Review

Animal Species Authentication in Dairy Products

Isabel Mafra 1,*, Mónica Honrado 2 and Joana S. Amaral 2,*

1 REQUIMTE-LAQV, Faculdade de Farmácia, Universidade do Porto, 4050-313 Porto, Portugal
2 CIMO, Instituto Politécnico de Bragança, 5300-253 Bragança, Portugal; monica-honrado@hotmail.com
* Correspondence: isabel.mafra@ff.up.pt (I.M.); jamaral@ipb.pt (J.S.A.)

Abstract: 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. They have relied mostly on electrophoretic, chromatographic and immunoenzymatic techniques. More recently, mass spectrometry, spectroscopic methods (near infrared (NIR), mid infrared (MIR), nuclear magnetic resonance (NMR) and front face fluorescence coupled to chemometrics), DNA analysis (real-time PCR, high-resolution melting analysis, next generation sequencing and droplet digital PCR) and biosensors have been advanced as innovative tools for dairy product authentication. Milk substitution from high-valued species with lower-cost bovine milk is one of the most frequent adulteration practices. Therefore, this review intends to describe the most relevant developments regarding the current and advanced analytical methodologies applied to species authentication of milk and dairy products.

Keywords: milk; dairy products; authenticity; analytical methods; adulteration

1. Introduction

In recent years, food authenticity has been considered as a core concern regarding the safety and quality control of food, with several regulatory agencies around the world increasingly devoting resources to this issue [1,2]. According to a 2013 report from the European Parliament, milk was ranked as one of the four ingredients/foods considered as the most common targets for economically motivated adulteration [3]. Milk and dairy products are highly nutritive foods, largely consumed by the general population, which play an important role in the diet of particular groups of consumers, namely children and pregnant women. Due to its high demand and value, frauds in the dairy industry have become a widespread problem, but also a real concern for many consumers and authorities, especially after the melamine scandal [4–8]. As for other food commodities, the labeling of dairy products is a key issue because the declared information must match the characteristics of the product, particularly in what concerns the used ingredients and production technology [6]. In dairy products, the detection of mislabeled and/or sub-standard products is of utmost importance for both economic and public health reasons [4–7]. Additionally, the introduction of milk from non-declared species might have health risks regarding the presence of allergens [9], as well as ethical implications due to religious practices or personal choices that avoid ingesting milk from certain species. Therefore, over the last decade, several methodologies have been proposed for the evaluation of quality and authenticity of dairy products aiming at consumer’s protection, as well as promoting fair competition and general confidence in the sector.

Several types of frauds have been reported to occur in the dairy industry, including the substitution of milk fat and/or milk protein, the substitution of milk from one species with a lower valued one, dilution with water, the addition of fillers, the addition of preservatives,
the addition of whey rennet, the application of undeclared processing methods and mislabe-
ling regarding the geographical origin [4–6,8]. Among them, the species of origin is one of
the most common frauds, namely the substitution of highly valued milks (such as sheep’s,
goat’s or buffalo’s) with cow’s milk, to reduce production costs and increase profits [5,8].
This fraudulent practice is frequently associated with seasonal oscillations and a lower
production yield of ovine, caprine and bubaline (or more exotic species such as camel or
donkey), which raises their economic value. The identification of animal species is also par-
ticularly important in the case of high-priced traditional products, such as cheeses labeled
with the European Union (EU) logos of protected designation of origin (PDO), protected
geographical indication (PGI) or traditional specialty guaranteed (TSG) [5,8]. Currently, in
the category of cheeses, there are 260 registered products in the EU geographical indications
register database (eAmbrosia), having either the logo of PDO (199), PGI (54) or TSG (7).
From the 22 countries of origin, Italy has the highest number of registered cheeses (56),
followed by France (55) and Spain (30). Several of these products, besides requiring the
use of milk from specific animal species, also specify the animal breed. For example, the
Spanish Manchego cheese must be produced from sheep’s milk of the Manchega breed and
the Portuguese Terrincho cheese produced from sheep’s milk of the Churra da Terra Quente
breed. Since PDO, PGI or TSG cheeses generally command higher prices than other similar
products without such labels, they are potential adulteration targets by the substitution of
milk from the specified species (or breeds) with others. This has been a driving force for
the development of novel methodologies that allow the specific identification of the animal
species, but also their quantitative determination in dairy products.

Considering that species substitution is one of the most relevant authenticity issues
in milk and dairy products, this review intends to provide an updated overview referring
to the most relevant and recent analytical advances for species authentication in dairy
products (Figure 1).

Figure 1. Graphical representation of analytical methods used for the authentication of animal species
in dairy products. Adapted from [16,72], reprinted from [16,72] with permission from Elsevier.

2. Protein-Based Methods

Protein-based techniques, including electrophoresis, chromatography and immuno-
chemical assays, are considered current methodologies for assessing the authenticity of
dairy products [5]. They are generally considered fast, high throughput and cost-effective, being suitable approaches for the analysis of animal species in raw milk. However, when applied to processed foods, their reliability might be compromised due to protein denaturation and consequent epitope modification, disabling the immunorecognition of proteins. In recent years, the developments of mass spectrometry (MS) platforms for protein analysis, characterization and quantification have provided alternative approaches that rely on marker peptides instead of whole proteins, being suitable alternatives to analyze processed products [10]. Nonetheless, MS methods require costly equipment and specialized personnel. Tables 1 and 2 present the summarized information on the reported protein-based methods applied to species authentication in dairy products.

2.1. Electrophoretic Techniques

Different works using electrophoretic techniques have been reported so far for the detection of milk adulteration, including the use of polyacrylamide gel electrophoresis (PAGE) or, most frequently, the use of isoelectric focusing (IEF) (Table 1). Although PAGE is generally effective, its main limitation concerns the complex band pattern obtained, with frequent overlap of bands that can lead to an equivocal interpretation of results. Pesic et al. [11] suggested the use of a native PAGE electrophoresis for the qualitative and quantitative analysis of bovine adulteration in ovine or caprine milk based on bovine β-lactoglobulins (β-LG) and α-lactalbumins (α-LA). This method was considered a fast and convenient alternative for the detection and estimation of milk adulteration. However, its application is limited to fresh milk mixtures since heat processing and pH can cause the denaturation of whey proteins, with β-lactoglobulins being remarkably affected particularly by severe heat treatments including ultra-high temperature (UHT).

A similar approach consisting of isoelectric focusing (IEF) of γ-caseins, namely γ2-and γ3-caseins obtained by plasminolysis of β-casein, is currently the reference method in the EU for the determination of cow’s milk caseins in ovine, caprine, and water buffalo cheeses [5,7,12–14]. In this method, the samples should be analyzed together with reference standards containing 0% and 1% cows’ milk, being considered positive if both bovine γ2- and γ3-caseins, or the corresponding peak area ratios, are equal to or greater than the level of the 1% reference standard [14]. The method can be used for detecting either raw or heat-treated cow’s milk and caseinate in fresh or ripened cheeses made of ewes’, goats’ and buffalos’ milk or their mixtures, though not being suitable for the detection of milk and cheese adulteration by heat-treated bovine whey protein concentrates [14]. It is also not adequate for species quantification, especially in ternary mixtures due to the similarities between some species, such as ovine and caprine [7,12,15]. In fact, the reference method fails in detecting goat’s milk in sheep’s cheese and milk. Additionally, other works demonstrated that the evaluation of cow’s milk casein in water buffalo cheese by IEF is sometimes uncertain due to the presence of interfering co-migrating bands that can result in false positives [12,15,16]. Recently, Caira et al. [16] used a proteomic approach to demonstrate that this false positive result was due to the water buffalo fragment β-casein(f100-209), which was also formed after plasminolysis of buffalo’s milk or dairy products and co-migrates in IEF with bovine γ2-casein. To avoid false positives due to a water buffalo casein band with an isoelectric point similar to that of bovine γ2-casein, Addeo et al. [15] proposed the use of IEF coupled to immunoblotting to detect the presence of cow’s milk in water buffalo cheeses. In this study, antipeptide antibodies were raised against three sequence stretches of bovine γ2-casein, with one of them, namely anti-β-casein-(106-110), showing to be highly specific for bovine proteins. The methodology proved to be successful in evaluating the authenticity of pure water buffalo milk and cheeses, with a limit of detection up to 0.25% bovine milk (v/v), which was lower than that described by the EU reference method (1%).

Capillary electrophoresis (CE) has been suggested as an alternative to gel electrophoresis-based methods for the authenticity assessment of dairy products because of its higher resolution power, low operation cost and high throughput [4,17] (Table 1). Somma et al. [17]
compared the efficiency of ultra-thin-layer IEF with capillary isoelectric focusing (cIEF) applied to the separation and identification of the main peptides arising from the hydrolysis of water buffalo and bovine β-caseins. Additionally, cIEF was used in combination with mass spectrometry for structural confirmation of the separated peptides. cIEF proved to be faster and more convenient because it does not require gel staining, though the cow-specific markers were only detectable at 5% cow’s milk addition in water buffalo’s milk, a value well above the sensitivity of the IEF method (0.5%). Nevertheless, both methods could be useful for detecting the fraudulent addition of cow’s milk to buffalo’s milk, which is very important in the production of Mozarella di Bufala cheese. More recently, Trimboli et al. [18] proposed the use of a routine CE method for human blood and urine protein analysis as a tool to authenticate ewe’s skimmed milk. The method was based on the separation of skimmed milk proteins and the use of a characteristic peak for ewe’s milk quantification in ovine/bovine skimmed milk mixtures, allowing us to detect a minimum amount of 5% of added cow’s milk with good linearity, precision and accuracy. A similar approach, using a routine CE method for blood analysis, was also attempted for detecting as low as 1% cow’s milk in buffalo’s milk and predicting the amount of fraudulently added milk by exploiting cow’s α-lactalbumin as a marker of adulteration [19]. Although most works dealing with the application of CE have been applied to milk mixtures, its use for the successful identification of animal species in cheese samples has also been demonstrated [20,21].

2.2. Immunochemical Techniques

Immunochemical methods are often used in the food industry for the qualitative and quantitative detection of food components and/or contaminants, being applied since the early 1980s to answer to the analytical demands in the dairy industry [7,13,22]. Essentially, an immunochemical assay consists of the reaction of an antigen with a specific antibody [13]. Therefore, immunochemical techniques provide highly specific and sensitive methods, being applied to a variety of complex food products. Compared with electrophoretic and chromatographic techniques, they are considered generally simpler, of lower cost, more sensitive and specific [7,13].

Enzyme-linked immunosorbent assay (ELISA) is the immunochemical technique most frequently used in dairy product analysis with diverse formats, including direct, indirect, sandwich and competitive, being applied to detect whey proteins and caseins (Table 1). ELISA are frequently used in the analysis of milk and dairy products because of their easy application in routine analysis, low-cost, speed and sensitivity. However, the selected antisera influences the specificity and sensitivity of the method, thus requiring specific antibodies capable of differentiating species, without providing false positives due to cross-reactivity with non-target species or other food ingredients [22–24]. This could be achieved by the use of novel immunoreagents obtained by antipeptide antibody technology, suitable for milk species identification [25]. The characteristics, advantages and limitations of antibody-based techniques for the assessment of dairy products authenticity have been reviewed by Pizzano et al. [13].

ELISA has been used for species authentication in milk and dairy products since the late 1980s [13]. Hurley et al. [26] described the development of an indirect competitive ELISA, using bovine immunoglobulin G (IgG) as a target, due to its high immunogenicity, to detect the presence of cow’s milk in other types of milk. The sensitivity of this technique was assayed using raw, pasteurized and previously frozen cow’s milk, concluding that high temperatures caused specific epitope modification. The detection limit in this method was 1 µg/mL of bovine IgG (0.1%), highlighting its high sensitivity without cross-reactivity with other species. Another study aiming at detecting cheese adulterations also targeting bovine IgG, but applying a sandwich ELISA, was performed by the same authors [23]. This methodology allowed further lowering the sensitivity to 0.001% of bovine milk in goat soft cheese and 0.01% of bovine milk in sheep and buffalo soft cheese.

ELISA targeting fairly thermostable proteins, such as caseins, has been proposed as a feasible alternative to detect adulterations in heat-treated milk and dairy products.
Among caseins, bovine β-caseins present a high specific antigenicity, not being affected by heat treatment and having a concentration more or less stable and independent of season, climatic and feeding conditions [27–29]. Therefore, different ELISA have become available in the format of commercial kits for routine surveillance tests. The performance of such kits has been evaluated in different studies showing their usefulness for qualitative purposes but exhibiting inconsistencies in quantitative determinations of cheese adulteration. In 2008, Costa et al. [30] evaluated two specific commercial ELISA kits to quantify the amount of cow’s and goat’s milk added to sheep’s milk and cheese and concluded that they were more successful in detecting the adulteration in milk than in cheeses. More recently, Zeleňáková et al. [31] tested the reliability of a commercial ELISA (RC-bovino from Zeu-Immunotec, Spain), concluding that the quantification of cow’s milk in sheep’s cheese was not exact, possibly due to modifications in the cheese matrix that take place during the manufacturing process. The same commercial ELISA kit was also used by Stanciuc et al. [32] to qualitatively detect the presence of cow’s milk in goat’s and sheep’s cheeses for confirmation of positive results obtained with a immunochromatographic method. From 73 tested samples from Romania, 67.3% of sheep’s cheeses and 79.7% of goat’s cheeses were adulterated by the addition of cow’s milk, suggesting the need to improve the quality control in the cheese industry. Another commercial kit (Casein ELISA set, SEDIUM R&D) was used by Zeleňáková et al. [33] to detect and quantify cow’s milk caseins in sheep’s milk and cheese, obtaining a calibration curve in the range of 0.5–50% using different mixtures of heat-treated milks. When applied to cheeses, the kit did not provide any relation between the presence of caseins and the increase in the cow’s milk proportion in the mixture, either using raw or pasteurized milk, concluding its inadequacy for cheese analysis. By contrary, the use of a sandwich ELISA kit (β-Lactoglobulin ELISA Set, SEDIUM R&D) targeting bovine β-lactoglobulin to detect adulterations in sheep’s milk and cheese was able to provide a quantitative analysis within 0.2–20 mg/kg [34].

