	Toxic-shock syndrome toxin		[84,92]
	egc	Enterotoxin gene cluster		[84,92,100,101]
	spa	*Staphylococcus aureus* protein A		[103-106]

Table 1. List of target genes used in conventional PCR protocols for the identification, detection and characterization of some of the most concerned foodborne pathogens. Assays either specifically developed or employing already existing protocols for milk- and dairy- borne pathogens are listed in columns "a" and "b", respectively.

2.3. Real time PCR

Conventional PCR-based detection requires post-amplification confirmative analyses, which, apart from the potential DNA carry-over, are time- and labour-consuming. In real time PCR, fluorescent dyes are used to directly monitor the amplification of the target DNA. Moreover, because fluorescence increases in direct proportion to the amount of specific amplicons, real time PCR can be used for quantification.

SYBR Green is one of the most frequently double-stranded (ds) DNA-specific dyes used in real-time PCR today. It is an asymmetric cyanine dye that can be excited with blue light with a wavelength of 480 nm and having an emission spectrum comparable to that of fluorescein with a maximum at 520 nm [108]. Being a DNA binding dye, SYBR Green allows the detection of any double-stranded DNA during the PCR. Strength and, at the same time, weakness of this system is that, being nonspecific, it can also bind any spurious product (dimers, artefacts etc.). To overcome this problem a melting curve analysis may be carried out at the end of the real time PCR amplification. The strand-specific methods have a higher specificity since they employ fluorophore-coupled nucleic acids to interact with reaction products, probing accumulating PCR products for the presence of the target sequence.

The most commonly used fluorogenic oligoprobes rely upon the fluorescence resonance energy transfer (FRET) between either fluorogenic labels or a fluorophore and a dark or blackhole non fluorescent quencher (NFQ), which disperses energy as heat rather than fluorescence [107]. The FRET spectroscopic process consists in an energy transfer between molecules separated by 10-100 Angstroms, which have overlapping emission and absorption spectra [107].The theory behind this non-radioactive induced dipole interaction process was developed by Förster [109]. The efficiency of this process mainly depends on the distance between fluorophores. Indeed, the sequence-specific signals are generated due to the PCR-product-dependent change in distance between fluorophores [108].

TaqMan probes contain two dyes, a reporter dye (e.g. 6- carboxy-fluorescein; FAM) at the 5′ end and a quencher dye (e.g. 6-carboxy-tetrametyl-rhodamine; TAMRA) at the 3′ end. The proximity of the quencher dye to the reporter in an intact probe allows the quencher to suppress, or "quench", the fluorescence signal of the reporter dye through FRET. If the target of interest is present, these probes specifically anneal between the forward and reverse primer sites. During the real time PCR amplification, the 5′ to 3′ nucleolytic activity of the Taq DNA polymerase cleaves the probe between the reporter and the quencher, only if the probe hybridizes to the target. Thereafter, the quencher is released from the fluorophor, which now fluoresces after excitation.

The signal increases in direct proportion to the amount of PCR product in a reaction [107]. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye. The average background fluorescence is usually measured for the first 10 cycles of the reaction and subtracted from each fluorescent reading, resulting in a standardized amplification plot of fluorescence intensity against cycle number for each reaction. The cycle threshold (Ct), defined as the first cycle in which there is a significant increase in fluorescence above a specified threshold, is then calculated and the fluorescence against the cycle number is plotted to obtain a curve that represents the accumulation of PCR products in function of time. Running several reactions containing dilutions of known amount of target DNA a standard curve can be created and used to quantify unknown amounts of target DNA [107].

Being time-saving (especially the "fast systems" and requiring reduced handling, avoiding the risk of carryover contaminations, real time PCR is revolutioning the clinical, food and

