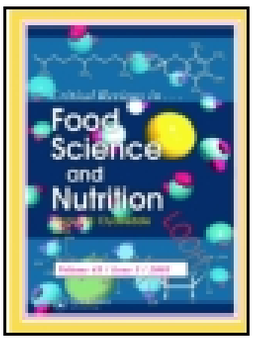

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Application of real-time PCR (qPCR) for characterization of microbial populations and type of milk in dairy food products

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ABSTRACT

Dairy foods represent an important sector of the food market for their nutritional qualities and their organoleptic characteristics, which are often linked to tradition and to region. These products are typically protected by labels such as PDO (Protected Designation of Origin) and PGI (Protected Geographical Indication). Real-time PCR (qPCR) is a fundamental tool in "Food Genomics;" a discipline concerned with the residual DNA in food, which, alongside traditional physical and chemical methods, is frequently used to determine product safety, quality and authenticity. Compared to conventional or "end-point" PCR, qPCR incorporates continuous monitoring of reaction progress, thereby enabling quantification of target DNA. This review describes qPCR applications to the analysis of microbiota, and to the identification of the animal species source of milk from which dairy products have been made. These are important aspects for ensuring safety and authenticity. The various applications of qPCR are discussed, as well as advantages and disadvantages in comparison with other analytical methods.

KEYWORDS

qPCR; dairy food products; authenticity; traceability; quality control

1. Introduction

The dairy food chain represents a significant section of the food market: world milk production in 2013 was 780 million tons and is progressively increasing. New Zealand and the EU were the two principal exporters in 2013, whereas Asia remained the main market for dairy products, accounting for some 55% of world import, followed by Africa with 15% (FAO 2013).

Milk and dairy products have remarkable nutritional benefit: they are sources of proteins, calcium, phosphorus, iodine, riboflavin and vitamins A and B12. A number of studies have shown that high consumption of milk and dairy products has protective effects against coronary heart disease, stroke, diabetes, colorectal and bladder cancers and dementia (Kliem and Givens 2011).

However, manufacture of dairy products involves hazards at all stages due to poor hygienic practices or deliberate fraud: contamination with pathogenic microorganisms can be cause of serious illness. The primary source of pathogen contamination is the farm: livestock species, their feed, and the environment are continuously incubators of microorganisms. Pathogenic species like *Campylobacter jejuni, Listeria monocytogenes, Salmonella* spp., *Yersinia enterocolitica*, and other microorganisms regularly involved in food poisoning, have been isolated from dairy-cattle faeces, bulk milk tanks, the dairy farm environment and animal feed (Papademas and Bintisis 2010). Contamination can occur also during post-farm activities, such as during milk storage, transportation and processing (Millogo, Svennersten Sjaunja, Ouédraogo, and Agenäs 2010). To prevent microbial contamination and improve the safety and quality of food products, the Scientific Committee on Veterinary Measures relating to Public Health (SCVPH), established by the European Commission, highlighted the importance of basing microbiological criteria on formal risk assessment and internationally approved principles. These criteria are described in the EC Regulation 2073/2005 (European Commission 2005) that established limits for microorganisms at specific points in the supply chains to improve hygienic conditions during manufacturing. For milk and dairy products, special attention was given to *Salmonella* and *Staphylococcus* for food safety and Enterobacteriaceae as an indicator of hygienic practices. In the United States, microbial standards for milk and milk products vary by state, but are based on guidance in the FDA Pasteurized Milk Ordinance (U.S. Dept. of Health and Human Services 2009). Specific information on the control of *Listeria monocytogenes* for ready-to-eat products are given in the United States Code of Federal Regulations (CFR) title 9 part 430 (U.S. Food and Drug Admin 2015).

In addition to safety, the quality of dairy products is an important issue: several cheeses are protected by labels introduced by the EU under Regulation 2081/1992 (European Commission 1992): Protected Designation of Origin (PDO), Protected Geographical Indication (PGI) and Traditional Specialties Guaranteed (TSG). This Regulation, enforced by EU

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Regulation 1151/2012 (European Commission 2012) has been gradually expanded internationally via bilateral agreements between EU and non-EU countries, ensuring that products genuinely originating in a specific region or produced according to traditional practice can be identified by the label. In the United States, there are foods that have some degree of protection for their regional designation, and for goods entering the US borders, the US Customs and Border Protection enforces NAFTA (North America Foreign Trade Agreement) Marking Rules. These are set forth in 19 CFR Part 102. Milk and dairy products are also subject to Standards of Identity for Milk and Cream, Cheese and Related Cheese Products, which are listed in CFR title 21, part 131–133 (United States Code of Federal Regulations) (U.S. Food and Drug Admin 2006).

The quality and safety of dairy products are strictly correlated: the milk composition determines the organoleptic properties of a dairy product, but can also be an important health issue. For example, many people are allergic to cows' milk. For these individuals, the consumption of milk from goats and sheep is advisable. However, cows' milk is usually less expensive and partial or total adulteration of goats and sheep milk with cows' milk is a common fraud (Fiocchi, Dahdah, Albarini, and Martelli 2015). Thus, operators and researchers working in the dairy field are involved in ensuring the maximum quality and safety of products, controlling both milk composition and microbial flora.

In 1988, the development of polymerase chain reaction (PCR) allowed the advent of "Food Genomics", the discipline concerned with analyzing the residual DNA of food to evaluate its composition, authenticity and safety. More recently, traditional end-point PCR has evolved into the more sophisticated qPCR that allows real-time continuous detection of DNA amplicons so that they are produced with precise quantification by means of continuous fluorescence detection. qPCR is based on three types of chemistry: i) double strand intercalating dye like SYBR Green (Ponchel et al. 2003); ii) hydrolysable fluorogenic probes (TaqMan probes) (Holland, Abramson, Watson, and Gelfand 1991) and iii) hybridization probes like FRET (Didenko 2001) probes and molecular beacons (Tyagi and Kramer 1996) (Figure 1).

Since 1988, many papers have been published describing the application of end-point and qPCR to food origin and composition. This paper is a critical review on the application of qPCR for assessing quality and safety of dairy foods through the detection and quantification of food borne pathogens, the

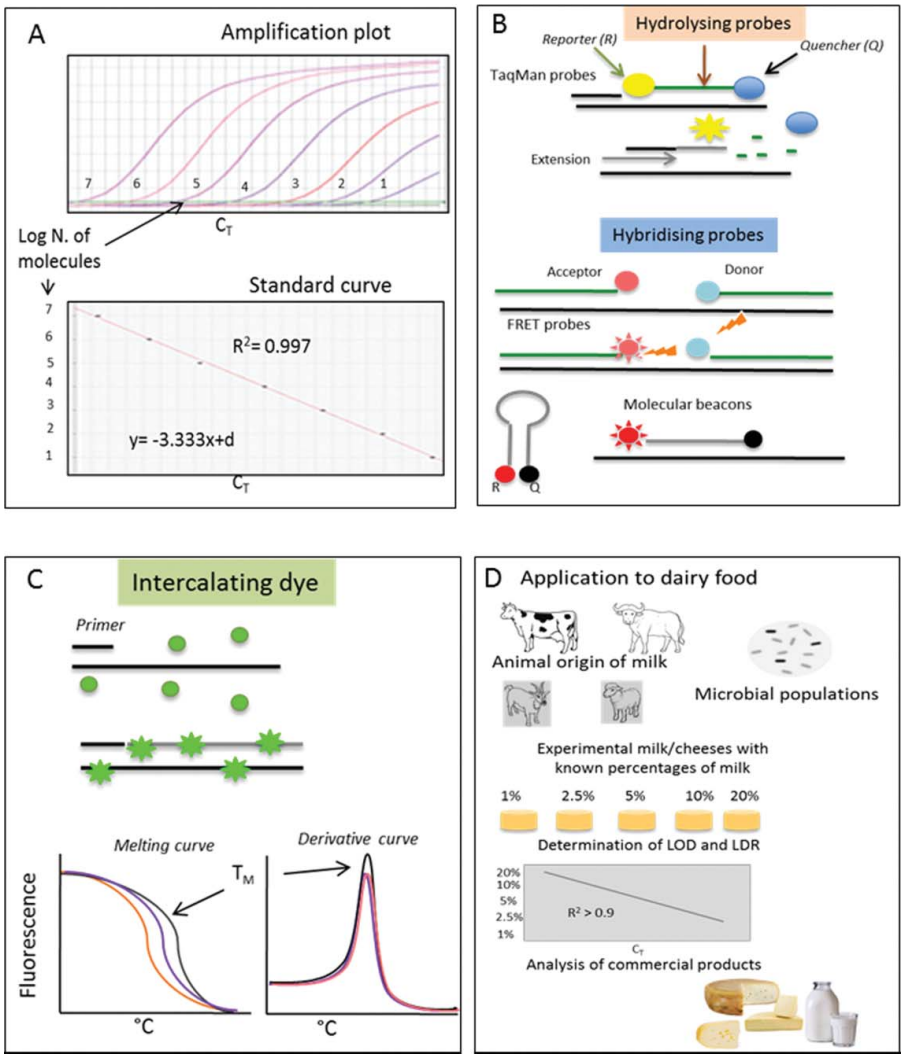

Figure 1. General features of qPCR and its applications to the analysis of dairy food products. C_T: threshold cycle.

characterization of microbial flora, and identification of the type of milk employed in manufacturing.