Lateral flow immunoassays (LFIA) are alternative tools very easy to handle by non-expert workers. Thus, they can be applied in-field for screening purposes and are appropriate to be used by the cheese industry to quickly check and control the genuineness of the milk used along its production chain. Recently, Galan-Malo et al. [35] developed and validated a rapid test based on LFIA able to detect down to 0.5% of cow’s milk in goat’s, sheep’s or buffalo’s milk without identifying any false-positives among over 146 negative assayed samples.

Although most available immunochemical assays concern the authentication of sheep’s, goat’s and buffalo’s milk and/or cheeses, some studies have addressed other animal species. Pizanno et al. [25] developed an ELISA based on the use of antipeptide antibodies raised against the 1–18 sequence stretch of cow’s β-casein to successfully detect the presence of low levels (0.5%, v/v) of cow’s milk fraudulently blended with high-valued donkey’s milk. An indirect competitive ELISA to detect cow’s milk in yak’s milk using a specific monoclonal antibody for bovine β-casein (mAb 1-9B) was developed by Ren et al. [36]. The method allowed detecting 10 µg/mL of bovine milk in yak’s milk and was not affected by any external factors such as temperature and milk treatment.

Table 1. Summarized information of examples on reported electrophoretic and immunochemical methods applied to species identification in dairy products.

| Method    | Target Species | Target Molecule                  | Type of Product         | Sensitivity                  | Reference |
|-----------|----------------|----------------------------------|-------------------------|------------------------------|-----------|
| Native PAGE | Cow            | Bovine β-lactoglobulin and α-lactalbumin | Milk mixtures         | 3% in caprine/bovine 5% in ovine/bovine | [11]      |
| IEF       | Cow            | γ2- and γ3-caseins               | Ewe’s and goat’s cheeses | - a                         | [12]      |
|           | Cow            | Bovine αs1-casein                | Donkey’s milk           | 5% of cow’s milk in donkey’s milk | [25]      |
| Method                        | Target Species | Target Molecule                     | Type of Product                                 | Sensitivity                                                                 | Reference |
|-------------------------------|----------------|-------------------------------------|------------------------------------------------|-----------------------------------------------------------------------------|-----------|
| IEF and immunoblot analysis   | Cow            | Bovine γ2-casein                    | Water buffalo milk and derived mozzarella cheese | 0.25% bovine milk in water buffalo mozzarella cheese                         | [15]      |
| CE                            | Cow, sheep, goat| Casein fractions and their breakdown products | Iberico-type cheeses made from cow, sheep or goat’s milk | -                                                                          | [20]      |
| Sheep and cow                 | Cow            | Bovine proteins                     | Sheep’s/cow’s milk mixtures                     | 5% of cow’s milk in ovine/bovine milk mixtures                              | [18]      |
|                               | Cow            | Bovine α-lactalbumin                | Cow’s milk in buffalo’s milk                    | 1% of cow’s milk (limit of quantification of 3.1%)                          | [19]      |
|                               | Cow            | α-lactalbumins and β-lactoglobulins | Goat’s and ewe’s cheeses                         | 1% (cow’s milk)                                                            | [21]      |
| Capillary IEF                 | Cow            | Products of plasmin hydrolysis of bovine and water buffalo β-casein | Water buffalo’s milk                           | 1% (cow’s milk)                                                            | [17]      |
| ELISA                         | Goat           | Caprine IgG                         | Sheep’s milk                                    | 0.5% (of goat’s milk in sheep’s milk)                                       | [28]      |
| Indirect Competitive ELISA    | Cow            | Bovine IgG                          | Goats’, sheep’s and buffalo’s milk              | 1.0 µg/mL of bovine IgG (0.1%)                                              | [26]      |
|                               | Cow            | mAb 1-9B                            | Yak’s milk                                      | 1% (10 µg/mL) of cow’s milk in yak’s milk                                   | [36]      |
| Competitive ELISA             | Cow            | Bovine β-casein                     | Donkey’s milk                                   | 0.5% of cow’s milk in donkey milk                                           | [25]      |
| Indirect ELISA                | Cow            | Bovine β-casein                     | Raw and heated goat’s milk                      | 2% of cow’s milk in goat’s milk                                             | [29]      |
|                               | Cow            | Bovine β-casein                     | Goat’s and sheep’s milk cheeses                 | - a                                                                        | [37]      |
| Sandwich ELISA                | Cow            | Anti-bovine IgG antibody            | Dairy products                                  | 0.001% cow’s milk in buffalo or sheep milk; 0.01% cow’s milk in goat’s milk; 0.001% in goat cheeses and 0.01% in buffalo and sheep cheeses | [23]      |
| ELISA kits                    | Cow and goat   | Bovine or caprine protein β-lactoglobulin | Ewe’s milk and cheese                          | ~0.2% of cow and goat’s milk in ewe’s milk                                  | [30]      |
|                               | Cow            | Bovine IgG                          | Sheep’s milk and cheese, and commercial “Bryndza” | 0.5% raw and 50% pasteurized cow milk in sheep’s milk; 0.5% raw and low pasteurized and 5% high pasteurized cow milk in sheep’s cheese | [31]      |
| Sandwich ELISA kit            | Cow            | Bovine β-lactoglobulin             | Sheep’s dairy products                          | 0.2 ppm (mg/kg)                                                            | [34]      |
| LFIA                          | Cow            | Specific bovine immunoglobulins (IgG) | Buffalo, sheep and goat raw milks              | 0.5% of cows’ milk                                                          | [35]      |
Table 1. Cont.

| Method                      | Target Species | Target Molecule          | Type of Product                          | Sensitivity                                      | Reference |
|-----------------------------|----------------|--------------------------|------------------------------------------|-------------------------------------------------|-----------|
| Optical immunoassay         | Cow            | Bovine k-casein          | Raw and pasteurized cow’s and goat’s milks | 0.04% (cow’s milk in goat’s milk)                | [38]      |
| QCM immunosensor            | Cow            | Bovine k-casein          | Cow’s and goat’s milks                   | 1 ppm (cow’s milk in goat’s milk)                | [39]      |

CE, capillary electrophoresis; ELISA, enzyme linked immunosorbent assay; IEF, isoelectric focusing; LFIA, lateral flow immunoassay; PAGE, polyacrylamide gel electrophoresis; QCM, quartz crystal microbalance; 2 not reported.

2.3. Chromatographic and Mass Spectrometry Techniques

Up until now, different chromatographic techniques, including either gas or liquid chromatography, have been applied to authenticate dairy products because of their relative simplicity and speed, as well as possibility of automation [7,22]. High-performance liquid chromatography (HPLC) with ultraviolet (UV) detection was firstly used for the separation of the different casein fractions, relying on both normal (NP) or reverse-phase (RP) columns to identify cow’s milk in goat’s and sheep’s milk [40–43]. However, UV detection has drawbacks related to low specificity in the presence of co-eluting peaks or interferents. Thus, during the past decade, the technological advances, mainly in the area of mass spectrometry (MS) detection, have steadily replaced UV detectors, whenever the detection of food frauds is concerned. Soft-ionization techniques, such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI), have made possible to accurately analyze proteins and peptides, therefore allowing their use as reliable biomarkers for dairy product authentication. Peptides as biomarkers present advantages over proteins, which are affected by thermal processing [44]. Owing to the specificity, fastness, sensitivity and high reproducibility of the mass spectra, several methodologies based on MALDI time-of-flight mass spectrometry (TOF MS) have been developed, so far, to obtain informative fingerprints of milk proteins towards dairy product authentication [45] (Figure 2).

![Figure 2. MALDI-TOF-MS-based identification of the proteotypic species WB β-CN (f1-28)4P and B β-CN (f1-25)4P as deriving from the CN fraction of WB milk containing 50% v/v B counterpart, which was preventively subjected to HA-based phosphoprotein enrichment and trypsinolysis. Reported is a partial view of the mass spectrum, showing well resolved (ΔM = +336 u), intense signals associated with the proteotypic species. WB β-CN (f1-28)4P (theor. MH+ = 3460.3); B β-CN (f1-25)4P (theor. MH+ = 3124.3). Reprinted from [16] with permission from Elsevier.](image-url)
Based on MALDI-TOF MS analysis of intact proteins of different milk species, Cozzolino et al. [46] suggested α-lactalbumin and β-lactoglobulin as markers for detecting cow’s milk added to sheep’s and buffalo’s milk or cheese. The authors also demonstrated the usefulness of the method in detecting the addition of powdered to fresh milk based on the presence of lactosylated forms originated by heat processing. The analysis of entire proteins by direct MALDI-TOF MS coupled to unsupervised statistical analysis was also successfully proposed for milk authentication by Di Girolamo et al. [45] and Nicolaou and Goodacre [47]. Identical results were obtained by Kuckova et al. [48] regarding the identification of the species of origin in milk, though the same was not verified when the method was applied to analyze commercial cheeses, which could be attributed either to protein profile modifications or to adulteration of ovine and caprine cheeses. Recently, Rau et al. [49] demonstrated the feasibility of MALDI-TOF MS combined with a small in-house validated database, containing more than 150 reference spectra of milk and cheese, as a rapid, easy and robust method to identify the species of origin in mozzarella and white brined cheeses. The direct protein extraction without applying a tryptic digestion step allowed performing the analysis in less than 30 min with reduced analytical costs.

Other approaches have relied on a bottom-up proteomic strategy, based on MS analysis of peptides obtained after enzymatic digestion [49–52]. Calvano et al. [50] reported several bovine-specific peptide markers in milk tryptic digests that can be useful for detecting adulterations by cow’s milk addition to goat’s or sheep’s milk. Since the detection of sheep’s milk adulterated with goat’s milk is a difficult task because of their similar protein profiles, two goat-specific peptide markers assigned to κ-casein were identified [50]. Caira et al. [51] used a MALDI-TOF MS method to simultaneously determine the presence of water buffalo’s and cow’s milk in Italian water buffalo’s mozzarella cheese. Since crossbreeding with other water buffalo breeds has been avoided in indigenous Mediterranean Italian buffalo, these animals generally exhibit reduced milk protein polymorphisms when compared to other international breeds. Therefore, hundreds of milk samples (Italian and from several other countries) were analyzed, aiming at identifying signature peptides associated with water buffalo origin for the authentication of PDO products [51]. Caseins were the target proteins owing to the identified differences between indigenous and international breeds, namely the unique presence of a β-CN A variant and an internally deleted αs1-CN (f35-42) variant in international water buffalo milk samples. The peptideomic approach allowed the identification of several tryptic signature peptides as molecular marker candidates to detect the addition of imported water buffalo’s milk in Italian PDO products, as well as adulterations with cow’s milk blending. The proposed methodology enabled the specific detection of international water buffalo and bovine caseins down to 2% and 0.78%, respectively. MALDI-TOF MS has also been proposed to detect the adulteration of water buffalo’s ricotta with bovine milk based on a specific peptide marker, corresponding to the region 149–162 of β-lactoglobulin, enabling its detection down to 5% [52]. Nardiello et al. [53] proposed the use of a nano LC–ESI-ion-trap tandem mass spectrometry (nano LC-ESI-IT-MS/MS) methodology combined with a database post-processing to validate peptide sequence assignments and determine the species of origin in milk samples. Bovine species-specific peptides originated from αS1-casein and β-lactoglobulin were identified as suitable authenticity markers with detection levels as low as 1%.

MALDI-TOF MS has also been referred to as a tool for selecting the most suitable peptide makers in further analysis by liquid chromatography coupled to mass spectrometry (LC-MS) [54–56]. In fact, LC-MS has been increasingly applied in food analysis owing to its powerful capacity in detecting and quantifying specific analytes in complex mixtures, offering particularly enhanced selectivity and sensitivity when multiple reaction monitoring (MRM) scanning is applied [57,58]. Cuollo et al. [54] used two techniques, namely MALDI-TOF MS and LC-ESI/MS, to detect specific signature peptides to differentiate cow’s, sheep’s, goat’s and water buffalo’s milks, with both approaches providing similar sensitivities (1% for caprine and 0.5% for the other species). αs1-CN (f8-22) peptide was selected as a convenient marker for cow’s, sheep’s and water buffalo’s milk, while
αs1-CN (f8-22) was for goat’s milk. MALDI-TOF MS data were tentatively used to perform quantitative analysis based on synthetically modified proteotypic peptides as internal standards, but accurate evaluation of caprine milk in quaternary mixtures was only achieved by LC-ESI-MS.

Sforza et al. [59] described an LC-MS method to evaluate the presence of cow’s milk in fresh sheep’s milk cheese targeting short marker peptides, namely αs1-CN (f1-23) and αs1-CN (f1-14), generated from proteolytic activities of the rennet enzyme chymosin and starter lactic acid bacteria, respectively. While the first peptide was degraded over time, thus being undetectable after long ageing periods, the second was frequently observed in cow’s milk cheeses. Despite this occurrence, the authors referred to its detection in hard cheeses aged for more than 30 months. Moreover, the degradation of αs1-CN (f1-23) peptide also led to other fragments that could be detected. The method allowed the detection of cows’ milk down to 1% in all the analyzed cheeses, demonstrating the usefulness of these two candidate biomarkers to assess the addition of cow’s milk in fresh sheep’s cheese [59].

Czerwenka et al. [60] developed an LC-MS method to detect the adulteration of cow’s milk in water buffalo’s milk and mozzarella cheese, targeting the whey β-lactoglobulin as an adulteration marker. Since this water-soluble protein is mainly present in the whey fraction and not in the cheese, the analyzed parts were the brine in which this type of cheese is usually sold, or in the exudate obtained after cheese centrifugation. The authors showed that sufficient amounts of β-lactoglobulin were present either in the brine or exudate, allowing the detection of adulterations with cow’s milk. The application of this method to assess 18 commercial samples of water buffalo mozzarella cheese allowed detecting three adulterated products. However, quantitative determination presented several pitfalls because of the variability the target the analyte between and within the two blended milks and the lack of an internal standard. Quantification of the fraudulent addition of bovine milk in the production of buffalo mozzarella PDO cheese was claimed by Russo et al. [57], based on UPLC-MS/MS exploiting the MRM mode, though the protein level in the studied cheeses was not taken into consideration. The use of MRM, as described in this study, allowed a highly selective and sensitive detection and quantification of the chosen proteotypic marker, even in complex matrices, by simultaneously monitoring both their parent and one or more product ions. The selection of the species-specific proteotypic marker—phosphorylated β-CN (f33-48) tryptic peptide—was performed by an untargeted LC-MS/MS analysis by means of a quadrupole TOF MS equipped with an ESI source (ESI-Q-q-TOF). Additionally, to select the best conditions for trypsin digestion, a preliminary study was conducted by MALDI-TOF MS. Overall, the method allowed targeting the marker peptides with high specificity, thus being adequate for the authentication of complex matrices such as dairy products [57].