environmental diagnostics. Moreover, depending on the detection platform utilised, it is very highthroughput being possible to process either 96 or 384 samples per run even in a multiplexing format. Simplex and multiplex real time PCR assays have been developed and used to identify and quantitatively detect directly and undirectly in milk and dairy products, *S. aureus*, *L. monocytogenes*, *Salmonella* spp., *E. coli* O157:H7 and *Campylobacter* spp. (Table 2). When the culture-independent approach is performed the availability of an appropriate DNA extraction and purification protocol is crucial. Milk and its derivatives, due to their intrinsic complexity (in terms of composition and structure) and the likely presence (in raw milk based products) of abundant background microflora, may significantly affect the efficiency of both the nucleic acid extraction and PCR amplification [66,86, 110-113]. Moreover, in such cases, it is of outmost importance to use standard curves appropriate to the specific case to be analysed. Fusco et al. [101] developed a TaqMan and a SYBR Green rt-PCR based assay targeting the enterotoxin gene cluster of *S. aureus*, regardless of its variants, for the rapid and reliable identification and quantitative detection of *egc*$^+$ *S. aureus* strains (i.e. *S. aureus* harbouring the *enterotoxin gene cluster*). In addition, given the well recognised role of *S. aureus* as one of the commonest aetiological agent of clinical and sub-clinical mastitis [114-116] and considering that milk and milk-based products contaminated with this pathogen are some of the food matrices more often involved in staphylococcal food poisoning [117-122], they evaluated the effectiveness of these novel assays in artificially and naturally contaminated raw milk [101]. To achieve these goals, standard curves were constructed using ten-fold dilutions of target pre-purified DNA, or DNA extracted from ten-fold dilutions of a *egc*$^+$ *S. aureus* strain in broth and in raw milk [101]. Moreover, in order to determine the diagnostic sensitivity, defined as a measure of the degree to detect the target pathogen in the biological matrix, and to assess the applicability of the assay in simulated staphylococcal food poisoning conditions (i.e enterotoxin positive *S. aureus* concentration above 10^5 cfu mL^{-1}), three standard curves in raw milk were constructed using log phase broth cultures of either a single *egc*$^+$ *S. aureus* strain, a mix of *egc*$^+$ *S. aureus* strains and a mix of *egc*$^+$ and *egc*$^-$ *S. aureus* strains harbouring (and not) some of the commonest enterotoxin genes associated to this syndrome [101]. Overall, the TaqMan assay revealed less sensitivity (limit of quantification=10^2 cfu equivalents per reaction mixture of *egc*$^+$ *S. aureus* either singly, in mix and in mix with *egc*$^-$ *S. aureus* strains) in milk than in DNA (pre-purified and not), thus highlighting the importance of using a standard curve in raw milk to accurately quantify *egc*$^+$ *S. aureus* in real raw milk samples [101].

Species	Target gene	Protein	Reference	
			a	b
Listeria monocytogenes	*hly*A	Listeriolysin	[123,124]	[125]
	*ssr*A	tmRNA	[126]	[127]
	*prf*A	Transcriptional regulator PrfA; listeriolysin positive regulatory protein	[128,129]	
	16S	16S rRNA	[130]	

Species	Target gene	Protein	Reference	
			a	b
	16S-23S rRNA IGS	Intergenic region spacer between the 16S and 23S rRNA	[131]	[132]
Staphylococcus aureus	*egc*	Enterotoxin gene cluster	[101]	
	*htr*A	High-temperature-requirement A protein	[133]	
	nuc	Thermostable nuclease	[134]	
Escherichia coli	*stx*1	Shiga-like toxin 1	[135]	
	*stx*2	Shiga-like toxin 2	[135,136]	
	eae	Intimin	[135]	[30]
	*stx1, stx*2	Shiga-like toxin 1 e 2	[137]	[30]
Campylobacter spp.	VS1	*C. jejuni* specific fragment	[138]	
Salmonella spp.	*ttr*RSBCA	Proteins involved in tetrathionate respiration	[139]	
	*inv*A	Invasion protein	[140]	

Table 2. List of target genes used in simplex and multiplex real-time PCR protocols for the identification, (quantitative) detection and characterization of some of the most concerned foodborne pathogens. Assays either specifically developed or employing already existing protocols for milk- and dairy- borne pathogens are listed in columns "a" and "b", respectively.