2. Characterization of microbiota in dairy products by means of qPCR

2.1. Detection of pathogen microorganisms and spoiling microorganisms

PCR-based techniques are routinely used to analyze pathogens, and to characterize the microbiota in fermented products such as cheese (Postollec et al. 2011); qPCR has made it possible, without the burden of long cultivation, to detect and quantify a wide variety of microorganisms. These include bacteria, fungi and viruses, with emphasis on the main foodborne pathogens (*Salmonella, Listeria monocytogenes, Escherichia coli* O157:H7, and *Staphylococcus aureus*) (Postollec et al. 2011). The technique has been used to detect *Enterobacter sakazakii* in infant formula and to quantify *Clostridium tyrobutyricum* spores in dairy products (López-Enríquez, Rodríguez-Lázaro, and Hernández 2007; Liu et al. 2006). Singh, Batish, and Grover (2012) developed a qPCR assay able to monitor the presence of *L. monocytogenes* and *Salmonella* spp. Indeed, pathogen contamination can be so low in certain dairy products that a pre-enrichment step is required to meet the microbiological criteria in national and international legislations for foodstuffs (Postollec et al. 2011). A few hours of pre-enrichment typically lowers the limit of detection (LOD) to <5 CFU/g for *Salmonella* and *Listeria monocytogenes* in milk and milk products (Perelle et al. 2004; Rossmanith, Krassnig, Wagner, and Hein 2006; O'Grady et al. 2008; Chen et al. 2010). A pre-enrichment step before qPCR can also permit the detection only of viable and cultivable microorganisms, as demonstrated by D'Urso et al. (2009), who counted only viable *L. monocytogenes* and *Salmonella* cells in yogurt. However, when an enrichment step is used, it is impossible to quantify the exact level of initial contamination. Thus, some authors have proposed alternatives to pre-enrichment for the detection and quantification of food pathogens. In fact, qPCR protocols without pre-enrichment steps are available for the detection of pathogens and other microorganisms in several food matrices, such as lettuce (Lee and Levin 2011), ground beef (Opet and Levine 2014) and fruit products (Wang et al. 2014). In dairy samples, the application of qPCR without pre-enrichment is described, in particular, for the detection of biogenic amine producing organisms, as discussed in the paragraph 2.2. Droplet digital PCR (ddPCR) was devised by Cremonesi et al. (2016) for simultaneous detection, without selective enrichment, of *Listeria* spp., *L. monocytogenes*, *Salmonella* spp., verocytotoxin-producing *E. coli* and *Campylobacter* spp. in cheese, using a single set of amplification conditions. ddPCR (a "third-generation PCR") provides absolute quantification of target DNAs without the need of a standard curve, and with a higher sensitivity than qPCR. A rapid qPCR assay combined with the use of sodium deoxycholate and propidium monoazide (PMA) was developed by Zhou et al. (2016) to detect viable *C. sakazakii* contamination in powdered infant formula, where the pathogens can be of particular hazard due to the specific consumer target of milk powder. A PMA-qPCR has been also developed to detect viable *Bacillus cereus* species in milk (Cattani et al. 2016). Only recently, Quigley et al. (2016) developed a qPCR-based method to quantify *Thermus* spp. This is not regarded as a typical cheese-associated genus, but it is responsible for the pink discoloration cheese defect.

As a risk assessment tool, qPCR has been used also to target specific genes which suggest the presence of coliforms in cheeses (Martin et al. 2010) and as an effective and sensitive screening tool for detecting *L. monocytogenes* in milk, cheese and other food samples (Kim et al. 2014).

Gore, Wakeman, Hull, and McKillip (2003) developed qPCR as nucleic acid sequence-based amplification (NASBA), based on molecular beacon, for assessing virulence and gene expression in three enterotoxigenic species: *B. amyloliquefaciens, B. cereus and B. circulans*, grown in artificially contaminated non-fat dried milk. Primers and a fluorescein-(FAM) labelled beacon are targeted to a 183 ribonucleotide-long segment within the *hblC* gene transcript of enterotoxigenic *Bacillus* spp encoding the L2 component of hemolysin BL (HBL). Maximal gene expression (for enterotoxin) was noted after 15 h of growth for *B. cereus*, and after 16 h for *B. amyloliquefaciens* and *B. circulans*, corresponding to a cell density of 5×10^7, 1.6×10^5 and 9.8×10^4 CFU/mL, respectively.

Thermophilic bacilli, such as *Anoxybacillus, Geobacillus* and *Bacillus*, are common sporogenic contaminants within processing milk powder producing factories. These bacteria are used as sanitary indicator in a production plant and specification limits of their number exist to ensure the milk powder quality. They are non-pathogenic bacteria, but beyond a certain limits can produce foul flavors and compromise product quality.

Rueckert, Ronimus, and Morgan (2005) have developed a method for the detection and enumeration of the total and viable vegetative cells and spore content of the thermophilic bacilli *Anoxybacillus flavithermus* in milk powder by TaqMan qPCR with DNase I so as to differentiate viable and non-viable cell. The method selectively enumerates the total and viable vegetative cell and total spore content of reconstituted milk within 90 min of sampling, by 16S rDNA qPCR. Quantification of thermophilic spores by qPCR of DNAs from the milk powders indicates on average the presence of spores 1.2 log units more than when measured by plate counting. The LOD of the method was 4.2×10^2 CFU/g of milk powder.

The same authors (Rueckert, Ronimus, and Morgan 2006) published a qPCR assay based on SYBR Green I for the enumeration of *Anoxybacillus, Geobacillus* and *Bacillus* sp. in milk powder by using degenerate oligonucleotide primers targeting small regions of 300 bp of the *spo0A* gene of *Bacillus* sp. This gene is present exclusively in endospore-forming bacteria as a unique sequence with only a single copy in the genome per bacteria. The assay showed linearity over a range of five orders of magnitude (from 10^7 to 10^3 CFU/mL) for vegetative cells of *A. flavithermus* strain C and *B. licheniformis* strain F when added to sterile reconstituted milk; the lower LOD was 8.0×10^1 CFU/mL. The results of the assay showed linearity when spores are in the range between 10^8 and 10^4/mL, with a LOD of approximately $6{,}4 \times 10^2$ spores/mL of milk.

A summary of the most recent applications of qPCR for the detection of pathogens and spoiling bacteria, also highlighting methodology and the genes targeted, is presented in Table 1.

Table 1. Some applications of qPCR in dairy microbiology. LOD: limit of detection; LDR: linear dynamic range; nd: not determined; RT-qPCR Reverse-Transcriptase qPCR.

Dairy matrix	Microorganism	Method	Target gene	LOD/LDR	Reference
		Pathogens or Spoilage microorganisms			
Spoiled yogurt, milk, cheese mousse	Yeasts, molds	RT-qPCR-SYBR Green	*act*	10^3 CFU/g	Bleve, Rizzotti, Dellaglio, and Torriani 2003
UHT milk	*Bacillus cereus* viable cells	PMA-qPCR-TaqMan	*HBL*	7.5×10^2 cfu/mL	Cattani et al. 2016
raw milk	*Salmonella* enterica	Enrichment + TaqMan-qPCR	*invA*	<3 CFU/25 g	Chen et al. 2010
Fluid milk, cheddar cheese and milk powder	*Bacillus licheniformis, Bacillus subtilis, Bacilluspumilus, Bacillus megaterium, Bacillus cereus, Geobacillus sp. Anoxybacillus flavithermus*	HRM	*V3- V6 16S rRNA*	Nd.	Chauhan et al. 2013
Soft cheeses	*Listeria* spp., *L. monocytogenes*, *Salmonella* spp., verocytotoxin-producing *E. coli*, *Campylobacterspp.*	ddPCR	*yccT, stx1, stx2, eae, prs, invA, inlA*	10^2 CFU/g	Cremonesi et al. 2016
Milk	*cos- and pac-type Streptococcus thermophilus phages*	TaqMan-qPCR	*orf1510, orf18*	nd	del Rio et al. 2008
Yogurt	*Salmonella enterica, Listeria monocytogene* viable cells	Filtration pretreatment + qPCR-SYBR Green	*inv, prfA*	10 bacterial cells/10 g	D'Urso et al. 2009
Artificially inoculated milk, curd, cheese, Natural cheeses	Histamine-producing lactic acid bacteria	qPCR-SYBR Green	*hdcA*	$2–4 \times 10^2$ CFU/mL	Fernandez et al. 2006
Milk	*Bacillus cereus, Listeria monocytogenes, Staphylococcus aureus*	HRM-qPCR	*gyrB, hly, nuc*	3.7×10^3 CFU/mL	Forghani et al. 2015
Non fat dried milk	*B. amyloliquefaciens, B. cereus and B. circulans*	qPCR-FAM beacon	*hblC*	nd	Gore, Wakeman, Hull, and McKillip 2003
Milk from grazing cows	*Staphylococcus aureus*	qPCR-SYBR Green	*16S rRNA*	nd	Hagi, Kobayashi, and Nomura 2010
Cheeses	*Staphylococcus aureus*	qPCR-SYBRGreen, TaqMan	*nuc*	20–300 CFU/2 g	Hein et al. 2001
Raw milk	*Staphylococcus aureus*	qPCR-SYBR Green	*nuc*	10 CFU/mL	Hein, Jørgensen, Loncarevic, and Wagner 2005
Milk, cheese	*Listeria monocytogenes*	qPCR-TaqMan	*iap*	7.2×10^4 CFU/mL	Kim et al. 2014
Cheese	*histamine-producing bacteria*	qPCR-SYBR Green	*hdc*	nd	Ladero, Linares, Fernandez, and Alvarez 2008
Cheese	*tyramine-producing bacteria*	qPCR-SYBR Green	*tdc*	10^2 CFU/g	Ladero, Fernandez, Cuesta, and Alvarez 2010
Cheese	*Putrescine-producing lactococci*	Multiplex-qPCR	*AGDI cluster*	7.78×10^1/g	Ladero et al. 2012
Infant formula	*Enterobacter sakazakii*	Enrichment + qPCR-TaqMan + qPCR-SYBR Green	*16S–23S rRNA ITS*	1.1 CFU/100 g	Liu et al. 2006
Raw milk, UHT whole milk	*Clostridium tyrobutyricum*	qPCR-TaqMan	*fla*	25 spores/25 mL	López-Enríquez, Rodríguez-Lázaro, and Hernández 2007
Milk	*bacteriophages of Lactobacillus delbrueckii*	qPCR-TaqMan	*mur*	10^5 PFU/ml	Martin et al. 2008
Dairy samples	Enterobacteriaceae	Enrichment + qPCR-SYBR Green	*lacZ*	1 cfu/mL	Martin et al. 2010
Milk and milk products	*Listeria monocytogenes*	Enrichment + Lightcycler-qPCR	*ssrA*	1e5 CFU/25 g	O'Grady et al. 2009
Raw milk	*Salmonella* spp.	Enrichment + Lightcycler-qPCR	*invA*	<5 cells/25 g	Perelle et al. 2004
Cream cheese, curd, milk powder,	Genera and species of spore-forming bacteria	Enrichment + multiparametric qPCR, TaqMan	*16S rRNA*+specific genes commercial biochip	≤ 1 spore/25 g *B. cereus*	Postollec et al. 2011
Cheese	*Thermus thermophilus*	qPCR-SYBR Green	*polymerase I*	nd	Quigley et al. 2016
Milk, fresh cheeses, ripened cheeses	*Listeria monocytogenes*	Enrichment-qPCR and RT-qPCR-TaqMan	IGS region between 16S and 23S rRNA genes	qPCR $10–10^3$ CFU/g, RT-qPCR 10^4e10^5 CFU/g	Rantsiou et al. 2008
Dairy products	*Listeria monocytogenes*	Enrichment + TaqMan-qPCR	*prfA*	1 CFU/15 g	Rossmanith, Krassnig, Wagner, and Hein 2006
Milk powder	*Anoxybacillus flavithermus*	TaqMan-qPCR	*16S rRNA*	$4,2 \times 10^2$ CFU/g	Rueckert, Ronimus, and Morgan 2005
Milk powder	*Anoxybacillus, Geobacillus and Bacillus sp.*	qPCR-SYBR Green	*spo0A*	80 CFU/mL 640 spores/mL	Rueckert, Ronimus, and Morgan 2006
Raw milk, pasteurized milk, ice cream	*Listeria monocytogenes, Salmonella*	Enrichment + duplex qPCR-SYBR Green/melting curve analysis	*hly, invA*	1 Log CFU/mL	Singh, Batish, and Grover 2012
Raw and pasteurized milk, icecream, soft cheese	*Escherichia coli* O157:H7	Enrichment + qPCR-Scorpion	*eae*	10^3 CFU/mL	Singh, Batish, and Grover 2009