Despite the claimed advantage of quantitative analysis by LC-MS methods, it must be referred that it mainly gives an estimation of the fraud extent since the protein content of milk is known to vary with different factors, with the breed and season being of most relevance [55–61]. Trying to overcome this aspect, Gunning et al. [58] proposed the use of MRM MS-targeting αS1-casein to detect the addition of cow’s milk to buffalo mozzarella cheese. The relative amounts of each species in binary mixtures were determined based on corresponding peptides arising from a corresponding protein strategy and the ratios of transition peak areas. Moreover, identical peptides with the same sequences in both species were used to establish the relative levels of both species of αS1-casein in the component mixtures. The method was applied in a survey of 28 products sold in UK retail and restaurants, enabling us to verify that almost 2/3 were suspicious of being adulterated with cow’s milk. An UHPLC-MS/MS method also exploiting MRM mode, using at least two transitions for each compound, has recently been reported by Ke et al. [62] to quantify cow’s whey and whole milk powder in goat’s and sheep’s milk products, including infant formula. This method allowed the simultaneous quantification of four caseins (β-CN, αs1-CN, αs2-CN, and κ-CN) and two whey proteins (α-lactalbumin, β-lactoglobulin) based on the detection of their signature peptides. Isotopic labeled signature peptides were used as internal stan-
standards to compensate the matrix effect. The method was successfully validated regarding several parameters. Calibration curves for the tryptic signature peptides presented good linearity, the limits of quantification were between 0.01–0.05 g/100 g for the target proteins and the method showed high precision, reproducibility and recovery rates. The analysis of 11 commercial samples of goat infant formula milk powder revealed some adulterations among the evaluated products [62].

Although proteomic approaches developed so far mostly rely on the target identification of marker peptides, recently an untargeted UHPLC–MS/MS high resolution MS (HRMS) combined with chemometrics, was proposed to discriminate among cow’s, goat’s and buffalo’s milk samples [63]. The approach allowed the identification of different marker compounds, suggesting β-carotene and ergocalciferol for cow’s and water buffalo’s milk identification, respectively. Moreover, the levels of octanoic, nonanoic and decanoic acids were found to be higher in goat’s than in cow’s and buffalo’s milk [63].

Recently, the development of ambient ionization techniques, such as direct analysis in real time (DART), enabled a high-throughput and easy analysis of food. The potential of this ionization technique coupled to HRMS and chemometrics was exploited for dairy product authentication, including the discrimination of cow’s, goat’s and sheep’s milk. Results showed that DART-HRMS analysis of the non-polar fraction of milk had a limited discrimination potential, probably due to the high variability in triacylglycerols (TAG) among each group of samples [64].

Although the application of both chromatographic and mass spectrometry techniques to dairy product authentication mainly relies on protein analysis, other compounds such as fatty acids and TAG have also been addressed for this purpose [65–67]. Bratu et al. [68] used GC-MS analysis of fatty acid methyl esters coupled to principal component analysis (PCA) to differentiate 25 different cheeses (including cow, goat and sheep). Although sample discrimination in 3 groups was achieved using 12 components, more studies should be performed comprising a higher number of samples, also including model cheeses made with mixtures of milk besides pure milk cheeses. Vieitez et al. [69] showed that the addition of cow’s milk to pure goat’s milk influences the TAG profile by determining the partition number (PN), which characterizes the molecular structure of TAG. The analysis of blends containing 10, 20 and 50% of cow’s milk showed that the addition of cow’s milk to goat’s milk affects the TAG profile by decreasing TAG with PN between 38 and 42, while increasing it with PN between 46 and 50. Of the 15 commercial samples evaluated, 3 presented a different TAG profile, suggesting their possible adulteration with cow’s milk. However, since there are many factors that can influence the TAG profile (breed, feeding regime, season, etc.) the study should be extended in order to further include a higher number of samples.

The summarized information about different chromatographic and mass spectrometry methods applied to species authentication in dairy products is presented in Table 2.

3. Spectroscopic Methods

In the last decade, different stakeholders have evidenced the need for less expensive, rapid and efficient methods for the detection of adulterations in dairy products. Accordingly, several spectroscopic methods have been developed and applied to dairy product authentication, including near infrared (NIR), mid infrared (MIR), front face fluorescence spectroscopy (FFFS), Fourier-transform infrared (FT-IR) and nuclear magnetic resonance (NMR) [6,70]. Spectroscopic techniques, in general, are considered as auspicious tools to detect adulterants in dairy products [6]. Compared with the reference methods, spectroscopic techniques present several advantages, such as fastness, simplicity, and a non-destructive nature, requiring few or no chemicals, making them suited for routine applications. However, these methods frequently require expensive equipment, extensive sample databases and chemometrics [6,7,71–73].
Table 2. Summarized information of reported chromatographic and mass spectrometry techniques applied to species identification in dairy products.

| Method       | Target Species                  | Target Molecule                                                                                                                                  | Type of Product       | Sensitivity                                      | Reference |
|--------------|---------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|--------------------------------------------------|-----------|
| HPLC-DAD     | Sheep, goat and cow             | Albumines (β-lactoglobulin, α-lactoalbumin and serum albumine), globulins (immunoglobulin: IgG, IgA and IgM), proteoso-peptones and lactoferrin | Milk and cheeses      | 3.92% (sheep’s milk in cheese)                   | [42]      |
|              |                                 |                                                                                                                                                    |                       | 2.81% (goat’s milk in cheese)                    |           |
|              |                                 |                                                                                                                                                    |                       | 1.47% (cow’s milk in cheese)                     |           |
|              | Buffalo and cow                 | β-lactoglobulin                                                                                                                                     | Creams                | 1% (cow’s milk in buffalo’s cream)               | [43]      |
| MALDI-TOF MS | Cow, buffalo, sheep, she-donkey and goat | Intact proteins                                                                                                                                   | She-donkey’s and goat’s milk | 0.5% (cow’s milk in She-donkey’s and goat’s milk) | [45]      |
|              | Goat, sheep and cow             | Caseins and proteose peptone                                                                                                                     | Milk                  | 2% (cow’s milk in goat’s and sheep’s milk)       | [47]      |
|              | Water buffalo and cow           | Four signature unphosphorylated peptides derived from β-CN A, i.e., (f49-68) Asn⁶⁸, (f1-28) Ser¹⁰, (f1-29) Ser¹⁰ and (f33-48) Thr⁴¹ and two from αs1-CN (f35-42), i.e., (f23-34) Met¹¹ and (f43-58) Val⁴⁴ | Mozzarella cheeses    | 0.78% (cow’s milk in PDO water buffalo’s cheeses) | [51]      |
|              | Cow and buffalo                 | Region 149–162 of bovine β-lactoglobulin                                                                                                          | Water buffalo’s ricotta PDO cheese | 5% (cow’s milk in buffalo’s cheese)              | [52]      |
|              | Goat                            | αs1-CN f8-22 and αs1-CN f4-22                                                                                                                    | Milk mixtures         | 0.5% (goat’s milk in milk mixtures)              | [54]      |
|              | Sheep, goat, buffalo and cow    | γ2-caseins and γ3-caseins in the four species; α-lactalbumins in bovine, buffalo and goat milk; β-CN fragments (98–207) in goat and ovine milk; β-lactoglobulin in goat milk, proteoso peptones p.p.8.1, in bovine milk and β-casein fragments (1–68) and (69–209) in buffalo milk | Fresh raw cow’s, buffalo’s, sheep’s and goat’s milk | 5% (cow’s milk in goat’s milk)                   | [74]      |
|              | Goat, sheep and cow             | Intact phospholipids                                                                                                                                | Milk                  | a[a]                                            | [75]      |
| LC-MS        | Sheep and cow                   | Fragments 1–14 and 1–23 from αs51 casein                                                                                                          | Fresh sheep’s milk cheeses | 1% (cow’s milk in sheep’s cheese)               | [59]      |
| LC-MS/MS     | Cow, buffalo, sheep and goat    | β-lactoglobulin variants A and or α-lactalbumin                                                                                                  | Buffalo’s, sheep’s and goat’s Italian ricotta cheese | 0.5% (cow’s whey in ricotta cheeses from the other species) | [55]      |
| LC-ESI-MS    | Goat                            | α1-CN f4-22 variant A and B                                                                                                                      | Milk mixtures         | a[a]                                            | [54]      |
| LC-ESI-MS/MS | -                               | Caseinomacropeptide (CMP) and pseudo-CMP                                                                                                           | Milk                  | 1 µg/mL (CMP and pseudo-CMP in milk)             | [76]      |
Techniques relying on infrared (IR) radiation have the advantage of allowing the analysis of samples, either in the solid or liquid state, which can provide specific spectra using selected frequency ranges [79]. Infrared spectroscopy is based on the measurement of the fundamental vibrations of molecules, with the collective effect from each functional group that has a specific vibrational frequency, resulting in a unique molecular fingerprint. Both mid-infrared (MIR, approximately from 400–4000 cm\(^{-1}\)) and near-infrared (NIR; approximately from 4000–14,000 cm\(^{-1}\)) have been applied to authenticate dairy products. FT-IR, considered as a fast biochemical fingerprinting technique, has already been described in the analysis of cheese quality, quality control of milk and cheese ripening process, as well as authenticity assessment [80]. FT-IR was proposed by Nicolaou et al. [72] to detect and quantify the percentage of cow’s milk adulteration in mixtures of different types of milk, namely goat, sheep and water buffalo’s milk, suggesting its potential applicability in the food industry. From a qualitative point of view, the spectra of cow’s and goat’s milk were very similar but showed quantitative differences that were mainly evidenced in sheep’s milk (Figure 3). FT-IR also allowed the discrimination of cow’s, sheep’s and water buffalo’s milks and their classification by hierarchical clustering and PCA on the basis of Euclidean distance and Ward’s algorithm [81]. Recently, FT-IR was employed to verify the species of origin of Halloumi cheese, a traditional Cypriot cheese that should be made either with goat’s or sheep’s milk. The interpretation of the obtained spectra was carried out by chemometric analysis using SIMCA software, enabling the differentiation of cow’s milk or goat/sheep’s milk products, with supervised orthogonal partial least squares discriminant analysis [82]. Unsupervised and supervised methods applied to FT-IR spectra to assess goat’s cheese and yogurt adulterated by cow’s milk addition at the levels of 10%, 15% and 20% were evaluated by Teixeira et al. [83]. Both approaches showed good results as they were able to distinguish the adulterated products. Moreover, the use of an interval partial least-square (iPLS) algorithm allowed the researchers to dramatically reduce the number of

---

### Table 2. Cont.

| Method                              | Target Species                  | Target Molecule                                                                 | Type of Product           | Sensitivity                        | Reference |
|-------------------------------------|---------------------------------|---------------------------------------------------------------------------------|---------------------------|-------------------------------------|-----------|
| HPLC-ESI-MS, MALDI-TOF MS and MS/MS | Goat                            | Variant D of caprine β-casein                                                   | Italian goat’s milk       | 0.001% (cow’s milk in buffalo’s cheese) | [56]     |
| UPLC-ESI-MS/MS                      | Cow and buffalo                 | β-casein f33-48 transitions                                                     | PDO buffalo’s mozzarella  | 0.01–0.05 g/100 g (cow’s whey and whole milk powder and goat’s milk infant formula) | [57]     |
| UHPLC-MS/MS                         | Goat, sheep and cow             | Caseins (β-casein, αs1-casein, αs2-casein, and κ-casein) and major whey proteins (β-lactoglobulin and α lactalbumin) | Cow’s milk whey, whole milk powder and goat’s milk infant formula | 0.3 mg/100 mg (cow’s lactoferrin in infant formulas) | [78]     |

ESI, electrospray ionization; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; UHPLC, ultra-high performance liquid chromatography; \(^a\) not reported.
variables which, according to the authors, may represent a step towards the development of cheaper portable devices.

![Fourier transform infrared spectra for pure cow, goat, and sheep milk. These spectra are offset to allow visualization of any difference. Reprinted from [72] with permission from Elsevier.

Brandão et al. [84] developed a front-face and time-resolved fluorescence method for a rapid screening of frauds in goat’s milk powder by the addition of cow’s milk powder. Compared with steady-state spectroscopy, time resolved fluorescence offers some advantages because it measures the time dependence (lifetimes, which are determined by fluorescence intensity decay) of the fluorescence instead of its emission intensity. Additionally, fluorescence lifetime is not altered by photo bleaching, it is independent from the fluorescence intensity and largely independent of fluorophore concentrations. The intensity levels of excitation and emission were measured at 315 nm and 468 nm, respectively, whose results showed increased intensity in samples related with increasing addition of cow’s milk powder. This study successfully demonstrated fluorescence lifetimes as a promising technique for the application in real-time assessment of frauds in goat’s milk powders, providing a potential tool for food authentication, particularly dairy products.

Synchronous fluorescence spectroscopy is another technique, generally combined with multivariate analysis, applied to detect adulterations in food. Velioglu et al. [85] exploited this technique to detect the addition of milk in buffalo’s milk and to discriminate both species. The developed method showed a limit of detection of 6% of cow’s milk and a good distinction between the spectra of both species. These differences were found in the range of 400–550 nm, with breaks of 10 nm, which were further analyzed using PCA to distinguish the two species and by partial least square (PLS) analysis to estimate the level of cow’s milk adulteration in buffalo’s milk samples [85]. This technique has also proved its usefulness in discriminating cow’s, goat’s, ewe’s and buffalo’s milk and estimate the level of cow’s milk addition in the case of samples classified as being binary mixtures [86].

4. DNA-Based Methods

DNA-based methods relying on polymerase chain reaction (PCR) have been widely applied to detect adulterations in foods from both plant [87] and animal [88–90] origins,
including dairy products [8,91] 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 [4]. During cheese making, these cells are concentrated and allow the isolation of DNA to discriminate the species.