2.4. Detecting stressed or injured pathogens: EMA and PMA PCR/real time PCR, reverse transcription PCR/real time PCR and NASBA

The complexity and variability of food composition as well as physical and/or chemical stresses that pathogenic microorganisms encounter in the environment, in foods and food preparation/production/storage processes, if inadequate or sub lethal, may result in incomplete inactivation [141,142]. Such injured or stressed bacteria are a potential risk since they can, under appropriate conditions, recover and regain or even enhance their pathogenicity [141,143-145]. Failure to detect injured pathogens can have adverse health effects as well as fatalities and economic losses. All these findings prompt the need for improved enumeration methods capable of discriminating among viable, dead, and injured microbial cells. Conventional culture based methods do not allow the enumeration of stressed or injured bacteria, as they use selective agents whose injured or stressed pathogens' cells, depending on the site and degree of damaging, are extremely sensitive [142]. The inadequateness of highly selective solid and liquid media remarkably complicates the detection of pathogenic bacteria in foods characterized by a complex and numerous background microflora, such as milk and milk-based products. The major drawback in using DNA-based assays to detect pathogenic microorganisms is that DNA is detectable in both viable, injured and dead cells of a given microorganism also after a long period of time [86,146]. However, DNA amplification techniques may be combined with the use of

molecules able to penetrate in dead or injured cells and bind to DNA making it insoluble so that it can be easily eliminated together with cell debris during genomic DNA extraction. Ethidium monoazide- (EMA) and propidium monoazide (PMA) conventional and real time PCRs have been applied to the detection and quantification of different food-borne pathogens [147-150]. PMA seems to have the important advantage over EMA of not penetrating living cells [149].

Stressed or injured pathogens cells may be quantitatively detected by PCR methods combined with a solid or a liquid based enumeration method in which stages of "revivification" have been introduced to restore and therefore bringing back in conditions of perfect viability and cultivability the greater number of stressed cells, not directly cultivable [79,123].

To address the need of detecting only living pathogens RNA may be detected rather than DNA [151-155].

Reverse Transcription (RT) PCR makes use of a reverse transcriptase, which, in presence of a complementary primer, can translate an RNA strand corresponding to a transcribed gene into complementary DNA (cDNA). The reaction is usually initiated by random oligonucleotide primers. Thereafter, the cDNA is used as template to amplify by PCR specific sequences using oligonucleotide primers and DNA polymerase under normal PCR or real time PCR conditions.

Another way to target mRNA as an indicator of cell viability is to employ the nucleic acid sequence-based amplification (NASBA). It is an isothermal nucleic acid amplification technology allowing the amplification of RNA or DNA targets (with a slight modification in the protocol) through a transcription process after the insertion of a T7 promoter, due to the concerted action of three enzymes: AMV Reverse Transcriptase for cDNA synthesis, RNase H to degrade the RNA in the heteroduplex RNA-DNA and T7 RNA polymerase to synthesize RNA from the T7 promotor [156].

Both the NASBA and the reverse transcription conventional and real time PCR techniques have been used for developing diagnostic tests to detect viable pathogenic microorganisms [157-159]. Since NASBA is performed in isothermal conditions, it does not require the use of a thermocycler. Therefore, it is less expensive than PCR and RT-real time PCR.

2.5. Biosensors, microarrays, micro and nano electro-mechanical-systems

Biosensors have been recognized as a means to provide a higher level of surveillance in a more automated and rapid manner. These analytical devices combine a biological sensing element (called receptor) with a chemical or physical transducer for selectively and quantitatively detecting a given compound. In complex matrices only the compound interacting specifically with the integrated biological component will generate the optical or electrical signal from the physical transducer, modulating the biosensors' selectivity [53]. Such specific interactions produce a physico-chemical change, detected and measured by the transducer that can output a signal proportional to the concentration of the target analyte, allowing for both real time

quantitative and qualitative measurements. The chemico-physical change detected by the transducer may be: 1) absorption or evolution of heat (thermometric or calorimetric biosensors), 2) changes in the distribution of charges causing an electrical potential to be produced (potentiometric biosensors), 3) movement of electrons produced in a redox reaction (amperometric biosensors), 4) light radiation or difference in optical properties between the reactants and products (optical biosensors) and, 5) effects due to the mass or intermolecular interaction of the reactants or products (piezo-electric biosensors) [53,160]. Enzymes, antibodies, DNA, receptors organelles and microorganisms as well as plant cells or tissues are frequently used as biological sensing elements [161].

