DNA-Based Animal Species Authentication in Dairy Products

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Milk is one of the most important nutritious foods, widely consumed worldwide, either in its natural form or via dairy products. Currently, several economic, health and ethical issues emphasize the need for a more frequent and rigorous quality control of dairy products and the importance of detecting adulterations in these products. For this reason, several conventional and advanced techniques have been proposed, aiming at detecting and quantifying eventual adulterations, preferentially in a rapid, cost-effective, easy to implement, sensitive and specific way. DNA-based methods relying on polymerase chain reaction (PCR) have been widely applied to detect adulterations in foods from both plant and animal origins, including dairy products because of their simplicity, high sensitivity and high specificity. They benefit from the high thermal stability of DNA molecules, which is particularly relevant when analysing processed foods, and are independent from immunochemical recognition, making them not susceptible to cross-reactivity. The ubiquity of nucleic acids in every type of cell and particularity in healthy mammary glands, which have high numbers of leucocytes and epithelial cells that are transferred to the milk, is another advantage to highlight. During cheese making, these cells are concentrated and allow the isolation of DNA to discriminate the species.

Keywords: authenticity ; DNA analysis ; PCR ; dairy products ; species detection

1. Introduction

For the successful application of polymerase chain reaction (PCR)-based methods, the extraction and isolation of DNA is a crucial task. In food matrices, the presence of hydrolytic enzymes may affect the DNA integrity and, consequently, its amplification [1]. A recent review details different aspects related to DNA extraction from dairy products as well as factors including processing, transport and handling, which may influence the applicability of DNA-based methods for the authentication of these products [2].

Several PCR-based methods have been widely applied to species identification in dairy products, namely PCR-RFLP (restriction fragment length polymorphisms), species-specific PCR, multiplex PCR and real-time PCR. Most of these methods rely on the amplification of mitochondrial genes because of their high number in animal cells, thus increasing the sensitivity of the assays. More recently, other DNA approaches such as high-resolution melting (HRM) analysis, droplet digital PCR (ddPCR), loop-mediated isothermal amplification (LAMP), next-generation sequencing (NGS) and biosensors have provided innovative alternatives for species authentication in dairy products.

2. PCR-RFLP

PCR followed by RFLP analysis relies on the amplification of a selected marker followed by digestion with restriction enzymes that recognize specific loci, providing species-specific fragment patterns. This technique has been long applied to food authentication, including dairy species identification due to its simplicity, low-cost and aptitude for routine analysis [3][4]. Plath et al. [5] reported the first PCR-RFLP method, targeting the β-casein gene and combined with polyacrylamide gel electrophoresis to identify bovine milk in ovine or caprine milk and cheeses. Since then, other PCR-RFLP methods coupled to agarose gel electrophoresis were further proposed to identify milk species in dairy products, targeting mostly casein [6][7] and *cytb* genes [8][9]. PCR-RFLP methods applied to dairy products provide mainly species differentiation, namely cow, sheep, goat and buffalo, although some methods allow achieving levels of detection [5][6].

3. Species-Specific PCR

Species-specific PCR is a standard technique that has been successfully applied to the species authentication of complex and processed foods, including dairy products, owing to its simplicity, high specificity and high sensitivity [2][3][4][10]. It relies

on the accurate design of primers to allow the amplification of a species-specific sequence based on end-point PCR. Different works have proposed the use of species-specific PCR followed by agarose gel electrophoresis for detecting milk species in dairy products, mainly cow, goat and sheep, but also other less commonly used such as buffalo, camel, mare and yak. The methods have been successfully applied to authenticate processed dairy products, namely pasteurized milk, freeze-dried milk, powder milk, UHT milk, fresh and aged cheeses, cream, yogurt and butter. Most works have relied on the amplification of mitochondrial DNA, with the 12S rRNA gene being the most frequent target, followed by the 16S rRNA, *cytb* and D-Loop regions. Generally, species-specific PCR methods allow reaching low sensitivity, down to levels in the range of 0.1–1%.