For the successful application of 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 [7]. 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 [8].

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. Table 3 presents the summarized information of reported methodologies based on DNA analysis for species authentication in dairy products.

Table 3. Summarized information of reported DNA-based methods applied to species identification in dairy products.

| Technique       | Target Species          | Application                  | Target Gene | Sensitivity                        | Reference |
|-----------------|-------------------------|------------------------------|-------------|------------------------------------|-----------|
| PCR-RFLP        | Cow, sheep and goat     | Milk and cheese              | β-casein    | 0.5% (cow’s milk in goat’s and sheep’s milk) | [92]      |
|                 | Cow, sheep, goat and buffalo | Meat and milk              | cytb        | - a                                | [93]      |
|                 | Buffalo, cow and sheep  | Milk                         | SSR marker and cytb | - a                                | [94]      |
|                 | Cow and buffalo         | Mozzarella cheeses           | α-, β- and κ-casein | 1% (cow’s milk in buffalo’s milk mozzarella cheese) | [95]      |
|                 | Cow and buffalo         | Milk and butter              | cytb        | 5% (cow’s milk in buffalo’s milk and butter) | [96]      |
|                 | Cow and buffalo         | Raw milk                     | cytb        | - a                                | [97]      |
|                 | Cow, goat, and sheep    | Raw and powder milks, pasteurized cream, and hard and semi-hard cheeses. | κ-casein    | - a                                | [98]      |
| Species-specific PCR | Sheep and goat       | Raw, thermally and process milk, milk mixtures and cheeses | 12S rRNA    | 0.1% (cow’s milk in sheep’s and goat’s milk) | [99]      |
|                 | Goat                    | Dairy products               | 12S rRNA    | 0.1% (goat’s milk in sheep’s milk)  | [100]     |
| Technique                      | Target Species                      | Application                          | Target Gene | Sensitivity                                      | Reference |
|-------------------------------|-------------------------------------|--------------------------------------|-------------|-------------------------------------------------|-----------|
| Cow and buffalo               | Mozzarella cheese                   |                                      | 12S rRNA    | 0.1% (cow’s milk in mozzarella cheeses)          | [101]     |
| Goat, sheep and cow           | Goat’s and sheep’s cheeses           |                                      | cytb        | 1% (cow’s milk in goat’s cheeses)                | [102]     |
| Goat and ovine                | Ovine cheeses                        |                                      | 12S rRNA    | 1% (goat’s milk in sheep’s cheeses)              | [103]     |
| Cow, goat and sheep           | Cheeses and other dairy products     |                                      | 12S rRNA    | 1% (cow’s milk in cheeses)                       | [37]      |
| Cow, sheep, goat and buffalo  | Raw and pasteurized milks and cheese| k-casein                             |             | 0.1% (cow’s milk in buffalo’s milk)              | [104]     |
| Multiplex PCR                 | Cow, goat and sheep                 | Mixture cheeses                      | 12S rRNA (cow, sheep and goat) and 16S rRNA (sheep)| 0.125 ng (DNA from the three species) 0.5% (cow’s milk in goat’s milk) | [105]     |
| Cow and sheep                 | Ovine cheeses                        |                                      | 12S rRNA (cow, sheep) and 16S rRNA (sheep) | 0.1% (cow’s milk in ovine cheeses)          | [106]     |
| Cow and goat                  | Goat cheeses                         |                                      | 12S rRNA    | 0.1% (cow’s milk in goat’s cheese)               | [107]     |
| Cow and yak                   | Raw, pasteurized, and sterilized milk mixtures |                    | 12S rRNA    | 0.1% (cow’s milk in yak’s milk)                  | [108]     |
| Cow and buffalo               | Raw and heat treated milks and cheeses |                                      | D-Loop      | 0.1% (both species in milk and cheese) 0.15 ng of buffalo’s and 0.04 ng cow’s DNA). | [109]     |
| Cow, goat and sheep           | Dairy products (butter, cheese, cottage cheese, cream, milk (fresh, UHT, powdered) and yogurt | mtDNA |                             | 1% (in two-species milk mixtures)          | [110]     |
| Cow and goat                  | Goat’s milk                          |                                      | mtDNA       | 0.5% (cow’s milk)                               | [111]     |
| Goat and cow                  | Goat’s cheese                        |                                      | 12S rRNA    | 0.5% (cow’s milk in goat cheese)                 | [112]     |
| Cow, sheep and goat           | Mono-species Sicilian dairy products | 12S rRNA (cow, goat) 12S rRNA and 16S rRNA (sheep) | 0.1% (milk all species in cheeses) | | [113]     |
| Cow, sheep and goat           | Goat’s milk products (aged cheese, fresh cheese, yogurt, UHT milk and powder milk) | 12S rRNA (cow and goat) and cytb (sheep) | 0.05 ng (DNA of each species) | | [114]     |
| Cow and goat                  | Milk powder                          |                                      | 12S rRNA    | 0.1% (cow’s milk in goat’s milk)                 | [115]     |
| Cow, camel, horse and goat    | Raw, freeze-dried, pasteurized and ultra-high temperature (UHT) milk | 16S rRNA (camel and cow) and D-Loop (horse and goat) | 0.1%, 0.2% and 0.5% (cow’s milk in raw milk and freeze-dried milk mixtures, pasteurized milk and UHT milk, respectively) | | [116]     |
| Cow, sheep and goat           | PDO Portuguese cheeses               | cytb                                 |             | - a                                             | [117]     |
Table 3. Cont.

| Technique                        | Target Species                  | Application                        | Target Gene | Sensitivity                      | Reference |
|----------------------------------|---------------------------------|------------------------------------|-------------|----------------------------------|-----------|
| Real-time PCR—SYBR Green dye     | Cow and buffalo                 | Mozzarella cheeses                 | cytb        | 0.1% (cow’s milk)                | [118]     |
| Cow and goat                     | UHT goat’s milk                 |                                    | 12S rRNA    | 0.5% (cow’s milk)                | [119]     |
| Cow, sheep and goat              | Goat’s milk products (aged cheese, fresh cheese, yogurt, UHT milk and powder milk) | 12S rRNA (cow and goat) and cytb (sheep) | 0.005 ng (DNA of each species) | [114]     |
| Cow and buffalo                   | buffalo yogurt                  |                                    | cytb        | 0.015 ng of DNA for both species | [120]     |
| Multiplex real-time PCR—SYBR Green dye | Cow, sheep, goat and buffalo | Milk mixtures and cheeses | 12S rRNA (cow and goat) and cytb (sheep and buffalo) | 0.1% (all species) | [121]     |
| Real-time PCR—TaqMan probes      | Goat and sheep                  | Raw and heat-treated milk mixtures  | 12S rRNA    | 0.5% (goat’s DNA) 0.6% (goat’s milk in raw and pasteurized mixtures) | [122]     |
| Cow and sheep                     | Raw and heat-treated milk mixtures |                                    | 12S rRNA    | 0.5% (cow’s milk in raw and pasteurized sheep’s milk) | [123]     |
| Cow                               | Fresh and processed meats, milks and cheeses | cytb | 35 pg cow’s DNA | [124]     |
| Bovine and buffalo                | Cheese samples                  | cytb                               | 2% (cow’s milk in buffalo’s milk) | [125]     |
| Cow and donkey                    | Raw, pasteurized and autoclaved milks | COI | 2% (cow’s milk in donkey’s milk) | [126]     |
| Bovine and buffalo                | Dairy products and meat         | cytb (cow) and 16S rRNA (buffalo)   | 1% (cow’s milk in buffalo cheese) | [127]     |
| Cow, goat, sheep and buffalo      | Dairy products                  | 12S rRNA                           | ≤25 ng (DNA of all species) | [128]     |
| Cow and goat                      | Milk powder                     | 12S rRNA                           | 0.1% (cow’s milk in goat’s milk) | [115]     |
| Camel                             | Milk mixtures                   | Heart development protein with EGF-like domain 1 (HEG1) (camel) Myostatin (mammalian species) | 1% (camel’s milk in cow’s milk) | [129]     |
| Multiplex real-time PCR—TaqMan probes | Cow and buffalo | milk | cytb | Allmilk: tRNA-Lys (cow), cytb (goat, sheep and buffalo) | [130]     |
| Cow, goat, sheep and buffalo      | Milk and cheeses                | Allcheese: β-actine (cow), prolactic receptor (sheep), growth hormone receptor (goat) | 0.32–32 ng of DNA of all species (Allmilk) | [131]     |
Table 3. Cont.

| Technique       | Target Species     | Application           | Target Gene | Sensitivity                                      | Reference |
|-----------------|--------------------|-----------------------|-------------|--------------------------------------------------|-----------|
| Cow and mare    | Dairy products     |                       | 12S rRNA    | 0.001 ng (DNA of cow milk, yogurt, and mare milk) | [132]     |
|                 |                    |                       |             | 0.005 ng (DNA of sour soup and Koumiss)          |           |
| Cow and goat    | Dairy and meat     | 12S rRNA              |             | 0.005 ng and 0.01 ng (DNA of goat’s milk and cheese, respectively) | [133]     |
|                 | products           |                       |             | 0.01 ng and 0.05 ng (DNA of cow’s milk and cheese, respectively) |           |
| Sheep and goat  | Dairy and meat     | 12S rRNA              |             | 0.001 ng and 0.01 ng (DNA of fresh and processed ovine meats, respectively) | [134]     |
|                 | products           |                       |             | 0.00025 ng, 0.005 ng and 0.01 ng (DNA of caprine meat, milk and cheese, respectively) |           |
| Camel and cow   | Dairy and meat     | 12S rRNA              |             | 1% (camel and cow milk in milk mixtures)          | [135]     |
|                 | products           |                       |             | 0.005–0.0025 ng (DNA of camel milk)              |           |
|                 |                    |                       |             | 0.05–0.001 ng (DNA of camel yogurt)             |           |
|                 |                    |                       |             | 0.001–0.0005 ng (DNA of camel milk beverage)    |           |
|                 |                    |                       |             | 0.00025–0.0001 ng (DNA of camel meat)           |           |
|                 |                    |                       |             | 0.0025–0.001 ng (DNA of cow milk)               |           |
|                 |                    |                       |             | 0.5–0.001 ng (DNA of cow yogurt)                |           |
|                 |                    |                       |             | 1–0.05 ng (DNA of cow cheese)                   |           |
|                 |                    |                       |             | 0.01 ng (DNA of cow acidic whey)                |           |
|                 |                    |                       |             | 0.001 ng (DNA of cow milk powder)               |           |
|                 |                    |                       |             | 0.0005–0.00025 ng (DNA of beef and beef jerky)  |           |
|                 |                    |                       |             | 0.005 ng (DNA of beef sausage)                  |           |
| HRM analysis    | Cow, sheep and     | Cheeses               | D-loop      | 0.1% (cow’s milk in mixed-milk)                  | [136]     |
|                 | goat               |                       |             |                                                  |           |
| Cow and buffalo | Buffalo dairy      | 12S rRNA and D-loop   |             | 1% (cow’s milk in mozzarella cheese)            | [137]     |
|                 | products           |                       |             |                                                  |           |
| ddPCR           | Cow and buffalo    | Mozzarella cheeses    | cytb        | 0.1% (cow’s milk in buffalo’s milk mozzarella cheese) | [138]     |
Table 3. Cont.

| Technique                     | Target Species                                    | Application                          | Target Gene | Sensitivity | Reference |
|-------------------------------|---------------------------------------------------|--------------------------------------|-------------|-------------|-----------|
| LAMP                          | Cow and buffalo                                   | Milk and meat mixtures               | D-loop      | 5% (cow’s milk in buffalo’s milk) | [139]     |
|                               | Cow and goat                                      | Milk and yogurt                      | cytb        | 2% (cow’s and goat’s milk)       | [140]     |
| NGS                           | Goat, sheep, cow and buffalo                      | Milk mixtures and cheeses             | 12S and 16S rRNA | .a         | [141]     |
| DNA biochip (microarray) kit  | Cow, pig, horse, donkey, sheep, goat, water buffalo, hare, rabbit, deer, chicken, turkey, ostrich, cat, and dog | Milk and meat mixtures, and dairy and meat products | 16S rRNA | 0.1% (Cow’s, goat’s and buffalo’s milk) | [142]     |
| DNA hybridization on microspheres | Cow, sheep and goat                              | Milk mixtures and yogurts            | cytb        | 0.01% (cow’s milk in goat’s yogurt and 0.05% (cow’s milk in sheep’s yogurt) | [143]     |
| Paper-based DNA biosensor     | Cow, sheep and goat                               | Milk mixture yogurts                 | cytb (cow and sheep) and prolactic receptor (sheep), | 0.01% of cow’s yogurt | [144]     |

ddPCR, droplet digital PCR; HRM, high-resolution melting; LAMP, loop-mediated isothermal amplification; NGS, next generation sequencing; SSR, simple sequence repeats; .a not reported.

4.1. 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 [88,89]. Plath et al. [92] 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 [95,98] and cytb genes [93,94]. 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 [92,95].

4.2. 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 [8,88,89,91]. It relies on the accurate design of primers to allow the amplification of a species-specific sequence based on endpoint 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 (Table 3). 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 (Table 3). 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. [105] 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. Following this work, Mafra et al. [106] 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 authors developed a duplex PCR with similar sensitivity and quantification range of cow’s milk in goat’s cheese [107]. 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. [110], who were able to simultaneous detect cow, sheep, goat, and water buffalo in dairy products. The applications of multiplex PCR to dairy product authentication are summarized in Table 3.

4.3. 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 [145]. 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 [88–90,146]. 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 [145]. As a result, most real-time PCR methods applied to dairy product authentication have used TaqMan probes (Table 3). 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) (Figure 4) (Table 3).

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. [130] to simultaneously detect cow’s and buffalo’s milks using specific fluorescent probes targeting the cytb gene of both species. Rentsch et al. [131] 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 [132], cow and goat [133], sheep and goat [134] and camel and cow [135]. The approaches were successfully applied to processed dairy products, achieving high sensitivities down to few pictograms of DNA (Table 3).
4.4. 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 us 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 [87,90,147–149]. HRM analysis targeting the mitochondrial D-loop region was able to discriminates 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 [136]. 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 [137].