Many biosensors for food diagnostic application in the food and drink industry are currently being developed [161-166]. Among these, biosensors that can detect DNA are of particular interest. Intrinsic features of the DNA molecule, such as the high specificity of base pairing between homologous strands of single-stranded DNA, as well as its predisposition to electrical, fluorescent and mechanical measurements, make it a highly specific sensing element, and render it useful for signal transduction in a wide range of DNA based biosensors [167,168].

Hybridization biosensors rely on the immobilization of a species- or strain- specific single stranded DNA probe onto the transducer surface. Due to the characteristic negative charge of DNA, the duplex formation can be detected for example by following the association of an appropriate hybridization indicator. Hybridization events between analyte and probe DNA may be translated through electrochemical, optical, or mechanical output signals [169]. As for other types of biosensors, high selectivity is crucial for the success of DNA hybridization devices. The specificity of these sensors depends primarily on the selection of the probe, and secondarily upon the hybridization conditions (mainly the temperature).

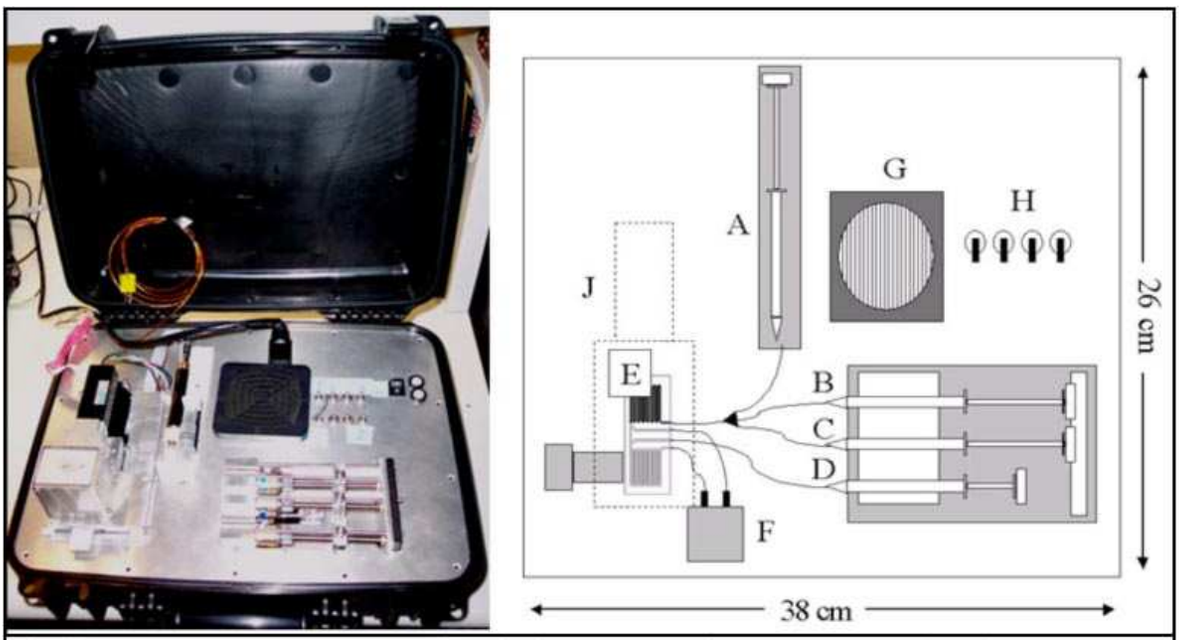
Microarrays, based on the Watson-Crick base pairing principle [4], consist of genetic sensors, the so-called "spots," each containing single strands of species- or strain- specific DNA sequences termed probes immobilized at pre-determined position at high density. The DNA sequence of a target organism's genetic sample, previously labelled (through PCR amplification), will hybridize with its complementary sequence on the microarray to form a stable structure. After washing away non-specifically bound targets, the array is scanned using laser light of a wavelength designed to trigger fluorescence in the spots where binding has occurred. A specific pattern of array spots will fluoresce, which is then used to infer the genetic makeup in the test sample [170]. Microarray analysis is an emerging technology that has the potential to become a leading trend in bacterial identification in the dairy and overall in the in food and drink industry, allowing both the detection and/or genotyping of pathogenic microorganisms [171-179].

Due to the robust nature of PCR and the high sensitivity that can be achieved through amplification of target DNA, PCR-based biosensing has been widely used [168,180].