Cheddar cheese	*lactoccocal bacteriophages*	qPCR-SYBR Green	orf6, capsid protein	1.4 phage/cm^2	Verreault et al. 2011
Infant formula	*Cronobacter sakazakii*	sodium deoxycholate-propidium monoazide-qPCR	*ITS*	4.4 × 10^2 CFU/g	Zhou et al. 2016
		Dairy interest microrganisms			
Hard coocked cheese during ripening	*Streptococcus thermophilus, Lactobacillus delbrueckii, and Lactobacillus helveticus*	qPCR-SYBR Green	*tuf*	nd	Achilleos and Berthier 2017
Natural whey starter	*Lactobacillus helveticus, Lactobacillus delbrueckii, Streptococcus thermophilus, Lactobacillus fermentum*	qPCR-SYBR Green	*pheS*	less 600 copies of DNA	Bottari et al. 2013
Emmental cheese	*Lactobacillus helveticus, Streptococcus thermophilus*	qPCR, RT-qPCR	*16S rRNA, tuf, groL*	2.6 × 10^3 – 1.2 × 10^3 copies/g	Falentin et al. 2012
Emmental cheese	*Propionibacterium freundenreichi, Lactobacillus paracasei*	qPCR, RT-qPCR-SYBR Green	*16S rRNA, tuf, groL*	qPCR: 1.3–2.6 × 10^3 genome copies/g, RT-qPCR: 2.1–4.3 × 10^3 copies/g	Falentin et al. 2012
Femented milk	*L. casei group, L. casei, L. paracasei, L. rhamnosus, S. thermophilus, L. acidophilus, L. delbrueckii, L. johnsonii*	qPCR-SYBR Green	*16S rRNA*	1.0 × 10^2 CFU/mL	Furet, Quénée, and Tailliez 2004
Commercial fermented milk, fresh cheese	*Streptococcus thermophilus, Lactobacillus delbrueckii, L. casei, L. paracasei, L. rhamnosus, L. acidophilus, L. johnsonii*	qPCR-SYBR Green	*16S rRNA*	5 × 10^2–4 × 10^3 CFU/ml	Furet, Quénée, and Tailliez 2004
Commercial probiotic product Simbiotic Drink	*Streptococcus thermophilus, Lactobacillus delbrueckii subsp. bulgaricus, Lactobacillus casei subsp. sasei, Lactobacillus acidophilus, Bifidobacterium lactis*	PMA-qPCR-SYBR Green	*16S rRNA*	1 × 10^3 CFU/mL	García-Cayuela, Tabasco, Peláez, and Requena 2009
Fermented milk	*Lactococcus cremoris*	qPCR-SYBR Green	*16S rRNA*	2 × 10^2 CFU/mL	Grattepanche, Lacroix, Audet, and Lapointe 2005
Yogurt	*Lact. acidophilus, Lact. brevis, Lact. delbrueckii subsp. bulgaricus, Lact. helveticus and Lact. reuteri*	qPCR-SYBR Green	*hsp60*	10^5 CFU/mL	Herbel et al. 2013
Milk, yogurt, commercial cheeses	*L. casei, L. paracasei, L. rhamnosus*	HRM	V1-V3 *16S* rRNA	nd	Iacumin et al. 2015
Dairy and probiotic products	*L. casei, L. paracasei, L. rhamnosus*	HRM	*groEL*	nd	Koirala et al. 2015
Camembert type cheese	*Penicillium camemberti, P. roqueforti*	qPCR-SYBR Green	ITS 1 region of rRNA, beta tubulin	0.25–4 mg mycelium/g	Le Dréan et al. 2010
Yogurt	*Lactobacillus delbrueckii* ssp. *Bulgaricus*, *Streptococcus thermophilus*	qPCR-SYBR Green	*CRISPR, mannitol-specific IIbC component gene*	10^4 CFU/mL	Miller, Dudley, and Roberts 2012
Cheese	*Corynebacterium casei*	qPCR-SYBR Green	*16S rRNA*	10^5 CFU/g	Monnet, Correia, Sarthou, and Irlinger 2006
Milk, cheese	*L. helveticus*	qPCR-SYBR Green	*pheS*	10 copies μL	Moser et al. 2017
Yoghurt	*Streptococcus thermophilus*	qPCR-TaqMan	*rimM*	10^2–10^3 CFU/mL	Ongol, Tanaka, Sone, and Asano 2009
Norvegian cheese	*Lc. lactis ssp. lactis*, *Lc. lactis ssp. cremoris*, *Leuconostoc mesenteroides*	HRM	V1-V3 *16S* rRNA	nd	Porcellato et al. 2012
Parmigiano Reggiano cheese	*L. casei, L. paracasei, L. rhamnosus*	HRM-qPCR	*spxB*	nd	Savo Sardaro et al. 2016
Ultrafiltration cheese, cheddar cheese, commercial cheeses	*Lactococcus lactis*	RT-qPCR-SYBR Green	*tuf, gapB, purM, cysK, ldh, cit, gyrA*	nd	Ulve et al. 2008
Artisanal cheeses	*Enterococcus gilvus*	qPCR-TaqMan	*pheS*	10^4 CFU/g	Zago, Bonvini, Carminati, and Giraffa 2009

2.2. Monitoring of useful microorganisms in dairy products

Lactic acid bacteria (LAB) are a composite family of bacteria responsible for milk fermentation which produces preservative agents and generates flavors in the products. Therefore their characterisation has been the subject of several studies using qPCR to follow their dynamic in dairy products. For example, Grattepanche, Lacroix, Audet, and Lapointe (2005) developed a qPCR protocol to quantify *Lactococcus (Lc) cremoris* ATCC 19257 in milk fermented by a mixed LAB culture; the qPCR employed SYBR Green I and primers 68FCa33/16SR308 that amplify a 33-nucleotide variable region of the *16S* rRNA gene of *Lc. cremori* ATCC 19257. The method allowed for the detection of concentrations as low as 2 CFU/reaction, with a LOD of about 2×10^2 CFU/mL of milk of *Lc cremoris* ATCC 19257.

Falentin et al. (2012) used qPCR and reverse transcription qPCR (RT-qPCR) targeting 16S rRNA to demonstrate the unexpected persistence of thermophilic LAB starters throughout the Emmental cheese ripening process. Herbel et al. (2013) and Miller, Dudley, and Roberts (2012) used qPCR to quantify probiotics and to monitor starter strains in yoghurt, similar to Furet, Quénée, and Tailliez (2004) and García-Cayuela, Tabasco, Peláez, and Requena (2009), who quantified LAB and bifidobacteria in fermented milks. Bottari et al. (2013) developed a multiplex qPCR to detect thermophilic LAB in natural whey starters, ending up with a test that was faster and more effective than other molecular methods (FISH and LH-PCR) and that could be used to follow the presence of thermophilic LAB species during ripening of dairy products. More recently, Moser et al. (2017) developed a qPCR method to detect and enumerate the thermophilic species *Lactobacillus helveticus* in milk and cheese during ripening by targeting the gene *pheS*, which encodes the alpha subunit of the phenyalanine-tRNA synthetase. Achilleos and Berthier (2017) compared qPCR to the culture-dependent plate counting method for absolute quantification of thermophilic starter LAB populations in dairy products, concluding that the use of both methods is advisable where plating can not provide a reliable quantification at species level. The combination of two methods is needed because each provides different information, being based on different principles and having different limitations.