4.5. 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 [150–152]. Recently, a ddPCR method targeting the cytb gene was developed to detect cow’s and buffalo’s milk in mozzarella cheese [138]. 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%). The authors concluded that, 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 [138].
4.6. 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 [87,153,154]. LAMP has also been applied for species identification in dairy products [139,140] (Table 3). 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 [139]. Kim and Kim [140] 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.

4.7. 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 [87,90,155,156]. 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 [141]. 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.

4.8. 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 this 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. [157] 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, the authors 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 [158]. Sardina et al. [159] described the use of SSR markers to discriminate among the most important Sicilian dairy goat breeds, aiming at the authentication of Girgentana dairy products. The authors identified three specific SSR markers that could be applied as a genetic traceability system of Girgentana dairy products, allowing the detection of adulterations due to Maltese and Derivata di Siria goat’s milk breeds.
5. (Bio)Sensors

Sensors are devices able to measure a physical quantity and convert it into a signal that can be read by an instrument. Chemical sensors measure chemical substances by chemical or physical responses, which can be designated as biosensors when using a biorecognition element [160]. The electronic tongue is an array device of non-specific and low-selective chemical sensors, possessing high stability and cross-sensitivity to different compounds. Dias et al. [161] developed a potentiometric electronic tongue with 36 cross-sensitivity sensors that was able to differentiate the five basic tastes (salty, sweet, acid, bitter and umami) and further detecting adulterations of goat’s milk with cow’s milk. Cross-validation of a model based on linear discriminant analysis of the recorded signal profiles allowed discriminating goat, cow and goat/cow raw skimmed milks with satisfactory sensitivity and specificity (over than 87% and 70%, respectively), suggesting its capacity in distinguishing the different species in various milk samples [161].

Biosensors base their principle on the direct recognition of a biological interaction between a receptor and the target molecule (proteins or DNA, immuno-or genosensors, respectively) by a transducer that produces a measurable signal. They can provide simple, fast, high-throughput, multitarget and low-cost detection, being considered as emerging and attractive tools for food analysis, with applications on GMO detection [154], food authentication [89] and allergen analysis [162]. Regarding dairy foods, recent studies have proposed both immunosensors [38,39] (Table 1) and genosensors [142,144] (Table 3) for species authentication. A miniaturized immunosensor with optical transduction based on ten planar silicon nitride waveguide Broad-Band Mach–Zehnder interferometers, targeting bovine k-casein, was developed by Angelopoulou et al. [38]. The approach provided the determination of cow’s milk in goat’s milk based on a competitive immunoassay, achieving a sensitivity of 0.04% (v/v) and a dynamic range of 0.1–1.0% (v/v) of cow’s milk in goat’s milk. The analytical performance of the proposed immunosensor was favorably compared with a competitive ELISA developed using the same monoclonal antibodies, but in a much shorter period of time (10 min) than ELISA (2 h). The immunoassay was considered a fast and sensitive tool, being suitable for incorporation into portable devices, thus having high potential for on-field applications [38]. Sakti et al. [39] developed an immunosensor with piezoelectric transduction (quartz crystal microbalance) for the detection of cow’s milk as an adulterant of goat’s milk. The method used a specific polyclonal antibody targeting a protein of 208 kDa (k-casein) as a marker of cow’s milk, not identified in goat’s milk, achieving a sensitivity of 1 ppm of cow’s milk.

Beltramo et al. [142] carried out a validation process for the low-cost and -density (LCD) array (MEAT 5.0 version) kit for food forensics based on a DNA biochip technology (microarray) that simultaneously detects 24 animal species, based on the analysis of PCR fragments (115–125 bp) of the 16S rRNA gene with specific capture probes. The LCD array kit was successfully validated to analyze mixtures of meats or milks, achieving limits of detection of 0.5% or 0.1%, respectively. Moreover, the assay did not show differences in the performance after analyzing heat treated mixtures, exhibiting high robustness regarding several key parameters and food ingredients.

Kounelli and Kalogianni [143] developed a DNA-based method that relied on hybridization of species-specific oligonucleotide on the surface of fluorescent microspheres, followed by flow cytometry analysis. The method consisted of DNA amplification with species-specific labeled primers targeting the cytb gene of each species (cow, sheep and goat), followed by hybridization of the single-strand biotinylated PCR products with species-specific oligonucleotide probes, carrying a NH$_2$ group at the 5′-end, which were attached to the surface of three different sets of carboxylated microspheres. The obtained hybrids were detected via a streptavidin–phycoerythrin conjugate, whose fluorescent signal is proportional to the DNA amount and achieved a sensitivity down to 0.01% of cow’s milk in goat’s milk and 0.05% in sheep’s milk. The method was successfully applied to detect milk species in milk mixtures and yogurts, exhibiting high reproducibility [143].
Recently, the same research group [84] developed a paper-based DNA biosensor for the detection of cow’s, sheep’s and goat’s in dairy products. Similarly to the above described approach, the method consisted of a first step of DNA amplification with biotin-labeled species-specific primers. Then, the single-strand biotinylated PCR products were hybridized with species-specific DNA probes carrying a poly-dA tail at one end and applied on the conjugate pad of the biosensor together with streptavidin-functionalized gold nanoparticles that provided the observation of the results by the naked eye. The biosensor revealed high specificity and high absolute (1.6 fmol of cow’s and goat’s and 3.1 fmol of sheep’s PCR products) and relative (0.01% of adulterant in yogurt) sensitivity, as well as good reproducibility.

6. Conclusions and Future Prospects

Species identification in milk and dairy products has been the subject of an increasing number of reports because of its importance regarding food authentication, but also in response to the growing consumers’ demands for label transparency. So far, several methodologies have been proposed to determine the species authentication in dairy products, relying on both proteins and DNA markers. Other techniques based on spectroscopy are also increasingly considered in the determination of food authenticity due to advantages related to sample preparation, rapidity, non-destructiveness, ease performance and potential for on-field use, although the need for expensive equipment, adequate databases and multicomponent analysis might restrain their use. The resumed advantages and drawbacks of the main techniques used for species authentication in dairy products are presented on Table 4. This information can be critically useful for selecting the method(s) for species authentication in dairy products, according to the intended application. One issue that should be specifically considered regards food processing since, depending on the selected analytical method, it might lead to false negative results as in the case of the immunochemical assays, or decreased sensitivity in the case of DNA-based methods and other techniques.

Of the protein-based methods, the proteomic approaches using MALDI-TOF MS have revealed a high number of advances in species identification in dairy products, particularly when combined with unsupervised statistical analysis. With the availability of databases with the reference spectra of milk and cheese proteins, the development of rapid and robust methods that do not require prior protein extraction and digestion is expected. It is also important to refer to the effectiveness of MALDI-TOF MS for selecting the suitable marker peptides for further bottom-up proteomic strategies, particularly by liquid chromatography coupled to tandem mass spectrometry. Despite the great technological advances in MS instrumentation and methods, the costly equipment and the need for specialized personal and databases are drawbacks that disable their wide application for routine analysis.

DNA-based methods have played an important role in species authentication of dairy products owing to their high specificity and sensitivity, simple performance and low/medium cost of analysis. Particularly, real-time PCR with specific probes targeting mtDNA markers has provided a high number of methods, with the advantages of multiplexing and quantitative analysis. More recently, ddPCR has provided promising alternative methods to real-time PCR, with the advantage of not requiring calibration curves for quantitative analysis, thus more advances in their application to authenticate dairy products being expected in the near future. The advent of high-throughput sequencing technologies has also shown applicability to dairy product authentication with the main advantage over Sanger sequencing of enabling multiple species identification in complex mixtures.

Biosensors are considered cutting-edge approaches for high-throughput, simple, fast and low-cost detection, with aptitude for multiplexing and on-site analysis. Despite their advantages, applicability to dairy species authentication, both as immuno- or genosensors, is still limited, being expected to increase in the near future. The combination of LAMP and biosensor is prospected to provide highly specific, sensitive and on-site analysis. However,
in biosensing analysis, considerable efforts are still required to provide quantitative analysis and applicability to processed foods.

Table 4. Summary of pros and cons of the main techniques applied for species identification in dairy products.

| Technique                              | Pros                                               | Cons                                      |
|----------------------------------------|----------------------------------------------------|-------------------------------------------|
| **Electrophoretic techniques**         | • Fast                                              | • Complex band pattern or co-migrating bands can lead to an equivocal interpretation of results |
|                                        | • Low cost                                          | • Inadequate for quantification, processed products and/or detecting adulteration by heat-treated bovine whey protein |
|                                        |                                                     | • Need of reference standards (IEF)       |
| **Immunochemical techniques**          | • Fast                                              | • Possible cross-reactivity leading to false positives |
|                                        | • Simple                                            | • Processing might lead to false negative results |
|                                        | • Low cost                                          | • Availability of specific antibodies     |
|                                        | • High sensitivity                                  |                                          |
|                                        | • Easy application in routine analysis              |                                          |
| **Chromatography coupled to mass spectrometry** | • High specificity                                  | • Costly equipment and maintenance       |
|                                        | • High sensitivity                                  | • Complex analysis                       |
|                                        | • Quantitative                                     | • Requires databases                     |
|                                        | • Possibility of multiplex                          | • Highly expertise technicians            |
|                                        |                                                     |                                          |
| **Spectroscopy**                       | • Fast                                              | • Requires a large database and chemometrics |
|                                        | • Simple                                            | • Expensive equipment (depending on the technique) |
|                                        | • High-throughput                                   |                                          |
|                                        | • Non-destructive                                   |                                          |
|                                        | • Capacity of portability (depending on the technique) |                                         |
| **PCR-RFLP**                           | • Simple                                            | • Not quantitative                       |
|                                        | • High specificity                                  |                                          |
| **Species-specific PCR**               | • Simple                                            | • Not quantitative                       |
|                                        | • High sensitivity                                  |                                          |
|                                        | • Possibility of multiplex                          |                                          |
| **Real-time PCR**                      | • High sensitivity                                  | • Moderate cost of equipment             |
|                                        | • High specificity                                  |                                          |
|                                        | • Quantitative                                     |                                          |
|                                        | • Possibility of multiplex                          |                                          |
|                                        | • Fast                                              |                                          |
| **Biosensors**                         | • Fast                                              | • Qualitative results                    |
|                                        | • User-friendly                                    | • Sensitivity can be low                 |
|                                        | • Low-cost                                          |                                          |
|                                        | • High-throughput                                   |                                          |
|                                        | • Potential of portability                          |                                          |
Funding: This work was funded by national funds (FCT, Fundação para a Ciência e Tecnologia) through the strategic funding of CIMO (UIDB/00690/2020) and LAQV-REQUIMTE (UIDB/50006/2020/UIDP/50006/2020). This study was also supported by the European Union through European Regional Development Fund (NORTE-01-0145-FEDER-000052) and SYSTEMIC (Knowledge Hub on Food and Nutrition Security, ERA-Net Cofund ERA-HDHL no. 696295). I. Mafra thanks FCT for funding through the Individual Call to Scientific Employment Stimulus (2021/03670/CEECIND). M. Honrado is grateful to FCT grant 2021.0819.BD, financed by POPH-QREN (subsidized by FSE and MCTES).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Downey, G. Advances in Food Authenticity Testing; Woodhead Publishing: Duxford, UK, 2016.
2. Amaral, J.S. Target and Non-Target Approaches for Food Authenticity and Traceability. Foods 2021, 10, 172. [CrossRef] [PubMed]
3. Committee on the Environment, Public Health and Food Safety. Draft Report the Food Crisis, Fraud in the Food Chain and the Control Thereof (2013/2091(INI)). Available online: https://www.europarl.europa.eu/doceo/document/ENVI-PR-519739_EN.pdf?redirect (accessed on 11 June 2021).
4. De la Fuente, M.A.; Juarez, M. Authentication assessment of dairy products. Crit. Rev. Food Sci. 2005, 45, 563–585. [CrossRef] [PubMed]
5. Amaral, J.S.M.I.; Pissard, A.; Pierna, J.A.F.; Baeten, V. Milk and milk products. In Foodintegrity Handbook; Morin, J.-F., Lees, M., Eds.; Eurofins Analytics France: Nantes, France, 2018; pp. 3–26.
6. Kamal, M.; Karoui, R. Analytical methods coupled with chemometric tools for determining the authenticity and detecting the adulteration of dairy products: A review. Trends Food Sci. Technol. 2019, 82, 77–95. [CrossRef]
7. Ortea, I.; O’Connor, G.; Maquet, A. Review on proteomics for food authentication. J. Proteom. 2016, 147, 212–225. [CrossRef]
8. Baptista, M.; Cunha, J.T.; Domingues, L. DNA-based approaches for dairy products authentication: A review and perspectives. Trends Food Sci. Technol. 2021, 109, 386–397. [CrossRef]
9. Villa, C.; Costa, J.; Oliveira, M.B.P.; Mafra, I. Bovine Milk Allergens: A Comprehensive Review. Compr. Rev. Food Sci. F 2018, 17, 137–164. [CrossRef]
10. Pizzano, R.; Nicolai, M.A.; Manzo, C.; Addeo, F. Authentication of dairy products by immunochemical methods: A review. Dairy Sci. Technol. 2011, 91, 77–95. [CrossRef]
11. Pesic, M.; Barac, M.; Vrivic, M.; Ristic, N.; Macej, O.; Stanojevic, S. Qualitative and quantitative analysis of bovine milk adulteration in caprine and ovine milks using native-PAGE. Food Chem. 2011, 125, 1443–1449. [CrossRef]
12. Spoljaric, J.; Mikulec, N.; Plavljanic, D.; Radeljevic, B.; Havranek, J.; Antunac, N. Proving the adulteration of ewe and goat cheeses with cow milk using the reference method of isoelectric focusing of gamma-casein. Mljekarstvo 2013, 63, 115–121.
13. Somma, A.; Ferranti, P.; Addeo, F.; Mauriello, R.; Chianese, L. Peptidomic approach based on combined capillary isoelectric focusing and mass spectrometry for the characterization of the plasmin primary products from bovine and water buffalo beta-casein. J. Chromatogr. A 2008, 1192, 294–300. [CrossRef] [PubMed]
14. Trimboli, F.; Costanzo, N.; Lopreato, V.; Ceniti, C.; Morittu, V.M.; Spina, A.; Britt, D. Detection of buffalo milk adulteration with cow milk by capillary electrophoresis analysis. J. Dairy Sci. 2019, 102, 5962–5970. [CrossRef]
15. Molina, E.; Ramos, M.; Amigo, L. Characterisation of the casein fraction of Iberico cheese by electrophoretic techniques. J. Sci. Food Agric. 2002, 82, 1240–1245. [CrossRef]
22. Reid, I.M.; O’Donnell, C.P.; Downey, G. Recent technological advances for the determination of food authenticity. *Trends Food Sci. Technol.* 2006, 17, 344–353. [CrossRef]