For PCR based-biosensing of bacteria optical methods of detection are widely used. In the case of the "real time PCR-based biosensor" the fluorescence emission is the measurable signal

allowing the translation of the DNA amplification. DNA-based biosensor technologies are in constant evolution. In particular, there is a growing tendency toward miniaturization of these systems [167]. In recent years, micro- and nano- fabrication technologies, originally developed for producing silicon-based chips for the microelectronics industry, have spread out in a variety of applications as chemical and biochemical tools, commonly referred to as Biomedical or Biological Micro (and nano)-Electro-Mechanical Systems (BioMEMS or bioNEMS). BioMEMS and devices have been used as biosensor for the detection of bacteria, and the resulting biochips, also known as lab-on-a-chip devices, incorporate multiple laboratory processes in a semi-automated, miniaturized format, allowing rapid, sensitive and real-time measurements [163,164,180-183]. Obvious advantages of the miniaturized integrated detection technologies include higher sensitivity, as well as reduced reagent and sample volumes, reducing associated costs and time to result.

An example of such useful devices is given by the integrated microfluidic platform, known as the "microFLUIDICS DESKTOP" (Figure 4), developed by Cady and co-workers [185] for detecting *Listeria monocytogenes* by real time PCR. Monolithic DNA purification/real-time PCR silicon chips (Figure 5) were fabricated utilizing standard semiconductor processing technologies. These chips incorporated a microfabricated DNA purification chamber with a second PCR amplification chamber, connected by microfluidic channels.



The "microFLUIDICS DESKTOP" includes integrated syringe pumps (A-D), Moog micro valve (F), cooling fan (G), LED-based fluorescence excitation/detection system (J and dotted outline), power toggle switches (H). The microfluidic purification/detection chip (E) is inserted into the unit directly above the thermoelectric heater cooler. The syringes are connected to the chip via TygonTM tubing (black lines) and contain the sample lysate (A), ethanol wash buffer (B), TE (C), and PCR master mix (D). The Moog micro valve (F) is also connected to the chip via tubing and controls pressurization and fluid flow through the chip outputs. The entire unit measures 36cm x 28cm x 15cm.

Figure 4. The "microFLUIDICS DESKTOP" [184,186].