LAB in food can be responsible for the production of biogenic amines, toxic substances that appear in foods and beverages as a result of amino acid decarboxylation. Consequently, histamine-producing LAB have been detected and quantified by qPCR in milk, curd and cheeses by Fernandez et al. (2006). A 174-bp fragment of the *hdcA* gene encoding histidine decarboxylase was used to determine the numbers of *Lactococcus* and *Enterococcus* histamine-producing bacteria. A positive correlation was observed between the presence or absence of histamine and the qPCR results; C_T values below 26 can be related to a histamine concentration higher than 500 mg/kg. This measure takes approximately 2 h for 96 samples. Also Ladero, Linares, Fernandez, and Alvarez (2008) evaluated the content of histamine and of histamine-producing bacteria in 80 different commercial cheeses made by different varieties of milk, different milk treatments, and different ripening periods, using HPLC and RT-qPCR, showing that results with the two methodologies correlate well. The gene *hdc* (histamine decarboxylase) was used to detect the presence of histamine-producing strains by RT-qPCR. C_T (threshold cycle) values were used to evaluate the levels of bacteria needed to produce high concentrations of histamine in dairy products: C_T below 28 have histamine concentrations greater than those recommended (100 mg/kg). Considering that tyramine is an abundant biogenic amine in fermented dairy products through the microbial decarboxylation of tyrosine, Ladero, Fernandez, Cuesta, and Alvarez (2010) developed a qPCR method that detects bacterial species involved in the process: *Enterococcus* sp, *Lactobacillus brevis* and *Lactobacillus curvatus*. The qPCR protocol used SYBR Green and primers targeting the *tdc* gene (tyrosine decarboxylase) during the manufacturing of a blue-veined cheese (Cabrales) at different stages during ripening, as well as in commercially available cheeses**.** Tyramine-producing bacteria from all three groups were detected by qPCR in all samples, though enterococci were always the prevalent group: 8.6×10^2 CFU/mL in the milk and their concentration increased to 2.4×10^4 CFU/g after the first month of ripening. *L. brevis* and *L. curvatus* showed concentrations below 10^2 CFU/g, which is outside the linear range for quantification.

Putrescine is another abundant biogenic amine in dairy products and is mainly produced through agmatine deaminase by LAB mostly belonging to *Enterococcus*, *Lactobacillus* and *Lactococcus lactis*. Ladero et al. (2012) developed a multiplex qPCR method based on the specific amplification of the agmantine deaminase cluster (AGDIc) that detected and quantified the three putrescine-producing taxonomic groups in twenty-nine commercially available cheeses from different parts of Europe. All groups were detected in all cheeses, though in different concentrations. Putrescine-producing lactococci were the most abundant group, with 7.78×10^1/g in industrial cow pasteurised semi-hard cheese to 1.92×10^8/g in the traditional raw blue veined soft cheese. Lactobacilli and enterococci were present at similar concentrations: from values below the linear range of quantification in some industrial cheeses to a maximum of 1.04×10^5/g in the traditional raw blue veined soft cheese.

Others cheese starter cultures have been monitored by qPCR. Mycelial dynamics during growth were studied by qPCR, targeting *Penicillium roqueforti* and *Penicillium camemberti* during cheese ripening (Le Dréan et al. 2010). Using DNA quantification they could measure fungal biomass from curd to ripened model cheese and Camembert-type cheeses. By RT-qPCR, Ulve et al. (2008) demonstrated metabolic activity of *Lactococcus* bacteria during Cheddar cheese ripening. They estimated the expression of seven genes involved in various metabolic activities, including glycolysis, purine metabolism and house-keeping genes, showing that lactococcal cells were still metabolically active in Cheddar cheeses after 6 months. Falentin et al. (2010) observed different behaviors, depending on time during Emmental cheese ripening, of the starter cultures *Lactobacillus paracasei* and *Propionibacterium freudenreichii*. By combining qPCR and RT-qPCR the authors were able to quantify and evaluate metabolic activities of the two species throughout Emmental cheese manufacturing and ripening. Minority microbial population were targeted using qPCR by Zago, Bonvini, Carminati, and Giraffa (2009), who detected specific sub-dominant populations of *Enterococcus gilvus*,

considered beneficial in cheeses, and also by Monnet, Correia, Sarthou, and Irlinger (2006) who studied the influence on cheeses of *Corynebacterium casei* populations.

2.3. Detection of bacteriophages

Infection of starters by bacteriophages is a significant problem in the dairy industry because phages slow the fermentation process and adversely impact product quality, resulting in delays and extra costs. Early phage detection in milk by qPCR is very useful and allows rapid decisions about the most suitable use of the milk when contaminated. A multiplex qPCR method was developed (del Rio et al. 2008) which makes use of TaqMan probes for quantitative detection and identification of *cos*- and *pac*-type *Streptococcus thermophilus* phages in milk. The limit of quantification (LOQ) was determined at 10^1 plaque forming units (PFU) per reaction.

To detect and quantify bacteriophages of *Lactobacillus delbrueckii*, Martin et al. (2008) performed a qPCR with TaqMan probes and primers specific to regions of the gene *mur*, encoding for the muramidase, which is conserved in all types of phages from this group. The LOD was 10^1 PFU per reaction, while the LOQ was proposed at 10^2 PFU per reaction which is equivalent to 10^5 PFU/mL of milk.

The environmental dissemination of phages (Verreault et al. 2011) was evaluated with qPCR measuring the presence of lactoccocal bacteriophages in the air of a dairy factory producing cheddar cheese with *Lactococcus lactis* strains as starter. The assay with qPCR was based on SYBR Green and primers specific to conserved regions of *orf6* for lactococcal phage group 936 and for the highly conserved major capsid protein for lactococcal phage group c2. Results from air samples showed that lactococcal phages of the two groups can be disseminated through the airborne route at levels up to 2.7×10^4 and 6.6×10^4 PFU/m^3 of air.

2.4. High Resolution Melting qPCR (HRM-qPCR)

Recently, several studies have reported the use of High Resolution Melting quantitative-PCR (HRM-qPCR) to monitor the dynamics of microbial growth and composition during fermentation and to investigate factors affecting microbial ecology in different substrates. HRM is a development of the PCR technique used to study DNA melting kinetics. With strict control of the temperature increase (sensitivity up to 0.01 °C/s), this approach monitors the fluorescence decrease of a double-stranded DNA-binding saturation dye. This phenomenon is represented normally as the first derivative of the melting curve, in which the melting temperature (T_M) and curve shape are influenced by amplicon length and GC content allowing the discrimination of even the smallest DNA variants, including single nucleotide polymorphisms (SNPs) and small insertions and deletions (INDELs) (Reed, Kent, and Wittwer 2007).

2.4.1. HRM-qPCR for detection of pathogenic microrganisms

HRM-qPCR methods are used for the identification of foodborne pathogens. Forghani et al. (2015) combined multiplex qPCR with HRM analysis for a specific simultaneous detection of *Bacillus cereus*, *Listeria monocytogenes* and *Staphylococcus aureus* in milk. The assay successfully detected *B. cereus* (*gyrB* gene), *L. monocytogenes* (*hly* gene) and *S. aureus* (*nuc* gene) in a single reaction; the average melting temperatures were 76.23 C°, 80.19 C° and 74.01°C, respectively. The sensitivities of the multiplex assay for detecting all three pathogens in milk were in the range 3.7×10^3 to 3.7×10^7 CFU/mL. Chauhan et al. (2013) analyzed 196 dairy mesophilic and thermophilic spore-forming bacteria isolated from raw and pasteurized fluid milk, cheddar cheese and milk powder samples. The authors developed an HRM analysis, using the V3 and V6 variable regions of gene 16S, providing a rapid approach to identifying the *Bacillus licheniformis* group, the *Bacillus subtilis* group, the *Bacillus pumilus* group, *Bacillus megaterium*, the *Bacillus cereus* group, *Geobacillus* species and *Anoxybacillus flavithermus* in dairy products.

2.4.2. HRM-qPCR for analysis of beneficial microorganisms

Porcellato et al. (2012) proposed one of the first applications of this method to characterize isolates of *Lactococcus (Lc) lactis ssp. lactis*, *Lc. lactis ssp. cremoris*, *Leuconostoc mesenteroides*, and different *Lactobacillus* species from Norwegian cheeses. The method determined the melting profiles of the 350bp V3 region of the amplified DGGE (denaturing gradient gel electrophoresis) bands of the 16S rRNA gene. Iacumin et al. (2015) extended this method to a wider collection of 201 strains belonging to the *L. casei* group (*L. casei, L. paracasei* and *L. rhamnosus*) proving its effectiveness in comparison with species-specific PCR for the discrimination of these very closely related species. Subsequently Koirala et al. (2015) and Savo Sardaro et al. (2016) extended the HRM-qPCR to target functional genes to monitor the active bacterial community involved in different stages of cheese ripening. Koirala et al. (2015) worked on the melting curve of a small hyper-variable region of the *Lactobacillus groEL* gene, which encodes a heat shock protein as a marker for genetic typing of the *L. casei* group, confirming the taxonomic assignment. Gene *groEL* codes for the 60-kDa group I chaperonin GrolEL which, because of its essential function, exhibits high sequence conservation across species (Goyal, Qamra, and Mande 2006). The *groEL* primers were designed for the conserved flanking region of the 110 bp sequence which has relatively high variability, obtaining a 150 bp PCR amplicon from *Lactobacillus* strains.