The use of two or more pairs of primers in the same reaction can allow the simultaneous detection of multiple species based on multiplex PCR. The development of duplex or multiplex PCR approaches has also been attempted for the simultaneous detection of different species in dairy products, resulting in faster and lower-cost authentication tools. Bottero et al. [11] developed a multiplex PCR method that was able to simultaneously identify cow, sheep and goat targeting the mitochondrial 12S rRNA and 16S rRNA genes, achieving a sensitivity of 0.5% of cow's milk in goat's milk. Mafra et al. [12] developed a duplex PCR method based of the measurement of band intensity of agarose gel electrophoresis that allowed detecting 0.1% of bovine milk in sheep's cheese and quantifying adulterations with bovine milk within 1–50%. Subsequently, the same researchers developed a duplex PCR with similar sensitivity and quantification range of cow's milk in goat's cheese [13]. Both approaches were successfully validated with blind cheeses and applied to commercial pure and mixture cheeses. Multiplex PCR assays have also been combined with capillary electrophoresis, as described by Gonçalves et al. [14], who were able to simultaneous detect cow, sheep, goat, and water buffalo in dairy products.

4. Real-Time PCR

Real-time PCR is based on monitoring the amplified target fragments along the amplification cycles with the use of fluorescent reported molecules. It provides several advantages over end-point PCR, namely higher sensitivity, specificity and reproducibility, as well as a low level of cross-contamination and reduced time of analysis. The capacity of quantifying the starting amount of a specific DNA target, which is intrinsic to its ability of measuring the target product at early stages of amplification (exponential), is a key advantage of real-time PCR [15]. Therefore, real-time PCR has been the technique of choice in many control and diagnostic laboratories for food analysis aiming at food authentication, GMO quantification and allergen analysis [3][4][16][17]. The use of DNA binding dyes, such as SYBR Green I, to monitor the real-time PCR amplification is the simplest and most economic approach, but it requires a melting curve analysis as a post-PCR verification of specificity. The hydrolysis fluorescent probes, such as the TaqMan™, designed to bind to a specific region of the target DNA have been preferred owing to the increased method specificity, but also to their relatively simple design and multiplexing capacity, without requiring melting curve analysis [15]. As a result, most real-time PCR methods applied to dairy product authentication have used TaqMan probes. Like for end-point PCR assays, real-time PCR assays have targeted mostly sequences of the mitochondrial 12S rRNA gene, followed by the *cytb* gene. The lowest relative sensitivities achieved with real-time PCR were similar to end-point PCR (0.1% for cow's milk in dairy products), though a much lower absolute detection was attained (down to 1–5 pg of milk DNA).

The use of multiple specific primer and probe sets targeting more than one species simultaneously has been particularly exploited in dairy product authentication. The first multiplex approach was proposed by Cottenet et al. [18] to simultaneously detect cow's and buffalo's milks using specific fluorescent probes targeting the *cytb* gene of both species. Rentsch et al. [19] developed two multiplex real-time PCR systems with TaqMan probes to simultaneously detect the main milk species targeting mitochondrial and nuclear genes, which were designated as Allmilk and Allcheese, respectively. Both systems were applied in the estimation of cow's milk of fresh and ripened model cheeses, with the nuclear systems revealing the highest specificity and quantitative performance. Later on, the same group of researchers developed three triplex real-time PCR methods with TaqMan probes targeting the 12S rRNA gene to simultaneously detect an endogenous control sequence and two species, namely cow and mare [20], cow and goat [21], sheep and goat [22] and camel and cow [23]. The approaches were successfully applied to processed dairy products, achieving high sensitivities down to few pictograms of DNA.

5. HRM Analysis

High-resolution melting (HRM) analysis is a post-PCR approach based on monitoring the gradual denaturation of double-stranded DNA of amplified fragments, allowing researchers to detect small nucleotide differences. It enables performing genotyping, gene mapping, allelic and single nucleotide variant discrimination, and barcode analysis. As a result, HRM has proven to be a rapid, simple and cost-effective tool, providing wide applicability in several research and diagnostic

areas, with particular emphasis for species differentiation from diverse food origins [16][24][25][26][27]. HRM analysis targeting the mitochondrial D-loop region was able to discriminate bovine, ovine and caprine species in cheeses. Moreover, it allowed detecting cow's milk down to 0.1% and estimating the ratio of goat to sheep milk [28]. The same group of researchers developed a duplex HRM method targeting the 12S rRNA gene to differentiate cow's and buffalo's milks, which allowed detecting cow's milk in Mozzarella cheese down to 1% and also estimating the ratio of bovine to buffalo milk [29].