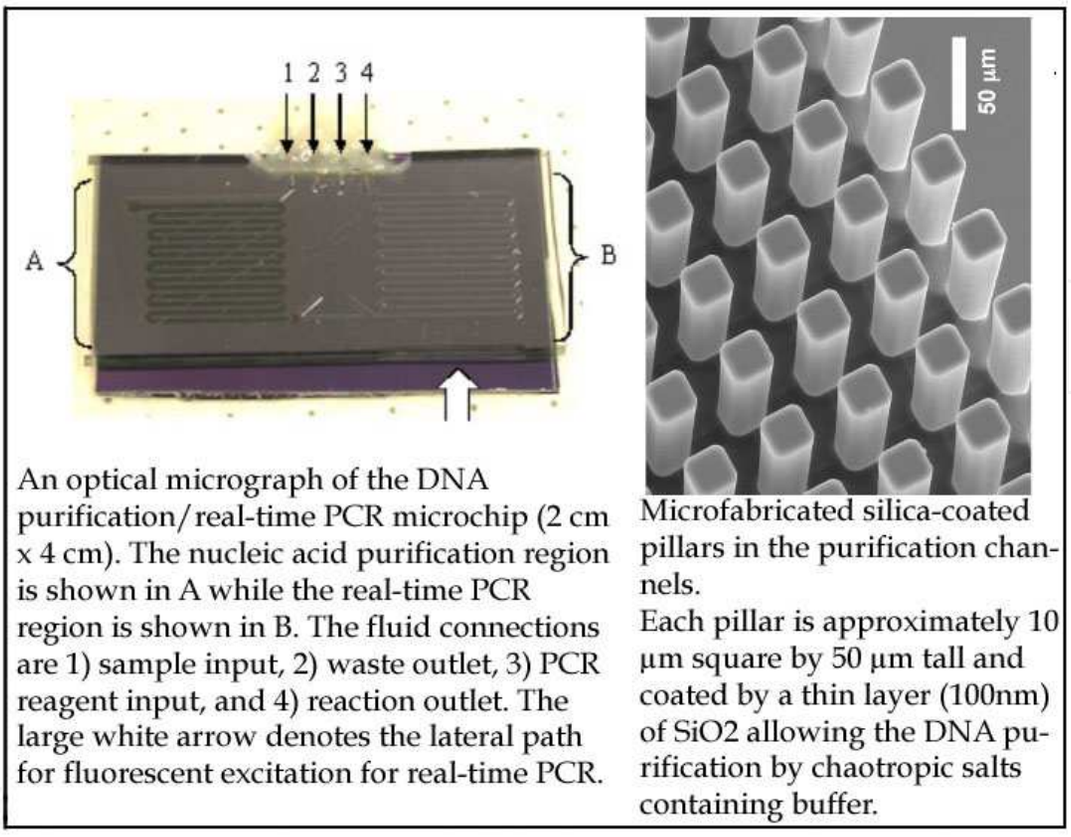


Figure 5. The monolithic DNA purification/real time PCR microchip [184-186].

The DNA purification section contained an array of 10 µm square pillars that were etched 50 µm deep in silicon to form a microfluidic channel. These pillars were coated with a thin layer (100 nm) of SiO2 that could be used for DNA purification in chaotropic salt-containing buffers [185]. Using an automated detection platform with integrated microprocessor, pumps, valves, thermocycler and fluorescence detection modules, microchips were used to purify and detect bacterial DNA by real-time PCR amplification using SYBR Green fluorescent dye. This system was able to both purify and quantify DNA from 10^7 to 10^4 cells by SYBR Green real-time PCR-based detection, with an average turnaround time of 45 min. The "microFLUIDICS DESKTOP" has been successfully used for the more specific TaqMan real-time PCR detection of *S. aureus* [186].

In an improvement over other systems, which are time consuming and require multiple laboratory instruments, this device provides a fully automated method capable of purifying DNA from bacterial cells and preparing samples for PCR-based detection.

Obvious benefits of such device include: reduced time to result; reduced amount of expensive reagents used; reduced handling, avoiding sample contaminations; the possibility to perform a multiplex assay for revealing various pathogenic microorganisms, by incorporating on-board multiple detectors; the possibility to further miniaturize a multifunctional integrated system, which can be developed as a truly portable device to be used for on-line and on-site rapid control of the whole food/beverage chain.

3. Typing

In diagnostic microbiology, besides identifying a pathogenic microorganism, it is of outmost importance to type it. Typing, or subtyping (synonymous used in the American literature) has been defined as: "Phenotypic and/or genetic analysis of bacterial isolates, below the species/subspecies level, performed in order to generate strain/clone-specific fingerprints or datasets that can be used, for example, to detect or rule out cross-infections, elucidate bacterial transmission patterns and find reservoirs or sources of infection in humans" [187]. Several typing methods are to date available for discriminating microorganisms at strain level: the choice should be based on their appropriateness for each specific purpose. Whatever typing method is chosen, it has to be evaluated and validated in respect to several performance criteria (stability, typeability, discriminatory power, epidemiological concordance, reproducibility, test population) and convenience criteria (flexibility, rapidity, accessibility, easy of use, cost, amenability to computerised analysis and incorporation of typing results in electronic databases) prior to use it in a given study [187]. Conventional typing methods, based on the phenotypic features of microorganisms, including biotyping, serotyping, antibiogram-based typing (antimicrobial susceptibility testing), phage and bacteriocin typing, sodium dodecyl sulphate-polyacrilammide gel electrophoresis (SDS-PAGE) of cellular and extracellular components, multilocus enzyme electrophoresis (MLEE), mass spectrometry (MS), matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS, Infrared or Raman spectroscopy, Fourier transform Infrared spectroscopy, etc. are being gradually overtaken by nucleic acid-based methods. The advent of the PCR technology and the development of bioinformatics tools have allowed implementing protocols to investigate the inter- and intra- specific heterogeneity of pathogenic microorganisms based on genotypic characters, which, unlike the phenotypic ones (on which rely conventional typing methods) are certainly the most reliable, being little or not influenced at all by exogenous factors. As for the detection and identification methods, nucleic acid based methods may be grouped into three main clusters including nucleic acid hybridization-, amplification- and fragment based typing methods and a third cluster including methodologies that combine all the above mentioned techniques. Herein, an excursus of the most important molecular methods used to type the five most concerned pathogenic bacteria will be reported.