Savo Sardaro et al. (2016) discriminated the *Lactobacillus casei* group by targeting the functional gene *spxB*. This encodes for pyruvate oxidase (POX), an enzyme that catalyzes the oxidation of pyruvate to acetyl phosphate. HRM analysis performed on 74 *L. casei* group strains provided the ability to distinguish *L. casei*, *L. rhamnosus* and *L. paracasei* species according to melting temperature. These authors also monitored the active bacterial community involved in different stages of cheese ripening. The retro-transcribed RNAs extracted from 6- and 24-months' old Parmigiano Reggiano were analyzed by HRM-qPCR, allowing the identification of metabolically active *L. casei* species and their relative quantification.

A summary of the most recent applications of qPCR for detection of beneficial microorganisms for dairy production is presented in Table 1, including methodology and gene target.

3. Identification and quantification of milk animal species in dairy products

The identification and quantification of the species origin of milk in dairy have two objectives: to protect people allergic to cows' milk and to defend product integrity from fraud. To maintain the label PDO and PGI designations, many cheeses should be made from milks other than cows'. Some PDO cheeses may be prepared with mixes of different milks: one example is Greek Feta, which must be made with sheeps' milk containing a maximum of 30% goats' milk (Mauropoulos and Arvanitoyannis 1999). In this case, the determination of percentages of the different milks needs to be done to assess conformity with regulations and with the product label.

3.1. DNA extraction from dairy products

The extraction of DNA in a quantity and quality suitable for amplification is a crucial step for the application of qPCR (and PCR in general) for identification and quantification of the animal species used for milks in dairy products. While the extraction of DNA from milk is straightforward (Pirondini et al. 2010), the methods become more difficult in processed products because of thermal and/or physical treatment; salting and ripening considerably reduce the quantity and integrity of DNA. Additionally, mammalian DNA is less abundant than microbial DNA, which renders detection by PCR more difficult. Pirondini et al. (2010) tested methods based on commercial kits including Wizard Resin (Promega), QIAamp DNA stool (QIAGEN), Charge Switch Forensic DNA Purification Kit (Invitrogen) and in-house procedures; the authors concluded that utilization of cetyltrimethylammonium bromide (CTAB) gave the best results both in terms of DNA yield and its potential for amplification. Nevertheless, the Wizard clean up kit is the method most employed in papers published in this area (Table 2).

To optimize DNA extraction protocols, Rentsch et al. (2013) introduced the "DNA index", expressing the PCR efficiency "in DNA extracted from" in fresh and matured cheeses. Using this approach, the authors demonstrated a dramatic decrease of mammalian DNA (96%) in cheese as it progressed from curd to matured products.

3.2. Simplex (sxqPCR) and duplex (dxqPCR) methods

In general, papers describing the detection and quantification of animal species milk by qPCR follows this common scheme: *i*) test of methods on mixes made with specific percentages of cows', sheep, goats' and buffalo milk, or experimental cheeses made with the same mixes to determine the LOD, LOQ and linear dynamic range (LDR) or the interval in which quantification of milk species is linear; *ii*) applying the method to test commercial products. A summary of applications of qPCR to analyse dairy products is presented in Table 3.

Table 2. Methods employed to extract DNA from dairy food products.

Method of extraction	Dairy matrix	Reference
Guanidinium isothiocyanate followed by purification with Silica Paramagnetic Particles	Fresh milk, condensed milk, cheese	Plath, Krause, and Einspanier 1997
Phenol/chloroform extraction followed by purification with Wizard® Genomic DNA Purification Kit Promega, Madison, WI, USA	Cheese	Branciari et al. 2000
DNeasy Blood and Tissue Kit QIAGEN GmbH, Hilden, Germany	Cheeses	Maudet and Taberlet 2001
EDTA/SDS method	Cheese	Rea et al. 2001
Phenol/chloroform method	Milk	Bania, Ugorski, Polanowski, and Adamczyk 2001
	Governing liquid of Mozzarella	Feligini et al. 2005
	Milk, cheese, yogurt, cream, butter	Gonçalves, Pereira, Amorim, and van Asch 2012
DNeasyTM Tissue Kit QIAGEN	Cheese	Bottero et al. 2003
Wizard DNA cleanup kit Promega	Milk, cheese	Lopez-Calleja et al. 2004 Lopez-Calleja et al. 2005a, 2005b
Guanidium hydrochloride method	Cheese	Mafra, Ferreira, Faria, and Oliveira 2004 Mafra, Roxo, Ferreira, and Oliveira 2007
Isolation kit Invisorb Spin Food I Invitek, Co., Berlin, Germany	Cheese	Maskova and Paulickova 2006
Phenol/chloroform extraction followed by purification with Nucleospin Food kit purification Macherey-Nagel, Duren, Germany	Cheese	Feligini et al. 2007
Invisorb Cell Mini Kit Invitek, Berlin, Germany	Milk, cheese	Lopparelli et al. 2007
Wizard Plus Miniprep DNA purification system Promega	Cheese Milk	Lopez-Calleja et al. 2007a Lopez-Calleja et al. 2007b Rentsch et al. 2013
Wizard Resin® Promega QIAampDNA Stool® Mini Kit QIAGEN Charge Switch Forensic® DNA Purification Kit Invitrogen, Carlsbad, CA, USA, Nucleo Spin® Food Macherey–Nagel, CTAB based method, SDS based method, Tween based method	Milk,cheese, butter, cream, yoghurt	Pirondini et al. 2010
DNeasy Blood and Tissue Kit QIAGEN	Milk Cheese	Dalmasso, Civera, La Neve, and Bottero 2011 Ganopoulos et al. 2013 Golinelli et al. 2014
DNeasyTM Tissue Kit QIAGEN	Cheese	Mayer, Bürger, and Kaar 2012
CTAB based method	Milk Milk, cheese, yogurt, cream, butter	Cottenet, Blancpain, and Golay 2011 Agrimonti, Pirondini, Marmiroli, and Marmiroli 2015

Table 3. Summary of qPCR methods for identification of animal origin of dairy products. sxqPCR: simplex qPCR; dxqPCR: duplex qPCR, txqPCR: triplex real time PCR; qxqPCR: quadruplex qPCR; LOD: limit of detection; LDR: linear dynamic range.

Dairy matrix	Method	Target sequence	Species considered	LOD/LDR	References
Raw and pasteurized goat/sheep milks	sxqPCR TaqMan	Mitochondrial 12S rRNA genes	Goat Sheep	0.5% goat milk 0.6–10% goat milk	Lopez-Calleja et al. 2007a
Raw and pasteurized cow/sheep milks	sxqPCR TaqMan	Mitochondrial 12S rRNA genes	Cow Sheep	0.5% cow milk 0.5–10% cow milk	Lopez-Calleja et al. 2007b
Mozzarella cheese	sxqPCR SYBR Green/ TaqMan	mitochondrial cyt *b* nuclear growth hormone GH	Cow Buffalo	0.1% cow milk 0.6–20% cow milk	Lopparelli et al. 2007
Cow/ buffalo milk mixes Commercial cheeses	dxqPCR TaqMan	Mitochondrial cyt *b*	Cow Buffalo	2% cow milk	Dalmasso, Civera, La Neve, and Bottero 2011
Milk	dxqPCR TaqMan	Mitochondrial cyt *b*	Cow Buffalo	0.01% cow and buffalo milk 0.01–100% cow and buffalo milk	Cottenet, Blancpain, and Golay 2011
Experimental cow/sheep/goat cheeses Commercial cheeses	qxqPCR TaqMan txqPCR TaqMan	Mitochondrial genes: tRNA Lys cow; cyt *b* sheep, goat, buffalo. Nulclear genes: β-actin gene cow; prolactine receptor gene sheep, specific insertion of a LINE-1 element in non coding region of GH goat	Cow Sheep Goat Buffalo	0.32% of DNA of each species 0.32–32% of DNA of each species	Rentsch et al. 2013
Liquid of governing of Mozzarella cheese	qPCR SYBR Green	Mitochondrial COI	Cow Buffalo	0.5% cow milk 0.5–30% cow milk	Feligini et al. 2007
Binary milk mixture: cow/sheep, cow/goat, cow/buffalo Experimental cheeses made with binary milk mixture above. Commercial products cheeses, yoghurt, cream, butter	qxqPCR SYBR Green	Mitochondrial 12S rRNA Mitochondrial cyt *b*	Cow Sheep Goat Buffalo	0.1% cow milk Milks: variable between 0.5–10% to 1–25% cow milk Cheeses: variable between 0.1–5% to 1–10% cow	Agrimonti, Pirondini, Marmiroli, and Marmiroli 2015
Commercial Feta cheeses	HRM-qPCR	Bovine D-loop Caprine D-loop Sheep $tRNA^{Lys}$	Cow Sheep Goat	0.1% cow milk C_T: 0.1–20% Fluorescence: 1–50%	Ganopoulos et al. 2013

Almost all papers which describe qPCR are conducted with primers designed on mitochondrial DNA because it is present in many copies per cell and therefore it is more easily amplifiable than nuclear DNA: the most common targets are the D-loop region, the gene for cytochrome b (*cyt b*) and the 12S gene (Table 3).

The first papers on the detection and quantification of animal species with qPCR in dairy products were published in 2007: they reported the use of TaqMan probes to quantify goats' (Lopez-Calleja et al. 2007a) or cows' (Lopez-Calleja et al. 2007b) milk in sheep milk using the 12S rRNA sequence as target. This system showed a LDR for raw and pasteurised goats' milks of 0.6%–10%, and of raw and pasteurised cows' milk of 0.5%–10%, with a coefficient of regression (R2) > 0.9 in all cases, and with a reported decrease in DNA levels in pasteurised milk, probably due to heat treatment.