23. Hurley, I.P.; Coleman, R.C.; Ireland, H.E.; Williams, J.H.H. Use of sandwich IgG ELISA for the detection and quantification of adulteration of milk and soft cheese. *Int. Dairy J.* 2006, 16, 805–812. [CrossRef]

24. Asensio, L.; Gonzalez, I.; Garcia, T.; Martin, R. Determination of food authenticity by enzyme-linked immunosorbent assay (ELISA). *Food Control* 2008, 19, 1–8. [CrossRef]

25. Pizzano, R.; Salimei, E. Isoelectric Focusing and ELISA for Detecting Adulteration of Donkey Milk with Cow Milk. *J. Agric. Food Chem.* 2014, 62, 5853–5858. [CrossRef] [PubMed]

26. Hurley, I.P.; Coleman, R.C., Ireland, H.E.; Williams, J.H.H. Measurement of bovine IgG by indirect competitive ELISA as a means of detecting milk adulteration. *J. Dairy Sci.* 2004, 87, 543–549. [CrossRef]

27. Hurley, I.P.; Ireland, H.E.; Coleman, R.C.; Williams, J.H.H. Application of immunological methods for the detection of species adulteration in dairy products. *Int. J. Food Sci. Technol.* 2004, 39, 873–878. [CrossRef]

28. Zelenakova, L.; Golian, J.; Ziaic, P. Application of ELISA tests for the detection of goat milk in sheep milk. *Milchwissenschaft* 2008, 63, 137–141.

29. Song, H.X.; Xue, H.Y.; Han, Y. Detection of cow’s milk in Shaanxi goat’s milk with an ELISA assay. *Food Control* 2011, 22, 883–887. [CrossRef]

30. Costa, N.; Ravasco, F.; Miranda, R.; Duthoit, M.; Roseiro, L.B. Evaluation of a commercial ELISA method for the quantitative detection of goat and cow milk in ewe milk and cheese. *Small Rumin. Res.* 2008, 79, 73–79. [CrossRef]

31. Zelenakova, L.; Židek, R.; Čanigová, M.; Ziarovska, J.; Zajác, P.; Marsalkova, L.; Fikselová, M.; Golian, J. Research And Practice: Quantification Of Raw And Heat-Treated Cow Milk In Sheep Milk, Cheese And Bryndza By ELISA Method. *Potravinarstvo* 2016, 10, 14–22. [CrossRef]

32. Stanciuc, N.; Rapeanu, G. Identification of adulterated sheep and goat cheeses marketed in Romania by immunocromatographic assay. *Food Agric. Immunol.* 2010, 21, 157–164. [CrossRef]

33. Zelenakova, L.; Židek, R.; Čanigová, M.; Ziarovska, J.; Zajác, P.; Marsalkova, L.; Fikselová, M.; Golian, J. Reliability of cow casein quantitation in sheep milk and cheese by ELISA method. *J. Food Phys.* 2010, 23, 22–26.

34. Zelenakova, L.; Židek, R.; Canigova, M. Optimization of ELISA method for detection of bovine beta-lactoglobulin in sheep milk and sheep milk products. *Milchwissenschaft* 2011, 66, 278–281.

35. Galan-Malo, P.; Mendiara, I.; Razquin, P.; Mata, L. Validation of a rapid lateral flow method for the detection of cows’ milk in water buffalo, sheep or goat milk. *Food Addit. Contam. Part A* 2018, 35, 599–604. [CrossRef] [PubMed]

36. Ren, Q.R.; Zhang, H.; Guo, H.Y.; Jiang, L.; Tian, M.; Ren, F.Z. Detection of cow milk adulteration in yak milk by ELISA. *J. Dairy Sci.* 2014, 97, 6000–6006. [CrossRef] [PubMed]

37. Lopez-Calleja, I.M.; Gonzalez, I.; Fajardo, V.; Hernandez, P.E.; Garcia, T.; Martin, R. Application of an indirect ELISA and a PCR technique for detection of cows’ milk in sheep’s and goats’ milk cheeses. *Int. Dairy J.* 2007, 17, 87–93. [CrossRef]

38. Angelopoulou, I.; Botsialas, A.; Salapatas, A.; Petrou, P.S.; Haasnoot, W.; Makarona, E.; Jobst, G.; Goustouridis, D.; Siafaka-Kapadai, A.; Raptis, I.; et al. Assessment of goat milk adulteration with a label-free monolithically integrated optoelectronic biosensor. *Anal. Bioanal. Chem.* 2015, 407, 3995–4004. [CrossRef]

39. Sakti, S.P.; Chabibah, N.; Ayu, S.P.; Padaga, M.C.; Aulani’am, A. Development of QCM Biosensor with Specific Cow Milk Protein Antibody for Candidate Milk Adulteration Detection. *J. Sens.* 2016, 17, 179–186. [CrossRef]

40. Veloso, A.C.A.; Teixeira, N.; Ferreira, I.M.P.L.V.O. Separation and quantification of the major casein fractions by reverse-phase high-performance liquid chromatography and urea-polyacrylamide gel electrophoresis—Detection of milk adulterations. *J. Chromatogr. A* 2002, 967, 209–218. [CrossRef]

41. Enge, G.; Elez, D.; Fondrini, F.; Bonizzi, I.; Feligini, M.; Aleandri, R. High-performance liquid chromatography of governing liquid to detect illegal bovine milk’s addition in water buffalo Mozzarella: Comparison with results from raw milk and cheese matrix. *J. Chromatogr. A* 2005, 1094, 169–174. [CrossRef]

42. Rodriguez, N.; Ortiz, M.C.; Sarabia, L.; Redaida, E. Analysis of protein chromatographic profiles joint to partial least squares to detect adulterations in milk mixtures and cheeses. *Talanta* 2010, 81, 255–264. [CrossRef]

43. Manzo, N.; Pizzolongo, F.; Montefusco, I.; Romano, A.; Masi, P.; Romano, R. Using whey proteins to detect the addition of bovine milk fat in buffalo cream destined for the butter-making process. *Food Control* 2017, 81, 164–167. [CrossRef]

44. Buckley, M.; Melton, N.D.; Montgomery, J. Proteomics analysis of ancient food vessel stitching reveals > 4000-year-old milk protein. *Rapid Commun. Mass Spectrom.* 2013, 27, 531–538. [CrossRef] [PubMed]

45. Di Girolamo, F.; Masotti, A.; Salvatori, G.; Scapaticci, M.; Muraca, M.; Putignani, L. A Sensitive and Effective Proteomic Approach to Identify She-Donkey’s and Goat’s Milk Adulterations by MALDI-TOF MS Fingerprinting. *Int. J. Mol. Sci.* 2014, 15, 13697–13719. [CrossRef] [PubMed]

46. Cozzolino, R.; Passalacqua, S.; Salemi, S.; Garozzo, D. Identification of adulteration in water buffalo mozzarella and in ewe cheese by using whey proteins as biomarkers and matrix-assisted laser desorption/ionization mass spectrometry. *J. Mass Spectrom.* 2002, 37, 985–991. [CrossRef] [PubMed]

47. Nicolaou, N.; Xu, Y.; Goodacre, R. MALDI-MS and multivariate analysis for the detection and quantification of different milk species. *Anal. Bioanal. Chem.* 2011, 399, 3491–3502. [CrossRef] [PubMed]
48. Kuckova, S.; Zitkova, K.; Novotny, O.; Smirnova, T. Verification of cheeses authenticity by mass spectrometry. J. Sep. Sci. 2019, 42, 3487–3496. [CrossRef] [PubMed]
49. Rau, J.; Korte, N.; Dyk, M.; Wenninger, O.; Schreiter, P.; Hiller, E. Rapid animal species identification of feta and mozzarella cheese using MALDI-TOF mass-spectrometry. Food Control 2020, 117, 107349. [CrossRef]
50. Calvano, C.D.; De Ceglie, C.; Monopoli, A.; Zambonin, C.G. Detection of sheep and goat milk adulterations by direct MALDI-TOF MS analysis of milk tryptic digests. J. Mass Spectrom. 2012, 47, 1141–1149. [CrossRef]
51. Cair, S.; Pinto, G.; Nicolai, M.A.; Chianese, L.; Addeo, F. Simultaneously tracing the geographical origin and presence of bovine milk in Italian water buffalo Mozzarella cheese using MALDI-TOF data of casein signature peptides. Anal. Bioanal. Chem. 2016, 408, 5609–5621. [CrossRef]
52. Russo, R.; Rega, C.; Chambery, A. Rapid detection of water buffalo ricotta adulteration or contamination by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. Rapid Commun. Mass Spectrom. 2016, 30, 497–503. [CrossRef]
53. Nardiello, D.; Natale, A.; Palermo, C.; Quinto, M.; Centonze, D. Milk authenticity by ion-trap proteomics following multi-enzyme digestion. Food Chem. 2018, 244, 317–323. [CrossRef]
54. Cuiolo, M.; Caira, S.; Fierro, O.; Pinto, G.; Picariello, G.; Addeo, F. Toward milk speciation through the monitoring of casein proteotypic peptides. Rapid Commun. Mass Spectrom. 2010, 24, 1687–1696. [CrossRef] [PubMed]
55. Camerini, S.; Montepeloso, E.; Casella, M.; Crescenzi, M.; Marianella, R.M.; Fuselli, F. Mass spectrometry detection of fraudulent use of cow whey in water buffalo, sheep, or goat Italian ricotta cheese. Food Chem. 2016, 197, 1240–1248. [CrossRef] [PubMed]
56. Galliano, F.; Saletti, R.; Cunsolo, V.; Foti, S.; Martella, D.; Bordonaro, S.; D’Urso, G. Identification and characterization of a new beta-casein variant in goat milk by high-performance liquid chromatography with electrospray ionization mass spectrometry and matrix-assisted laser desorption/ionization mass spectrometry. Rapid Commun. Mass Spectrom. 2004, 18, 1972–1982. [CrossRef] [PubMed]
57. Russo, R.; Severino, V.; Mendez, A.; Lliberia, J.; Parente, A.; Chambery, A. Detection of buffalo mozzarella adulteration by an ultra-high performance liquid chromatography tandem mass spectrometry methodology. J. Mass Spectrom. 2012, 47, 1407–1414. [CrossRef] [PubMed]
58. Gunning, Y.; Fong, L.K.W.; Watson, A.D.; Philo, M.; Kemsley, E.K. Quantitative authenticity testing of buffalo mozzarella via alpha(s1)-Casein using multiple reaction monitoring mass spectrometry. Food Control 2019, 101, 189–197. [CrossRef]
59. Sforza, S.; Aquino, G.; Cavastrò, V.; Galaverna, G.; Mucchetti, G.; Dossena, A.; Marchelli, R. Proteolytic oligopeptides as molecular markers for the presence of cows’ milk in fresh cheeses derived from sheep milk. Int. Dairy J. 2008, 18, 1072–1076. [CrossRef]
60. Czerwenka, C.; Muller, L.; Lindner, W. Detection of the adulteration of water buffalo milk and mozzarella with cow’s milk by liquid chromatography-mass spectrometry analysis of beta-lactoglobulin variants. Food Chem. 2010, 122, 901–908. [CrossRef]
61. Fuselli, F.; Tidona, F. Foreign milk in sheep’s, goat’s and water buffalo milk cheeses. In Handbook of Cheese in Health: Production, Nutrition and Medical Science; Preedy, V.R., Watson, R.R., Patel, V.B., Eds.; Wageningen Academic Publishers: Wageningen, The Netherlands, 2013; pp. 397–411.
62. Ke, X.; Zhang, J.S.; Lai, S.Y.; Chen, Q.; Zhang, Y.; Jiang, Y.R.; Mo, W.M.; Ren, Y.P. Quantitative analysis of cow whole milk and whey powder adulteration percentage in goat and sheep milk products by isotopic dilution-ultra-high performance liquid chromatography-tandem mass spectrometry. Anal. Bioanal. Chem. 2017, 409, 213–224. [CrossRef]
63. Jia, W.; Dong, X.Y.; Shi, L.; Chu, X.G. Discrimination of Milk from Different Animal Species by a Foodomics Approach Based on High-Resolution Mass Spectrometry. J. Agric. Food Chem. 2020, 68, 6638–6645. [CrossRef]
64. Hrbek, V.; Vaclavík, L.; Elich, O.; Hajšlová, J. Authentication of milk and milk-based foods by direct analysis in real time ionization-high resolution mass spectrometry (DART-HRMS) technique: A critical assessment. Food Control 2014, 36, 138–145. [CrossRef]
65. Blasi, F.; Lombardi, G.; Damiani, P.; Simonetti, M.S.; Giua, L.; Cossignani, L. Triacylglycerol stereospecific analysis and linear inverse analysis for milk speciation. J. Dairy Res. 2013, 80, 144–151. [CrossRef] [PubMed]
66. Chmilenko, F.A.; Minaeva, N.P.; Sidorova, L.P. Complex chromatographic determination of the adulteration of dairy products: A new approach. J. Anal. Chem. 2011, 66, 572–581. [CrossRef]
67. Cossignani, L.; Pollini, L.; Blasi, F. Invited review: Authentication of milk by direct and indirect analysis of triacylglycerol molecular species. J. Dairy Sci. 2019, 102, 5871–5882. [CrossRef] [PubMed]
68. Bratu, A.; Mihailescu, M.; Hangau, A.; Chira, N.A.; Todesca, M.C.; Rosca, S. Gas Chromatography Coupled with Chemometric Method for Authentication of Romanian Cheese. Rev. Chim.-Buchar. 2012, 63, 1099–1102.
69. Vieitez, I.; Irigaray, B.; Callejas, N.; Gonzalez, V.; Gimenez, S.; Arechavala, A.; Grompone, M.; Gambaro, M. Composition of fatty acids and triglycerides in goat cheeses and study of the triglyceride composition of goat milk and cow milk blends. J. Food Compos. Anal. 2016, 48, 95–101. [CrossRef]
70. Karoui, R.; De Baerdemaecker, J. A review of the analytical methods coupled with chemometric tools for the determination of the quality and identity of dairy products. Food Chem. 2007, 102, 621–640. [CrossRef]
71. Lyuyks, D.M.A.M.; Van Ruth, S.M. An overview of analytical methods for determining the geographical origin of food products. Food Chem. 2008, 107, 897–911. [CrossRef]
72. Nicolaou, N.; Xu, Y.; Goodacre, R. Fourier transform infrared spectroscopy and multivariate analysis for the detection and quantification of different milk species. J. Dairy Sci. 2010, 93, 5651–5660. [CrossRef]
73. Domingo, E.; Tirelli, A.A.; Nunes, C.A.; Guerreiro, M.C.; Pinto, S.M. Melamine detection in milk using vibrational spectroscopy and chemometrics analysis: A review. Food Res. Int. 2014, 60, 131–139. [CrossRef]