Acronyms such as RAPD- (Random Amplified Polymorphic DNA), DAF- (DNA Amplification Fingerprinting) and AP-PCR (arbitrarily Primed PCR), define a set of methodologies having, as lowest common denominator, the possibility to amplify anonymous DNA sequences by using a single oligonucleotide as a primer for the Taq Polymerase [188,189]. Under conditions allowing the annealing of the primer at several points, a complex banding pattern should be obtained that could be characteristic for each strain. Methods such as REP- (Repetitive Extragenic Elements Palyndromic), ERIC- (Enterobacterial repetitive Intergenic Consensus Sequence), BOX-, VNTRs- (Variable Number of Tandem Repeats) PCR, being based on the detection of repetitive regions of the genomic DNA, varying in length and number, have been individuated as more reliable and robust than RAPD-PCR to type pathogens [188,190-192].

Classical Restriction Fragment Length Polymorphism (RFLP) analysis combines the restriction endonuclease analysis with a Southern hybridization step. By this technique, genomic restriction fragments are separated by gel electrophoresis and then transferred to membranes, where they are hybridized with a labelled probe for specific DNA fragments that are present in the bacterial genome. Microbial strains are thereafter discriminated based on the size and number of restriction fragments that are homologous to the probe. A variation of RFLP, which is also one of the most used nucleic acid hybridization based method to type pathogenic bacteria, is ribotyping. This technique, originally used to establish phylogenetic relationships [193], is based on the restriction enzyme digestion of chromosomal DNA followed by Southern hybridization with a probe specific to conserved regions of the rRNA coding sequence. The resulting banding patterns are analysed to sort isolates into ribotypes also establishing the relatedness of isolates [193]. Using an automated ribotyping system, the RiboPrinter® (Qualicon Inc., Wilmington, Del., U.S.), this assay has been shortened from five days to eight hours. It has been demonstrated that the discriminatory power of this technique can considerably increase by using multiple ribopatterns to determine the overall ribotype of isolates [194]. Ribotyping has been widely used to characterise milk- and dairy- borne isolates of *L. monocytogenes* [195-200], *S. aureus* [201,202], *E. coli* O157:H7 [203,204], *Salmonella* spp. [205] and *Campylobacter* spp. [206,207]. rDNA based fingerprints can be obtained also by ARDRA (Amplified rDNA restriction analysis). In this case, bacterial rRNA gene(s) are amplified by PCR and digested with restriction endonuclease enzyme(s). The resulting restriction fragments are then resolved by gel or capillary electrophoresis to obtain a fingerprint [208]. However, although ARDRA is faster and easier to perform, it has a lower discriminatory power than ribotyping, because smaller areas of the rRNA operon are targeted. Other methods are available to obtain restriction maps providing strain-specific fingerprints of pathogenic bacteria. PCR-RFLP, based on the amplification of a given gene or operon, coupled with the digestion with appropriate endonuclease enzymes and electrophoresis of the resulting restriction fragments, has been used to characterise milk- and dairy- borne *S. aureus* [84,100,209], *L. monocytogenes* [2010-212], *E. coli* O157:H7 [213], *Salmonella* spp. [214,215] and *Campylobacter* spp. [216,217] isolates.

Restriction endonuclease analysis of genomic DNA fragments separated by pulsed-field gel electrophoresis (REA-PFGE) has become the "gold standard" for molecular typing [187,218]. By this technique, intact genomic DNA, obtained by performing the DNA extraction and purification of microbial cells imbibed in low melting agarose, is digested by rare-cutting endonuclease enzymes. The resulting restriction fragments, usually fewer than 30 ranging in size between 20 and 600 kilobasepairs, are then separated in agarose gels by the periodic alternation of the angle of the electric's field direction through PFGE, thus obtaining banding patterns that can be compared for each isolate to discriminate the different pulsotypes. Despite its lower convenience criteria, as it is more laborious, time consuming and expensive (it requires skilled labour, specialised equipment and expensive restriction endonucleases) than the other molecular typing techniques to date available, REA-PFGE has superior performance criteria (mainly discriminatory power and reproducibility) due to both the quality and quantity of banding patterns obtainable, which in turn are due to the intrinsic nature of the REA-PFGE. PulseNet (www.cdc.gov/pulsnet), which is a network of health and food regulatory laboratories created by the the Center for Disease Control and