Mozzarella is a typical Italian cheese certificated as PDO if produced exclusively from buffalo (*Bubalus bubalis*) milk. Mozzarella cheese may be made with bovine milk or bovine/buffalo milk mixtures, but then it cannot be sold as PDO. However, traces of cows' milk are detected very frequently in PDO Mozzarella and the substitution of buffalo with cows' milk is one of the most common frauds in the dairy sector (Dalvit, De Marchi, and Cassandro 2007). Detection of cows' milk below 1% in Mozzarella is not necessarily an index of deliberate fraud but rather of likely accidental contamination.

qPCR with SYBR Green was used to detect and quantify bovine DNA in the governing liquids of experimental Mozzarella cheeses made from fixed ratios of cow and buffalo milks using a DNA barcode system (Hebert, Cywinska, Ball, and deWaard 2003) based on cytochrome oxidase subunit 1 (COI). The method showed good sensitivity (LOD: 0.5% for cows' milk) but the relationship between cycle threshold ($C_{T)}$) and percentage of cows' milk in governing liquid was not linear and thus quantitation was not reliable (Feligini et al. 2007).

Attempts to quantify cows' milk directly in Mozzarella cheese were conducted by Lopparelli et al. (2007) comparing SYBR Green and TaqMan chemistry; as endogenous control, the sequence of the nuclear gene for growth hormone (GH), common to buffalo and cows were used, whereas specific primers and probes for cow' was designed for the mitochondrial gene for cytochrome b (mt *cytb*). The two methods gave comparable efficiency and LDR on DNA extracted from milk, but TaqMan probes significantly extended the LDR (0.6–20% of cows' milk in cheese analysis), and therefore was preferred for analysis of commercial PDO and other Mozzarella cheeses. Cows' milk was detected in 79.7% of samples analysed and was quantified in 37.5% of them, finding levels of contamination varying from 0.6 to 20%. Identification of cows' milk in Mozzarella cheese was also conducted using SNPs in the sequence of the mitochondrial gene *cytb* (Dalmasso, Civera, La Neve, and Bottero 2011). The method used one pair of primers designed for sequences of this gene which are conserved in cows and buffalo; two specific probes differing in a single base permitted an LOD as low as 2% of cows' milk. This method, when applied to 52 commercial Mozzarella cheeses, confirmed the label information for 46, while four samples labelled as "pure buffalo" showed the presence of cows' milk and, in the other two, only cows' milk was detected.

Feta is a soft white cheese ripened in brine and traditionally produced in Greece. With Regulation No 1829/2002 (European Commission 2002), the EU established that only cheeses

produced in the mainland of Greece or the island of Lesbos, and made with pure sheep milk or mixed with no more than 30% of goats' milk, may be labelled as Feta. Cheeses similar, but produced in other regions or countries of the eastern Mediterranean Sea, often made partly or wholly with cows' milk cannot be labelled as Feta.

3.3. HRM-qPCR

HRM was used for qualitative detection of bovine milk in commercial samples of Feta (PDO), samples of sheep-goats' cheeses (non PDO) and one sample allegedly made only with goats' milk (Ganopoulos et al. 2013). Targeting the mitochondrial bovine and caprine D-loop sequences and the ovine gene for tRNA-leu, the assay had an LOD of 0.1% of bovine milk, thus confirming the labelling of all the commercial cheeses except for one which showed small traces of bovine milk. To quantify goats' milk in Feta cheeses, the same authors exploited the relationships between emitted fluorescence after dissociation of amplicons and their quantity, as previously reported by Mader, Ruzicka, Schmiderer, and Novak (2011). HRM conducted on ovine cheeses which contained increasing amounts of goats' milk allowed the development of a standard curve with a high correlation ($R^2 = 0.945$) between emission of fluorescence and the goats' milk content. This relationship was exploited in eight commercial and two reference Feta cheeses (100% sheep milk and 70%–30% sheep-goats' milk), and three sheep-goats' cheeses. Dissociation profiles of amplicons show that only one Feta cheese contained >30% of goats' milk.

3.4. Multiplex qPCR

Over the last years, qPCR has evolved from sxqPCR, with a unique target and one pair of primers, to multiplex (mxqPCR), with two or more targets and primer pairs, thus opening the analysis to a higher number of samples with a considerable saving of time and cost.

Rentsch et al. (2013) experimented with two mxqPCR qPCR methods, based on TaqMan probes, to quantify cows', sheep and goats' milk in cheeses. The two methods were based on: *i*) the mitochondrial target sequences bovine gene tRNA-lys and caprine, ovine and buffalo *ctyb* (method I), and *ii)* nuclear target genes: bovine beta actin, ovine prolactin receptor and a caprine specific insertion of a LINE-1 element in the 5′-noncoding region of the growth hormone receptor gene (Method II). The performance of the two methods was exhaustively evaluated in a ring trial involving eleven laboratories which quantified the proportions of cows', sheep and goats' milk in two fresh and two ripened cheeses. The collaborative trial was conducted with three standard DNAs: one extracted from meat and two derived from fresh (CFC) and ripened (CTS) cheeses. Method II, using meat DNA as standard, underestimated cows' milk; the authors suggested this was due to the reduced amounts in milk of cows' somatic cells compared to goats and sheep, the estimation of which were closer to the expected proportions. The utilization of matrix-adapted standard DNA (MAS-DNA) significantly improved the results for cows' milk but had only small effects on estimation of goats' and sheep milk proportions, both in fresh and in ripened cheeses. Method I overestimated cows' milk in all types of cheese with all the three standard DNAs, but MAS-DNA allowed a more accurate estimation of goats' and sheep milk proportions.

Method I, based on meat and MAS-DNA, was used for detection of cows' milk in 34 market cheeses, declared as made with goats', sheep and buffalo milk, and for quantification of milk from all these species. Both types of standard DNA allowed confirmation of the same number of cheeses as conforming with labeling; however with MAS-DNA a greater number of samples containing >10% of undeclared goats' milk was detected. Because the cheese composition was unknown, the authors could not assess which method was more accurate.

Cottenet, Blancpain, and Golay (2011) employed a duplex qPCR (dxqPCR) targeting the gene *cytb* to assess the level of cross-contamination between cows' and buffalo milks in different locations of India, Pakistan and China. The authors examined 79 cows' and 39 buffalo milks and discovered the highest level of cross contamination was in Indian milks, where contaminant levels of cows' or buffalo milks were > 20%. No cross contamination was found in cows' milk from China, where buffalo breeding is limited, or in Pakistan buffalo milks.

A quadruplex qPCR (qxqPCR) based on SYBR Green has been described by Agrimonti, Pirondini, Marmiroli, and Marmiroli (2015) to discriminate cows', goats', sheep and buffalo DNA in milk mixes and cheeses. Specific primers for the four species were designed on genes for ribosomal RNA 12S and *cytb*. This method was tested on binary mixes of cows' milk combined with milk from each of the other species and also tested on cheeses produced with the same mixes, giving an LOD of 0.1% of cows' milk in all matrices considered. The method was applied to test the composition of 26 commercial dairy products, comparing the results with those obtained with sxqPCR. Almost equal results were obtained (80%) with qx and sxPCR, while sxqPCR indicated that a higher percentage of products conformed to label statements compared with qxqPCR (77% vs 61%), which failed to detect some varieties of milk.

In the same paper, the authors explored the possibility of quantifying cows' milk in milk mixes and cheeses with qxqPCR, exploiting a possible relationship between the percentages of cows' milk and peak area under the derivative of the dissociation curve of the relative amplicon. For milk mixes, the results were highly satisfactory, with $R^2 > 0.9$ over a wide range of cows' milk percentages (0.5–10% or 1–25%); while for cheeses R^2 was > 0.9 in the only test reported (Agrimonti, Pirondini, Marmiroli, and Marmiroli 2015).

4. qPCR vs other methods

"Food genomics" based on the analysis of residual DNA of raw materials in foods supported by the emerging "omic" tools is currently considered the most innovative option to complement and enforce the concept of dairy food quality and safety but routine use of these methods is still difficult due to costs and the technical expertise required (Yeung 2012). For these reasons, conventional molecular methods are often required, and qPCR can be the good candidate.

In the previous paragraphs we have described the application of qPCR to identify the species origin of dairy products

and to characterize microbial population of milks, cheeses and other products. The application of qPCR is limited to these two important issues but it is not used to identify the geographical origin of products, type of processing, or addition of chemical adulterant such as melamine. These problems require application of sophisticated physico-chemical methods which are reported in the literature and have been reviewed exhaustively by Kamal and Karoui (2015).

4.1. Analysis of dairy microbiota

The utilization of qPCR and culture-independent techniques to characterize food microbiota has been dramatically increasing to overcome the limitations of traditional culture-based methods, such as long wait times to results, incapacity of discovering non-cultivable bacteria species and viable but non-cultivable (VBNC) cells (Sohier et al. 2014). Because of these limits, traditional microbiological methods provide a limited description of the food microbiota and a very simplistic, often biased, view of the physiological state of microbial populations in which several subpopulations characterized by various levels of "viability" and metabolic activity may coexist (Davey 2011).

On the other hand, qPCR may detect residual bacterial DNA from dead cells, which would cause false positive results that lead to unnecessary product recalls and economic losses (Wang and Levin 2006; Pathak et al. 2012), or in an overestimation of microbial load. Several methods have been developed to distinguish dead and viable cells, mostly based on treatment with PMA or ethidium monoazide. These dyes can permeate the membranes of dead cells and covalently bind to genomic DNA making it non-amplifiable (Fittipaldi, Nocker, and Codony 2012; Soejima, Minami, and Iwatsuki 2012). However, these methods are not always able to completely reduce PCR signalling due to dead cells and efforts should be made to improve functionality. (Seinige, Krischek, Klein, and Kehrenberg 2014). An alternative approach to distinguish dead cells is the quantification of mRNA, but this is hampered by the low abundance of the transcripts, less than 10%, in the total amount of RNA (Schmieder, Lim, and Edwards 2012).