6. ddPCR

Droplet digital PCR (ddPCR) is a breakthrough technology based on partitioning individual amplifications into separate compartments using droplets or chambers, providing accurate quantification of target DNA. ddPCR enables ultrasensitive and absolute DNA quantification without the need of a standard curve, which is an advantage over real-time PCR. It has been applied to clinical diagnostics, pathogen detection and food analysis, particularly gene-edited plants, GMO detection and authentication of meat products [30][31][32]. Recently, a ddPCR method targeting the *cytb* gene was developed to detect cow's and buffalo's milk in mozzarella cheese [33]. The method provided a sensitivity down to 0.1% of cow's milk in cheese, which was identical to real-time PCR, but higher than end-point PCR, IEF and HPLC-UV (0.5–1%). Despite the need for qualified personnel, the costs of ddPCR are comparable to those of the official IFE method and real-time PCR, considering it as an effective tool to detect adulterations at trace levels [33].

7. LAMP

Loop-mediated isothermal amplification (LAMP) is a technique that relies on the design of a set of primers that allow specific, sensitive and rapid detection of a DNA target under isothermal conditions. LAMP enables visual monitoring, providing simple, cost-effective and field applications. It is the most widely used isothermal amplification technique, being applied to food safety evaluation regarding foodborne pathogens, food allergens, GMO detection and botanical/animal species authentication [24][34][35]. LAMP has also been applied for species identification in dairy products [36][37]. A LAMP method was developed to specifically target the D-loop region and visually detect up to 5% of cow's milk/meat in mixtures with buffalo counterparts [36]. Kim and Kim [37] proposed a duplex LAMP method for the on-site detection of cow's and goat's milk using a portable fluorescence device. The method achieved a sensitivity of 0.1 and 1 pg of cow's and goat's DNA, respectively, and 2% for both species in milk mixtures.

8. NGS

Next-generation sequencing (NGS) technologies have revolutionised the mode of analysing DNA by providing high-speed sequencing and multiple/parallel reads, with a resultant marked reduction in cost per base. It is becoming a standard approach in many research areas, including applications to food analysis, such as foodborne microorganism detection and food authentication [16][24][38][39]. Despite the high potential of NGS for food authentication, its application to dairy foods is still limited. NGS with ion torrent technology targeting three regions of two mitochondrial genes enabled the identification of milk species in dairy products, namely goat, sheep, cow and buffalo [40]. Additionally, NGS identified different dairy species mitotypes and the presence of human DNA as a possible marker to verify the level of hygiene of dairy products.

9. Fingerprint Techniques

In addition to the demonstrated feasibility of DNA-based methods for species authentication in dairy products, they have also been challenged to identify particular breeds associated with premium dairy products. For the purpose, non-target fingerprint techniques, such as randomly amplified polymorphic DNA (RAPD), have been exploited. RAPD is a simple and economical technique that uses a single arbitrary primer to generate band fingerprint profiles. After assaying several RAPD primers, Cunha et al. [41] identified two of them capable of differentiating milks of adulterant breeds of Serra da Estrela sheep breeds used to produce PDO cheeses. To overcome the problems of low reproducibility associated with RAPD and to be able to detect adulterant breeds in PDO cheeses, researchers identified discriminatory bands that, based on their sequence, were designated as sequenced characterized amplified region markers (SCAR). The design of new SCAR primers to amplify small fragments allowed the development of a PCR-SCAR method that could be effectively applied to identify a common milk adulterant breed of Serra da Estrela PDO cheese.

Microsatellites or simple sequence repeats (SSR) are fingerprint DNA markers that rely on PCR amplification with a set of primers to target tandem repeated motifs of 2–6 bp flanked by highly conserved sequences. The different numbers of repeats in the microsatellite region are the identified polymorphisms. The high polymorphic degree and reproducibility of

SSR markers allow species identification, but mostly breed/variety or even individual identification, thus being particularly useful in food traceability studies [42]. Sardina et al. [43] described the use of SSR markers to discriminate among the most important Sicilian dairy goat breeds, aiming at the authentication of Girgentana dairy products..

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