Prevention [1], uses PFGE for typing foodborne pathogens, allowing a better tracking and earlier detection of possible common source outbreaks [219,220]. As several similar initiatives have been developed in other countries (129 laboratories from 70 countries trained on PulseNet methods in Latin America, Canada, Europe, the Middle East, Asia Pacific) the PulseNet international (www.pulsenetinternational.org) has been developed. Networks like these rely upon standardization of REA-PFGE and regular quality assessment through appropriate ring trials for all participating laboratories, in order to warrant consistently comparable data.

Informative polymorphic banding patterns can be obtained by amplified fragment length polymorphism (AFLP) allowing the differentiation with a high discriminatory power (as it involves, like REA-PFGE, the whole genome) even of phylogenetically closely related bacteria without any prior information on their genomes [110, 221]. This multi-locus fingerprinting technique combines the reliability of RFLP analysis with the flexibility and robustness of PCR using restriction site/adaptor-specific primers under stringent conditions [110, 222]. Semi-automated versions of this technique may be obtained by the fluorescent AFLP (fAFLP) using capillary array systems, fluorescently labelled primers and adequate analysis software [223], which provide digitised and complex DNA fingerprints, covering nearly the whole genome [110]. AFLP has been used to type *Salmonella* spp., *E. coli* O157, *Campylobacter* spp., *S. aureus* and *L. monocytogenes* [203,223-228].

Typing of pathogenic microorganisms can be achieved by PCR based methods. Conventional and, more proficiently, real time PCR, either in simplex or (better) in multiplex format of enterotoxins' and/or other virulence factors' encoding genes may provide valuable information on the potential pathogenicity of a given isolate (paragraph 2.2 and 2.3). The same achievement can be reached by using the microarray technology (described above).

PCR amplification and sequencing analysis of (usually) seven house-keeping genes is the basics of the Multilocus Sequence Typing (MLST), which, being based on sequence data, is an unambiguous procedure to characterize isolates of bacterial species [229,230]. If virulence and virulence-associated genes are used this technique is referred to as Multi-Virulence Locus Sequence Typing (MVLST) [231]. Since MLST relies upon specific nucleotide base changes to type microorganisms, it is easiest to perform (it can be automated by using an automated pipetting platform and an automated sequencer) and analyse unlike other typing procedures which compare complex fingerprints rather than sequence data and allelic profiles. Of course, high quality sequences results are essential in this method. Being a highly reproducible and reliable technique, by which results, i.e. strings of digits representing different alleles, are easily and unequivocally exchangeable, as for REA-PFGE, a multilocus sequence network has been developed (www.mlst.net).

4. Conclusion

As it emerges from the reading of the present chapter, a plethora of nucleic acid based methods are to date available for the detection, identification and typing of milk- and dairy-

borne pathogenic microorganisms. However, none of these is perfect, harbouring wikenesses besides strenghts such as suboptimal reproducibility, sensitivity or discriminatory power. It can be inferred that the choice of the most appropriate method should rely upon the specific needs and, of course, on the available equipment for carrying out the task. Nucleic acid-based methods are gradually replacing traditional methods to identify, detect and type pathogenic microorganisms in milk and dairy products. Several efforts are being made to overcome limitations of these methods, mainly related to their sensitivity and accuracy besides the effectiveness in respect to the complex target food matrices, and achieve the validation and standardization of these approaches, which are basic requirements to become reference methods. As concerns identification and detection systems, the major perspective in the nearest future is the possibility to use portable miniaturized integrated devices allowing the rapid and reliable detection and quantification of pathogenic microorganisms directly from food. Such detection platform will decrease the risk of contaminating the food/feed/beverage supply, preventing a wide dissemination of contaminated foods and the possibility of a disease outbreak, as well as facilitate real-time preventive measures along the whole food/feed/beverage chains. On the other side, next generation sequencing and other user-friendly nucleic acid-based automated platform will provide promising way of achieving the genetic typing of milk- and dairy- borne pathogenic microorganisms.

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