In addition to qPCR, other culture independent methods include: denaturing gradient gel electrophoresis (DGGE) (Muyzer, de Waal, and Uitterlinden 1993), temporal temperature gradient gel electrophoresis (TTGE) (Yoshino, Nishigaki, and Husimi 1991), single strand conformation polymorphism (SSCP) (Dong and Zhu 2005) and analysis of *16S* gene libraries (von Wintzingerode, Göbel, and Stackebrandt 1997). As compared with qPCR, these methods allow for a more extensive characterization of food microbiota, whereas only DGGE is semi-quantitative. Moreover, resolution with gel electrophoresis is often unsatisfactory, which may cause an over or under estimation of the microbial diversity in the food samples (Quigley et al. 2011).

The knowledge of food microbiota has dramatically increased in recent years through development of next generation sequencing (NGS), which can produce thousands or even millions of sequences from an environmental sample. NGS has provided exhaustive databases of microbial taxa present in food samples, as well a robust inventory of microbial genes regulated under different environmental conditions (Mayo et al. 2014). Application of NGS to dairy food led to the identification of dominant microbial populations in milks and cheeses, as well as those pathogenic species which derive from cow infections (Mayo et al. 2014). An exhaustive "dairy microbial genome" was constructed by Almeida et al. (2014) that includes 137 species and 67 genera of bacteria, with 117 of them having draft genomes.

Though qPCR can not compete in terms of data collection with NGS, the two approaches are complementary: high throughput methods such as NGS provides an extensive overview of food microbiota but with only relative information on the abundance of each species, whereas qPCR compensates for this with precise quantification, though at low throughput. Moreover, though the costs for NGS have been considerably reduced, the enormous amount of data generated requires high bioinformatics skills and algorithms to effectively analyze the data (Muir et al. 2016). The choice between a shotgun (NGS) or a targeted (qPCR) approach depends mostly on the practical needs of researchers or of stakeholders.

Though not comparable with NGS or DGGE, the informative capacity of qPCR can be significantly increased in the mxqPCR configuration, which targets more sequences simultaneously (Bottari et al. 2013). When *16S* RNA is targeted via cDNA quantification, RT-qPCR allows quantification of any sub-dominant populations due to the abundance of ribosome transcripts as compared to ribosome gene copies. It also allows monitoring of the metabolic activity of bacterial communities in foods (Nakayama et al. 2007). RT-qPCR is complementary to other molecular approaches such as microarray (expression array) and RNA sequencing (RNA seq). Expression array allows an extensive analysis of the transcriptome of a certain organism, but it is only considered as semiquantitative; therefore, the data generated by microarray usually need to be validated by RT-qPCR. An example of a microarray coupled with RT-qPCR is the work of Liu and Ream (2008) applied to the transcriptome of *L. monocytogenes* during growth in ultrahigh temperature processed skim milk. RNAseq is a NGS platform (Wang, Gerstein, and Snyder 2009) with the same considerations: it may provide an extensive picture of the transcriptome, but skills and costs needed are not always justified or available in a routine practice.

4.2. Analysis of milk animal species

Methods alternative to qPCR that have been used to characterise the animal origin of milks are summarised in Table 4. A scored comparison of qPCR vs other methods is shown in Table 5, based on parameters including LOD, costs, time of execution, use of toxic reagents, personnel training and the possibility of result visualization "in the field" (off/on line).

The official method of European Commission established by EC Regulation No 273/2008 (European Commission 2008) is aimed at the detection of bovine γ globulin by isoelectric focusing (Table 4). This method has an LOD below the limit fixed by the EU for dairy products made with milks other than cows' milk, but it is laborious, requires trained personnel and makes use of toxic acrylamide (Table 5).

Immunological methods have been proposed by some authors (Addeo et al. 2009; Hurley, Coleman, Ireland, and Williams 2004; Hurley, Coleman, Ireland, and Williams 2006)

Table 4. Methods other than qPCR for determination of animal and plant species in dairy food products.

Differentiation of animal milks				
Scope	Dairy matrix	Method	Results	Reference
		Immunology		
Differentiation of cow milk from goat, buffalo and sheep milk	Milk mixes	Detection of bovine IgG by indirect Competitive enzyme-linked immunosorbent assay ELISA	Detection limits of the assay are 1.0 μg/mL of bovine IgG, or 0.1% vol/vol adulteration with cows' milk	Hurley, Coleman, Ireland, and Williams 2004
Differentiation of cow milk from goat, buffalo and sheep milk	Milk mixtures, cheeses made with the same mixtures	Detection of bovine IgG by sandwich ELISA	Detection limits in milk were 0.001% cows' milk adulteration of sheep or buffalo milk, and 0.01% cows' milk adulteration of goat milk. Detection limits in soft cheese were 0.001% in goat cheese and 0.01% in sheep or buffalo cheese	Hurley, Coleman, Ireland, and Williams 2006
Differentiation between cow and buffalo milk	Buffalo milk and Mozzarella	Immunisoelectric focusing of ã-caseins after plasminolysis.	LOD: 0.25% of cow milk. LDR 0.25–10% of cow milk	Addeo et al. 2009
		Electrophoresis		
Differentiation between cow, buffalo, sheep and goat milk	Milk mixtures, cheeses	Isoelectric focusing of γ-caseins after plasminolysis.	LOD: 0.5% of cow milk	European Commission 2008
Differentiation between cow and goat milk	Milk mixtures and cheeses.	Identification of specific β-lactoglobulin and α-lactalbumin by capillary electrophoresis.	LOD: 2% of cow milk in mixtures and 4% in cheeses.	Cartoni, Coccioli, Jasionowska, and Masci 1999
		Determination of physicochemical properties		
Differentiation between cow and goat milk	Fermented dairy beverages	Comparison of fats, total solids and proteins.	Fats resulted higher in goat milk beverages Total solids and proteins resulted higher in cow milk beverages	Gomes et al. 2013
Differentiation between sheeep and cow milk	PDO polish cheeses: – Oscypek cheese made with sheep and cow milk; – Gazdowski cheese made with cow milk	Determination of total solids and proteins in dry matter: higher in Oscypek cheese	Total solids and proteins resulted higher in Oscypek cheese.	Kędzierska-Matysek et al. 2014
		Chromatography/Mass spectrometry		
Differentiation between cow and buffalo and sheep milk	Milk mixtures	Characterization of β-lactoglobulin and α-lactalbumin by matrix-assisted laser desorption/ionization time-of flight mass spectrometry MALDI-TOF MS.	LOD: 10% of cow milk in sheep milk and 5% in buffalo milk.	Cozzolino et al. 2001
Differentiation between cow and buffalo and sheep milk	Mozzarella and Pecorino cheeses	Characterization of â-lactoglobulin and α-lactalbumin by MALDI-TOF MS.	LOD: 2% of sheep milk in Mozzarella cheese; 10% of cow milk in Pecorino cheese	Cozzolino, Passalacqua, Salemi, and Garozzo 2002
Differentiation between cow and buffalo milk	Milk mixtures, cheeses, governing liquid	Identification of specific β-lactoglobulin by high-performance liquid chromatography HPLC.	LOD: $<$ 0.5% of cow milk	Enne et al. 2005
Differentiation between cow and sheep milk	Cheeses produced with cow and sheep milk	Characterization of casein peptides by liquid chromatographyLC/MS	LOD: $<$ 1% of cow milk	Sforza et al. 2008
Differentiation and quantitation of cow and buffalo milk	Buffalo milk and Mozzarella cheeses	Characterisation of â-lactoglobulin by LC-MS	Rapid detection of cow milk, quantitation only aproximative	Czerwenka, Műller, and Lindner 2010
Differentiation between sheep, goat and cow milk.	Cheeses made with ovine, bovine and caprine milks	Determination of chromatogaphic profile of bovine, ovine and caprine proteins by HPLC	LOD: 3.92, 2.81 and 1,47% of sheep, goat and cow milk respectively	Rodríguez, Ortiz, Sarabia, and Gredilla 2010
Differentiation between cow and buffalo milk	Cow and buffalo mozzarella	Identification of specific buffalo and cow peptide derived from y-casein by liquid chromatography accurate mass quadrupole time-of-flight mass spectrometry with electrospray ionization LC-ESIQTOF MS/MS.	Identification of bovine marker in a linear dinamic range 0.001–10% of bovine milk	Russo et al. 2012
Differentiation between bovine and caprine milk	Cheese made with bovine caprine milk	Analysis of fatty acid profiles by GC	Cheeses containing goat milk have: higher content of short- and medium-chain fatty acids, and long chain polyunsaturated fatty acids -lower content of long-chain of fatty acid	Queiroga et al. 2013

		Sensory evaluation		
Differentiation between bovine and ovine milk	Bovine and ovine cheese	Sensory evaluation.	Ovine cheese has colour and harness higher than bovine cheese	Lteif, Olabi, Kebbe Baghdadi, and Toufeili 2009
Differentiation between cow and goat milk	Fermented beverages made with cow and goat milk and mixtures of the two.	Sensory evaluation.	No difference were found	Gomes et al. 2013
Adulteration of goat cheese with cow milk	Goat cheese	Sensory evaluation.	LOD: 10% of cow milk	Golinelli et al. 2014
		Spectroscopy		
Differentiation between sheep genotypes Comisana and Sicilo-Sarde	Milk	Front face fluorescence spectroscopy: emission spectra of aromatic aminoacids and nucleic acids	Good discrimination between milks produced from Comisana and those collected from Sicilo-Sarde sheeps	Zaïdi et al. 2008
Differentiation and quantitation of cow, goat and sheep milk	Milk and milk mixtures	Fourier transform infrared spectroscopy	Accurate detection and quantification of adulteration of sheep, goat, and cow milks at percentages > 8%	Nicolaou, Xu, and Goodacre 2010