74. Sassi, M.; Arena, S.; Scalon, A. MALDI-TOF-MS Platform for Integrated Proteomic and Peptidomic Profiling of Milk Samples Allows Rapid Detection of Food Adulterations. J. Agric. Food Chem. 2015, 63, 7093. [CrossRef]

75. Calvano, C.D.; De Ceccoli, C.; Aresta, A.; Facchini, L.A.; Zambonin, C.G. MALDI-TOF mass spectrometric determination of intact phospholipids as markers of illegal bovine milk adulteration of high-quality milk. Anal. Bioanal. Chem. 2013, 405, 1641–1649. [CrossRef][PubMed]

76. Motta, T.M.C.; Hoff, R.B.; Barreto, F.; Andrade, R.B.S.; Lorenzini, D.M.; Meneghini, L.Z.; Pizzolato, T.M. Detection and confirmation of milk adulteration with cheese whey using proteomic-like sample preparation and liquid chromatography-electrospray-tandem mass spectrometry analysis. Talanta 2015, 120, 498–505. [CrossRef][PubMed]

77. Bernardi, N.; Benetti, G.; Haouet, N.M.; Sergi, M.; Grotta, L.; Marchetti, S.; Castellani, F.; Martin, G. A rapid high-performance liquid chromatography-tandem mass spectrometry assay for unambiguous detection of different milk species employed in cheese manufacturing. J. Dairy Sci. 2015, 98, 8405–8413. [CrossRef][PubMed]

78. Zhang, J.S.; Lai, S.Y.; Cai, Z.X.; Chen, Q.; Huang, B.F.; Ren, Y.P. Determination of bovine lactoferrin in dairy products by ultra-high performance liquid chromatography-tandem mass spectrometry based on tryptic signature peptides employing an iso-labeled winged peptide as internal standard. Anal. Chem. Acta 2014, 829, 33–39. [CrossRef][PubMed]

79. Hruzikova, J.; Milde, D.; Krajancova, P.; Ranc, V. Discrimination of Cheese Products for Authenticity Control by Infrared Spectroscopy. J. Agric. Food Chem. 2012, 60, 1845–1849. [CrossRef]

80. Cirak, O.; Icyer, N.C.; Durak, M.Z. Rapid detection of adulteration of milks from different species using Fourier Transform Infrared Spectroscopy (FTIR). J. Dairy Sci. 2018, 88, 222–225. [CrossRef][PubMed]

81. Cirak, O.; Teixeira, J.L.D.; Carames, E.T.D.; Baptista, D.P.; Gigante, M.L.; Pallone, J.A.L. Rapid adulteration detection of yogurt and cheese samples according to species’ origin. Food Sci. Nutr. 2020, 8, 3262–3273. [CrossRef]

82. Teixeira, J.L.D.; Carames, E.T.D.; Baptista, D.P.; Gigante, M.L.; Pallone, J.A.L. Rapid adulteration detection of yogurt and cheese made from goat milk by vibrational spectroscopy and chemometric tools. J. Food Compos. Anal. 2021, 96, 103712. [CrossRef]

83. Teixeira, J.L.D.; Carames, E.T.D.; Baptista, D.P.; Gigante, M.L.; Pallone, J.A.L. Rapid adulteration detection of yogurt and cheese made from goat milk by vibrational spectroscopy and chemometric tools. J. Food Compos. Anal. 2021, 96, 103712. [CrossRef]

84. Brandao, M.P.; Neto, M.G.; dos Anjos, V.D.; Bell, M.J.V. Detection of adulteration of goat milk powder with bovine milk powder by front-face and time-resolved fluorescence. Food Control 2017, 81, 168–172. [CrossRef]

85. Velioglu, S.D.; Ercioglu, E.; Boyaci, I.H. Rapid discrimination between buffalo and cow milk and detection of adulteration of buffalo milk with cow milk using synchronous fluorescence spectroscopy in combination with multivariate methods. J. Dairy Res. 2017, 84, 214–219. [CrossRef][PubMed]

86. Teixeira, J.L.D.; Carames, E.T.D.; Baptista, D.P.; Gigante, M.L.; Pallone, J.A.L. Rapid adulteration detection of yogurt and cheese made from goat milk by vibrational spectroscopy and chemometric tools. J. Food Compos. Anal. 2021, 96, 103712. [CrossRef]

87. Grazina, L.; Amaral, J.S.; Mafra, I. Botanical origin authentication of dietary supplements by DNA-based approaches. Compr. Rev. Food Sci. Food Saf. 2020, 19, 1080–1109. [CrossRef][PubMed]

88. Mafra, I.; Ferreira, I.M.P.L.V.O.; Oliveira, M.B.P.P. Food authentication by PCR-based methods. Eur. Food Res. Technol. 2008, 227, 649–665. [CrossRef][PubMed]

89. Amaral, J.S.; Meira, L.; Oliveira, M.B.P.P.; Mafra, I. Advances in authenticity testing for meat speciation. In Advances in Food Authenticity Testing; Downey, G.; Olive, E., Eds.; Woodhead Publishing Ltd.: Sawston, UK; 2016; pp. 369–414.

90. Fernandes, T.J.R.; Amaral, J.S.; Mafra, I. DNA barcode markers applied to seafood authentication: An updated review. Crit. Rev. Food Sci. 2021, 61, 3904–3935. [CrossRef]

91. Kalgoggianni, D.P. DNA-based analytical methods for milk authentication. Eur. Food Res. Technol. 2018, 244, 775–793. [CrossRef]

92. Plaza, A.; Krause, I.; Einspanier, R. Species identification in daily products by three different DNA-based techniques. Z. Lebensm. Unt-Forsch. A 1997, 205, 437–441. [CrossRef]

93. Lanzilao, I.; Burgalassi, F.; Fancelli, S.; Settimelli, M.; Fani, R. Polymerase chain reaction-restriction fragment length polymorphism analysis of mitochondrial cyt b gene from species of dairy interest. J. Anim. Int. 2005, 88, 128–135. [CrossRef]

94. Abdel-Rahman, S.M.; Ahmed, M.M.M. Rapid and sensitive identification of buffalo’s, cattle’s and sheep’s milk using species-specific PCR and PCR-RFLP techniques. Food Control 2007, 18, 1246–1249. [CrossRef]

95. Otaviano, A.R.; Lima, A.L.F.; Laureano, M.M.M.; Sena, J.A.D.; de Albuquerque, L.G.; Tonhati, H. beta-casein gene polymorphism permits identification of bovine milk mixed with babuline milk in mozzarella cheese. Genet. Mol. Biol. 2008, 31, 902–905. [CrossRef]

96. Abdelfatah, E.N.; El-Araby, I.E.; Mohamed, A.A. Identification of species adulteration in raw milk and dairy products using polymerase chain reaction—Restriction fragment length polymorphism. System 2015, 15, 332–338.

97. Ewida RM, E.-M.D. Species adulteration in raw milk samples using polymerase chain reaction-restriction fragment length polymorphism. Vet. World 2018, 11, 830–833. [CrossRef][PubMed]

98. Vafin, R.R.; Gallstryn, A.G.; Tyulkin, S.V.; Gilmanov, K.K.; Yurova, E.A.; Semipyatniy, V.K.; Bigaeva, A.V. Species identification of ruminant milk by genotyping of the K-casein gene. J. Dairy Sci. 2022, 105, 1004–1113. [CrossRef][PubMed]

99. Lopez-Calleja, I.; Gonzalez, I.; Fajardo, V.; Rodriguez, M.A.; Hernandez, P.E.; Garcia, T.; Martin, R. Rapid detection of cows’ milk in sheeps’ and goats’ milk by a species-specific polymerase chain reaction technique. J. Dairy Sci. 2004, 87, 2839–2845. [CrossRef]
100. Lopez-Calleja, I.; Gonzalez, I.; Fajardo, V.; Martin, I.; Hernandez, P.E.; Garcia, T.; Martin, R. Application of polymerase chain reaction to detect adulteration of sheep’s milk with goats’ milk. *J. Dairy Sci.* 2005, 88, 3115–3120. [CrossRef]

101. Lopez-Calleja, I.; Alonso, I.G.; Fajardo, V.; Rodriguez, M.A.; Hernandez, P.E.; Garcia, T.; Martin, R. PCR detection of cows’ milk in water buffalo milk and mozzarella cheese. *Int. J. Dairy Sci.* 2005, 15, 1122–1129. [CrossRef]

102. Maskova, E.; Paulickova, I. PCR-based detection of cow’s milk in goat and sheep cheeses marketed in the Czech Republic. *Czech J. Food Sci.* 2006, 24, 127–132. [CrossRef]

103. Diaz, I.L.C.; Alonso, I.G.; Fajardo, V.; Martin, I.; Hernandez, P.; Lacarra, T.G.; de Santos, R.M. Application of a polymerase chain reaction to detect adulteration of ovine cheeses with caprine milk. *Eur. Food Res. Technol.* 2007, 225, 345–349. [CrossRef]

104. Reale, S.; Campanella, A.; Merigioni, A.; Pilla, F. A novel method for species identification in milk and milk-based products. *J. Dairy Res.* 2008, 75, 107–112. [CrossRef]

105. Bottero, M.T.; Civera, T.; Nucera, D.; Rosati, S.; Sacchi, P.; Turi, R.M. A multiplex polymerase chain reaction for the identification of cows’, goats’ and sheep’s milk in dairy products. *Int. Dairy J.* 2003, 13, 277–282. [CrossRef]

106. Mafra, I.; Ferreira, I.M.P.L.V.O.; Ferreira, I.M.P.L.V.O.; Oliveira, B.P.P. A novel approach to the quantification of bovine milk in ovine cheeses using a duplex polymerase chain reaction method. *J. Agric. Food Chem.* 2004, 52, 4943–4947. [CrossRef] [PubMed]

107. Mafra, I.; Roxo, A.; Ferreira, I.M.P.L.V.O.; Oliveira, M.B.P.P. A duplex polymerase chain reaction for the quantitative detection of cows’ milk in goats’ milk cheese. *Int. Dairy J.* 2007, 17, 1132–1138. [CrossRef]

108. Bai, W.L.; Yin, R.H.; Zhao, S.J.; Dou, Q.L.; Yang, J.C.; Jiang, W.Q.; Zhao, Z.H.; Luo, G.B. Rapid detection of bovine milk in yak milk using a polymerase chain reaction technique. *J. Dairy Sci.* 2009, 92, 1354–1360. [CrossRef]

109. De, S.; Brahma, B.; Polley, S.; Mukherjee, A.; Banerjee, D.; Gohain, M.; Singh, K.P.; Singh, R.; Datta, T.K.; Goswami, S.L. Simplex and duplex PCR assays for species specific identification of cattle and buffalo milk and cheese. *Food Control* 2011, 22, 690–696. [CrossRef]

110. Goncalves, J.; Pereira, F.; Amorim, A.; van Asch, B. New Method for the Simultaneous Identification of Cow, Sheep, Goat, and Water Buffalo in Dairy Products by Analysis of Short Species-Specific Mitochondrial DNA Targets. *J. Agric. Food Chem.* 2012, 60, 10480–10485. [CrossRef] [PubMed]

111. Rodrigues, N.P.A.; Givisiez, P.E.N.; Queiroga, R.C.R.E.; Azevedo, P.S.; Gebreyes, W.A.; Oliveira, C.J.B. Milk adulteration: Detection of bovine milk in bulk goat milk produced by smallholders in northeastern Brazil by a duplex PCR assay. *J. Dairy Sci.* 2012, 95, 2749–2752. [CrossRef] [PubMed]

112. Gololini, L.P.; Carvalho, A.C.; Casesa, R.S.; Lopes, C.S.C.; Deliza, R.; Paschoalin, V.M.F.; Silva, J.T. Sensory analysis and species-specific PCR detect bovine milk adulteration of frescal (fresh) goat cheese. *J. Dairy Sci.* 2014, 97, 6693–6699. [CrossRef] [PubMed]

113. Tortorici, L.; Di Gerlando, R.; Tolone, M.; Mastrangelo, S.; Sardina, M.T. 12S rRNA mitochondrial gene as marker to trace Sicilian mono-species dairy products. *Livest. Sci.* 2016, 193, 39–44. [CrossRef]

114. Di Pinto, A.; Terio, V.; Marchetti, P.; Bottaro, M.; Mottola, A.; Bossolo, G.; Bonerba, E.; Ceci, E.; Tantillo, G. DNA-based approach for species identification of goat-milk products. *Food Chem.* 2017, 229, 93–97. [CrossRef]

115. Liao, J.; Liu, Y.F.; Ku, T.; Liu, M.H.; Huang, Y. Qualitative and quantitative identification of adulteration of milk powder using DNA extracted with a novel method. *J. Dairy Sci.* 2010, 103, 1657–1663. [CrossRef]

116. Deng, L.; Li, A.L.; Gao, Y.; Shen, T.; Yue, H.T.; Miao, J.; Li, R.R.; Yang, J. Detection of the Bovine Milk Adulterated in Camel, Horse, and Goat Milk Using Duplex PCR. *Food Anal. Method* 2020, 13, 560–567. [CrossRef]

117. Guerreiro, J.S.; Fernandes, P.; Bardsley, R.G. Identification of the species of origin of milk in cheeses by multivariate statistical analysis of polymerase chain reaction electrophotERIC patterns. *Int. Dairy J.* 2012, 25, 42–45. [CrossRef]

118. Lopparelli, R.M.; Cardazzo, B.; Balzan, S.; Giaccone, V.; Novelli, E. Real-time TaqMan polymerase chain reaction detection and quantification of cow DNA in pure water buffalo mozzarella cheese: Method validation and its application on commercial samples. *J. Agric. Food Chem.* 2007, 55, 3429–3434. [CrossRef] [PubMed]

119. Dabrowska, A.; Malecka, E.; Bania, J.; Zelazko, M.; Szoltysek, M.; Chrzanowska, J. Quality of UHT goat’s milk in Poland evaluated by real-time PCR. *Small Rumin. Res.* 2010, 94, 32–37. [CrossRef]

120. Marchetti, P.; Mottola, A.; Tantillo, G.; Castrica, M.; Di Pinto, A. Short communication: Detection of undeclared presence of bovine milk in buffalo yogurt. *J. Dairy Sci.* 2011, 104, 4056–4061. [CrossRef]

121. Agrimonti, C.; Pirondini, A.; Marramioli, M.; Marmioli, N. A quadruple PCR (qPCR) assay for adulteration in dairy products. *Food Chem.* 2015, 187, 58–64. [CrossRef]

122. Lopez-Calleja, I.; Gonzalez, I.; Fajardo, V.; Martin, I.; Hernandez, P.E.; Garcia, T.; Martin, R. Quantitative detection of goats’ milk in sheep’s milk by real-time PCR. *Food Control* 2007, 18, 1466–1473. [CrossRef]

123. Lopez-Calleja, I.; Gonzalez, I.; Fajardo, V.; Martin, I.; Hernandez, P.E.; Garcia, T.; Martin, R. Real-time TaqMan PCR for quantitative detection of cows’ milk in ewes’ milk mixtures. *Int. Dairy J.* 2007, 17, 729–736. [CrossRef]

124. Zhang, C.L.; Fowler, M.R.; Scott, N.W.; Lawson, G.; Slater, A. A TaqMan real-time PCR system for the identification and quantification of bovine DNA in meats, milks and cheeses. *Food Control* 2007, 18, 1149–1158. [CrossRef]

125. Dalmasso, A.; Civera, T.; La Neve, F.; Bottero, M.T. Simultaneous detection of cow and buffalo milk in mozzarella cheese by Real-Time PCR assay. *Food Chem.* 2011, 124, 362–366. [CrossRef]

126. Dalmasso, A.; Sacchi, P.; Bottero, M.T. Development of a real-time PCR assay for the detection of cow and donkey milk. *Eur. Food Res. Technol.* 2012, 235, 47–52. [CrossRef]
127. Drummond, M.G.; Brasil, B.S.A.F.; Dalsecco, L.S.; Brasil, R.S.A.F.; Teixeira, L.V.; Oliveira, D.A.A. A versatile real-time PCR method to quantify bovine contamination in buffalo products. *Food Control* 2013, 29, 131–137. [CrossRef]

128. Di Domenico, M.; Di Giuseppe, M.; Rodriguez, J.D.W.; Camma, C. Validation of a fast real-time PCR method to detect fraud and mislabeling in milk and dairy products. *J. Dairy Sci.* 2017, 100, 106–112. [CrossRef] [PubMed]

129. Wang, Z.Y.; Li, T.T.; Yu, W.J.; Qiao, L.; Liu, R.; Li, S.S.; Zhao, Y.; Yang, S.M.; Chen, A.L. Determination of content of camel milk in adulterated milk samples by normalized real-time polymerase chain reaction system based on single-copy nuclear genes. *J. Sci. Food Agric.* 2020, 100, 3465–3470. [CrossRef]

130. Cottenet, G.; Blancpain, C.; Golay, P.A. Simultaneous detection of cow and buffalo species in milk from China, India, and Pakistan using multiplex real-time PCR. *J. Dairy Sci.* 2011, 94, 3787–3793. [CrossRef]

131. Rentsch, J.; Weibel, S.; Ruf, J.; Eugster, A.; Beck, K.; Koppel, R. Interlaboratory validation of two multiplex quantitative real-time PCR methods to determine species DNA of cow, sheep and goat as a measure of milk proportions in cheese. *Eu. Food Res. Technol.* 2013, 236, 217–227. [CrossRef]

132. Guo, L.; Qian, J.P.; Guo, Y.S.; Hai, X.; Liu, G.Q.; Luo, J.X.; Ya, M. Simultaneous identification of bovine and equine DNA in milks and dairy products inferred from triplex TaqMan real-time PCR technique. *J. Dairy Sci.* 2018, 101, 6776–6786. [CrossRef]

133. Guo, L.; Ya, M.; Hai, X.; Guo, Y.S.; Li, C.D.; Xu, W.L.; Liao, C.S.; Feng, W.; Cai, Q. A simultaneous triplex TaqMan real-time PCR approach for authentication of caprine and bovine meat, milk and cheese. *Int. Dairy J.* 2019, 95, 58–64. [CrossRef]

134. Guo, L.; Yu, Y.; Xu, W.L.; Li, C.D.; Liu, G.Q.; Qi, L.M.G.; Luo, J.X.; Guo, Y.S. Simultaneous detection of ovine and caprine DNA in meat and dairy products using triplex TaqMan real-time PCR. *Food Sci. Nutr.* 2020, 8, 6467–6476. [CrossRef]

135. Hai, X.; Liu, G.Q.; Luo, J.X.; Guo, Y.S.; Qian, J.P.; Ya, M.; Guo, L. Triplex real-time PCR assay for the authentication of camel-derived dairy and meat products. *J. Dairy Sci.* 2020, 103, 9841–9850. [CrossRef]

136. Ganopoulos, I.; Sakaridis, I.; Argiriou, A.; Madesis, P.; Tsafaris, A. A novel closed-tube method based on high resolution melting (HRM) analysis for authenticity testing and quantitative detection in Greek PDO Feta cheese. *Food Chem.* 2013, 141, 835–840. [CrossRef] [PubMed]

137. Sakaridis, I.; Ganopoulos, I.; Argiriou, A.; Tsafaris, A. High resolution melting analysis for quantitative detection of bovine milk in pure water buffalo mozzarella and other buffalo dairy products. *Int. Dairy J.* 2013, 28, 32–35. [CrossRef]

138. Cutarelli, A.; Fulgione, A.; Fraulo, P.; Serpe, F.P.; Gallo, P.; Biondi, L.; Corrado, F.; Citro, A.; Capuano, F. Droplet Digital PCR (ddPCR) Analysis for the Detection and Quantification of Cow DNA in Buffalo Mozzarella Cheese. *Animals* 2021, 11, 1270. [CrossRef] [PubMed]

139. Deb, R.; Sengar, G.S.; Singh, U.; Kumar, S.; Alyethodi, R.R.; Alex, R.; Raja, T.V.; Das, A.K.; Prakash, B. Application of a Loop-mediated Isothermal Amplification Assay for Rapid Detection of Cow Components Adulterated in Buffalo Milk/Meat. *Mol. Biotechnol.* 2016, 58, 850–860. [CrossRef] [PubMed]

140. Kim, M.J.; Kim, H.Y. Direct duplex real-time loop mediated isothermal amplification assay for the simultaneous detection of cow and goat species origin of milk and yogurt products for field use. *Food Chem.* 2018, 246, 26–31. [CrossRef]

141. Ribani, A.; Schiavo, G.; Utzeri, V.J.; Bertolini, F.; Geraci, C.; Bovo, S.; Fontanesi, L. Application of next generation semiconductor based sequencing for species identification in dairy products. *Food Chem.* 2018, 246, 90–98. [CrossRef]

142. Beltramo, C.; Riina, M.V.; Colussi, S.; Campia, V.; Maniaci, M.G.; Biondetti, C.; Trisorio, S.; Modesto, P.; Peletto, S.; Acutis, P.L. Validation of a DNA biochip for species identification in food forensic science. *Food Control* 2017, 78, 366–373. [CrossRef]

143. Kounelli, M.L.; Kalogianni, D.P. A sensitive DNA-based fluorometric method for milk authenticity of dairy products based on spectrally distinct microspheres. *Eu. Food Res. Technol.* 2017, 243, 1773–1781. [CrossRef]

144. Bougadi, E.T.; Kalogianni, D.P. Paper-based DNA biosensor for food authenticity testing. *Food Chem.* 2020, 322, 126758. [CrossRef]

145. Navarro, E.; Serrano-Heras, G.; Castano, M.J.; Solera, J. Real-time PCR detection chemistry. *Clin. Chim. Acta* 2015, 439, 231–250. [CrossRef] [PubMed]

146. Villa, C.; Costa, J.; Mafra, I. Lupine allergens: Clinical relevance, molecular characterization, cross-reactivity, and detection strategies. *Compr. Rev. Food Sci. Food Saf.* 2020, 19, 3888–3915. [CrossRef] [PubMed]

147. Drumli, B.; Cichna-Markl, M. High resolution melting (HRM) analysis of DNA—Its role and potential in food analysis. *Food Chem.* 2014, 158, 245–254. [CrossRef] [PubMed]

148. Grazina, L.C.J.; Amaral, J.S.; Mafra, I. High-Resolution Melting Analysis as a Tool for Plant Species Authenticity. In *Crop Breeding: Genetic Improvement Methods*; Tripodi, P., Ed.; Springer: New York, NY, USA, 2021; pp. 55–73. [CrossRef]

149. Pereira, L.; Gomes, S.; Barrias, S.; Fernandes, J.R.; Martins-Lopes, P. Applying high-resolution melting (HRM) technology to olive oil and wine authenticity. *Food Res. Int.* 2018, 103, 170–181. [CrossRef] [PubMed]

150. Kosir, A.B.; Domsar, T.; Stebih, D.; Zel, J.; Milavec, M. Digital PCR as an effective tool for GMO quantification in complex matrices. *Food Chem.* 2019, 294, 73–78. [CrossRef]

151. Shehata, H.R.; Li, J.P.; Chen, S.; Redda, H.; Cheng, S.M.; Tabujara, N.; Li, H.H.; Warriner, K.; Hanner, R. Droplet digital polymerase chain reaction (ddPCR) assays integrated with an internal control for quantification of bovine, porcine, chicken and turkey species in food and feed. *PloS ONE* 2017, 12, e0182872. [CrossRef]

152. Zhang, H.W.; Li, J.; Zhao, S.B.; Yan, X.H.; Si, N.W.; Gao, H.F.; Li, Y.J.; Zhai, S.S.; Xiao, F.; Wu, G.; et al. An Editing-Site-Specific PCR Method for Detection and Quantification of CAO1-Edited Rice. *Foods* 2021, 10, 1209. [CrossRef]

153. Huang, T.Z.; Li, L.Z.; Liu, X.; Chen, Q.; Fang, X.E.; Kong, J.L.; Draz, M.S.; Cao, H.M. Loop-mediated isothermal amplification technique: Principle, development and wide application in food safety. *Anal. Methods* 2020, 12, 5551–5561. [CrossRef]
154. Plácido, A.; Amaral, J.S.; Costa, J.; Fernandes, T.J.R.; Oliveira, M.B.P.P.; Delerue-Matos, C.; Mafra, I. Novel strategies for genetically modified organism detection. In *Genetically Modified Organisms in Foods*; Watson, R.R., Preedy, V.R., Eds.; Academic ress: London, UK, 2016; pp. 119–131.

155. Haynes, E.; Jimenez, E.; Pardo, M.A.; Helyar, S.J. The future of NGS (Next Generation Sequencing) analysis in testing food authenticity. *Food Control* **2019**, *101*, 134–143. [CrossRef]  

156. Mayo, B.; Rachid, C.T.C.C.; Alegria, A.; Leite, A.M.O.; Peixoto, R.S.; Delgado, S. Impact of Next Generation Sequencing Techniques in Food Microbiology. *Curr. Genom.* **2014**, *15*, 293–309. [CrossRef]  

157. Cunha, J.T.; Ribeiro, T.I.B.; Rocha, J.B.; Nunes, J.; Teixeira, J.A.; Domingues, L. RAPD and SCAR markers as potential tools for detection of milk origin in dairy products: Adulterant sheep breeds in Serra da Estrela cheese production. *Food Chem.* **2016**, *211*, 631–636. [CrossRef]  

158. Fanelli, V.; Mascio, I.; Miazzi, M.M.; Savoia, M.A.; De Giovanni, C.; Montemurro, C. Molecular Approaches to Agri-Food Traceability and Authentication: An Updated Review. *Foods* **2021**, *10*, 1644. [CrossRef] [PubMed]  

159. Sardina, M.T.; Tortorici, L.; Mastrangelo, S.; Di Gerlando, R.; Tolone, M.; Portolano, B. Application of microsatellite markers as potential tools for traceability of Girgentana goat breed dairy products. *Food Res. Int.* **2015**, *74*, 115–122. [CrossRef] [PubMed]  

160. Meshram, B.D.; Agrawal, A.K.; Adil, S.; Ranvir, S.; Sande, K.K. Biosensor and its Application in Food and Dairy Industry: A Review. *Int. J. Curr. Microbiol. App. Sci.* **2018**, *7*, 3305–3324. [CrossRef]  

161. Dias, L.A.; Peres, A.M.; Veloso, A.C.A.; Reis, F.S.; Vilas-Boas, M.; Machado, A.A.S.C. An electronic tongue taste evaluation: Identification of goat milk adulteration with bovine milk. *Sens. Actuat. B-Chem.* **2009**, *136*, 209–217. [CrossRef]  

162. Costa, J.; Fernandes, T.J.R.; Villa, C.; Oliveira, M.B.P.P.; Mafra, I. Advances in food allergen analysis. In *Food Analysis: Innovative Analytical Tools for Safety Assessment*; Spizzirri, U.G., Cirillo, G., Eds.; Scrivener Publishing LLC: Beverly, MA, USA, 2017; pp. 305–360.