Identification of plant adulterants				
Scope	Dairy product	Method	Results	Reference
		Chromatography/Mass spectrometry		
Adulteration with soy and pea proteins	Skimmed milk powder	Determination of protein patterns by LC-MS	Unequivocal identification of vegetable proteins added to milk at 5%	Cordewener et al. 2009
Identification of non milk fats fish, peanut, corn, olive and soy oils	Milk	Gas Chromatography GC	Correct classification of 94 samples with 10% of adulteration.	Gutierrez et al. 2009
Adulteration with soy milk	Milk powder	Detection of the soybean sugar stachyose by HPLC	LOD: 5% of soy milk	Sharma, Rajput, Poonam Dogra, and Tomar 2009
Adulteration with soy, pea, brown rice and hydrolized wheat protein	Milk powder	Classification of milk proteins by ultrahigh-performance liquid chromatography UHPLC	Identification of vegetable proteins at 1 and 3%	Jablonski, Moore, and Harnly 2014
		Sensory evaluation		
Adulteration with olive oil	Cheese	Sensory evaluation.	Lower score for cheeses with olive oil	Felfoul et al. 2015
		Spectroscopy		
Identification of vegetable oils	Butter	Front face fluorescence spectroscopy: source of the fats	LOD: 5% of vegetable oils	Ntakatsane, Liu, and Zhou 2013
Detection and quantification of soy milk in cow-buffalo milk	Milk	Attenuated total reflectance Fourier transform infrared spectroscopy	LOD: 2% of soy milk	Jaiswal et al. 2015

Table 5. Fuzzy logic-like comparison of some methods employed for analyzing dairy food products.

	Biochemical/Molecular methods				Chemical analytical methods		
		Immunological assays					
Discrimination criteria	Real time PCR	ELISA	IIEF	IEF	MS	HPLC	GC
[a]Determination of animal origin	Yes (10)	Yes (10)	Yes (10)	Yes (10)	Yes (10)	Yes (10)	Yes (10)
[b]Determination of geographical origin	No (0)	No (0)	No (0)	No (0)	Yes (10)	Yes (10)	Yes (10)
[c]LOD[(1)]	0.1% (8)	0.001–0.01% (8)	0.25% (8)	0.25% (8)	0.1% (8)	0.5% (8)	2% (5)
[d]Cost of equipment	Medium (5)	Low (10)	Low (10)	Low (10)	High (0)	Medium (5)	Medium (5)
[e]Cost of consumables (per analysis)	Medium (5)	Medium (5)	Medium (5)	Medium (5)	Medium (5)	High (0)	High (0)
[f]Personnel training	Medium (5)	Medium (5)	High (0)	High (0)	High(0)	High (0)	High (0)
[g]Need of polluting reagents	Low (10)	Low (10)	High (5)	High (5)	High (5)	High (5)	High (5)
[h]Time for execution[(2)]	Medium (5)	Medium (5)	Long (0)	Long (0)	Short (10)	Short (10)	Short (10)
[i]Type of measure	On-line (10)[(3)]	Off-line (0)	Off-line (0)	Off-line(0)	On-line (10)	On-line (10)	On-line (10)
Total score	58	53	38	38	58	558	55

Ranking scores were attributed following criteria:
[a]yes or no (yes = 10, no = 0)
[b]yes or no (yes = 10, no = 0)
[c]low (from 0.001 to 1%), medium (>1%) (low = 8, medium = 5)
[d]low (<10.000 $), medium (from 10.000 to 50.000 $), high (>50000 $) (low = 10, medium = 5, high = 0)
[e]low (<5 $), medium (from 5 to 20 $), high (>20 $) (low = 10, medium = 5, high = 0)
[f]medium or high (medium = 5, high = 10)
[g]low or high (low = 10, high = 0)
[h]short (< 2 h), medium (from 2 to 6 h), long (> 6 h) (short = 10, medium = 5, long = 0)
[i]on-line or off-line (on-line = 10, off-line = 0)
Abbreviations: IIEF = immunoisoelectrofocusing, IEF = isoelectrofocusing; MS = mass spectrometry; HPLC = High-performance liquid chromatography
[(1)]percentage of cow milk detected
[(2)]Includes sample preparation and analysis
[(3)]http://www.thermofisher.com/it/en/home/cloud.html

(Table 4); these techniques are highly sensitive (LOD between 0.001% and 0.5%), do not require toxic reagents and are relatively simple, but they are relatively costly since the antibodies are expensive and have a short life (Table 5).

Other methods are based on determination of fats, solids and proteins of milk and have successfully differentiated cows' and goats' milks (Gomes et al. 2013) and cows' and sheep milks (Kędzierska-Matysek et al. 2014); however, few examples are reported in the literature and those few are criticised for the use of polluting reagents, the time it takes to conduct the analysis, and for the fact that these characteristics are dependent not only on genotype but also on animal breed, feed and lactation period (Kamal and Karoui 2015) (Table 4).

Sensorial analysis is also employed in some cases (Golinelli et al. 2014) but the LOD (10% of cows' milk) is significantly higher than that of qPCR and other methods that will be discussed below (Table 4). Gomes et al. (2013) were not able to differentiate cows' and goats' milks by sensory evaluation.

Alternatives to qPCR for identification of cows' and/or the other milk are methods based on gas and liquid chromatography, alone or coupled with mass spectrometry, for detection of specific peptide markers derived from milk protein like β-lactoglobulin, α-lactalbumin or casein (Table 4). All of these methods have been used to detect the kind of milk in cheeses, but the LODs reported in different papers are very variable: from 0.001% (Russo et al. 2012) to 10% of bovine milk in milk or cheeses (Cozzolino, Passalacqua, Salemi, and Garozzo 2002). In several cases they are consistently higher than those reported in experiments conducted using qPCR. Other drawbacks of these methods are the high cost of equipment, especially mass spectrographs, and the use of toxic reagents (Table 5).

In terms of sensitivity qPCR is highly competitive with methods mentioned above, with LODs typically reported as <1% of all kinds of milk (Table 5). This is due to the improved stability in technological processes of DNA compared to proteins; moreover, DNA is not influenced by the animal breeding. QPCR can be employed to find traces of vegetable DNA for detection of adulteration with low quality plant milk, fats or proteins, but to date, no papers have been published using this application. The detection of adulteration with milks of plant origin is dependent on other methodologies (Table 4).

Whereas undeclared milk in dairy products can easily be determined with qPCR or other methods, the quantification of the type of milk remains a difficult challenge. The use of qPCR for quantification of cows' milk in dairy product is criticised by Mayer, Bürger, and Kaar (2012) because the quantity of DNA in milk depends also on the number of animal somatic cells, used as milk quality index, which can vary from 1–4 × 10^5 up to several million cells/mL, according to season and health of the animal. Another criticism is that DNA is only a marker of the allergens that are contained in cows' milk, or in another food, but the procedure does not detect the allergen itself. On the other hand, the direct detection of the allergen suffers from similar limitations (Mayer, Heidler, and Rockenbauer 1997). Moreover, proteins are more sensitive to denaturation than DNA during food processing (Hurley, Coleman, Ireland, and Williams 2006), though a reduced DNA signal has also been observed in ripened cheese (Rentsch et al. 2013) as discussed in the paragraph 3.1.

Recently, because of their relative simplicity in acquiring data and fairly low costs, spectroscopic techniques have been used to determine authenticity and to detect adulteration in dairy products. In particular, Nicolaou, Xu, and Goodacre (2010) proposed the use of Fourier transform

mid infrared spectroscopy to directly analyse binary and tertiary mixes of cows, goats' and sheep milk (Table 4). The spectra, combined with multivariate statistical analysis, provided good quantification of adulterated milk in the analysed mixes; however, this method is limited due to the high absorption of mid infrared in water that can confound interpretation (Kamal and Karoui 2015). Data are still not available on the application of this methodology to cheeses or other dairy products.

Conclusions

This review shows how qPCR has been widely utilized to analyze microbial populations in dairy food products because of its advantages over traditional culture-based methods and in spite of the rise of NGS, it is still a highly valuable approach. qPCR has also been employed to assess the animal origin of milks for dairy processing, for which it is competitive in terms of sensitivity, costs, and speed of execution when compared with classical biochemical and molecular methods (ELISA, IEF, IF) or chemical analytical methods (HPLC, MS, GC). Moreover qPCR method is more "sustainable" and environmentally-friendly because of a limited need for toxic reagents and energy. Table 5 reports a compendium of strengths and weaknesses of qPCR in a fuzzy logic-like comparison with other methods. The main drawback in comparison with classical chemical analytical methods is the inability to assess the geographical origins of dairy food products.

The issue of quantification of any kind of milk in a dairy food product is still considered a challenge for qPCR, as it is for other methods, because of the great variability of animal somatic cells in milks, the degradation of proteins during processing and of the environmental influence on the chemical composition of milk.

However, with the stability of DNA, qPCR becomes an elective platform in assessing the animal species contributing to dairy food products (determination of animal origin in Table 5). Recent advances, like ddPCR and HRM, will give qPCR a step forward in the accuracy and speed of analysis. With next generation equipment, there will be the possibility of using the platform on-line, more successfully meeting the needs of industry.

Globally considering all the scores in Table 5, qPCR was among the first biochemical and molecular methods and is comparable to more-assessed and widely-used chemical analytical methods. Improvements to some of the current drawbacks, such as inability to determining the geographical origin of dairy food products, the cost of the equipment, the speed of execution (although hindered by isolation of DNA for which extraction-less methods will be a real breakthrough), qPCR will rapidly become an elective method for traceability of dairy food products.

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Conflict of interest

The authors have declared no conflict of interest.

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