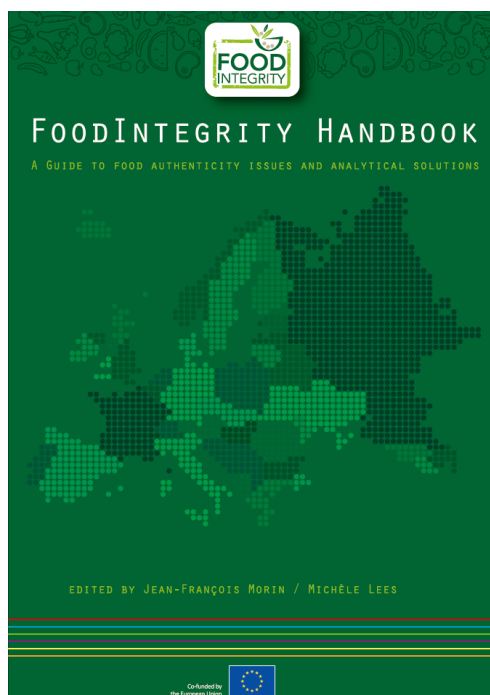


# FOODINTEGRITY HANDBOOK

## *A GUIDE TO FOOD AUTHENTICITY ISSUES AND ANALYTICAL SOLUTIONS*

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# Milk and milk products

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## General overview of the products

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Milk is a nutritious food that plays an important role in the diet of particular groups, such as the new-born, children, the elderly and pregnant women. In addition to those groups, milk is consumed worldwide by a large part of the population, either alone or in the form of dairy products. According to the most recent data available from the Food and Agriculture Organization [1], the world production of milk increased from 724 million tonnes in 2010 to 798 million tonnes in 2018. Considering the data for cow, goat, sheep, camel and buffalo whole fresh milk, currently, Asia is the main producer in the world, mainly due to a high production of buffalo milk besides that of cow's milk. However, cow's milk remains the most consumed worldwide, corresponding to 82.6 % of the total fresh milk production in 2016. Europe is the major producer of this milk (32.7 % of world production in 2016), with most of it being produced in the European Union (EU). The dairy sector is of great importance to the EU since its value (close to 55 billion EUR) represents around 15 % of the total EU agricultural output (average 2011-2013) [2]. Although Germany and France are the most significant producers, followed by the United Kingdom, the Netherlands, Poland and Italy, a striking feature in the EU dairy sector is that milk is produced in every single Member State, without exception. The EU dairy industry is renowned for the quality of its products, being considered a major player in the world dairy market and a leading exporter of many dairy products, most notably cheeses [3]. In the EU, approximately 50 % of milk is used for cheese production, though a wide variety of other products is also produced, such as butter, yogurts, ice creams, among others. In 2013, the EU produced 9.3 million tonnes of cheese, 46.2 million tonnes of fresh dairy products, 2.1 million tonnes of butter, 1.1 million tonnes of skimmed milk powder (SMP) and 0.7 million tonnes of whole milk powder (WMP) [2]. In addition to these, a wide range of new products is nowadays being offered by the dairy industry, from products targeting special groups of consumers (such as products with low lactose content or lactose free, for lowering blood cholesterol, etc.) to dairy-based ingredients for other food industries.

# 1. Product Identity

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## 1.1. Definition of the product and manufacturing process

According to the Codex Alimentarius, milk is the normal mammary secretion of milking animals, without either addition to it or extraction from it, intended for consumption as liquid milk or for further processing, while a milk product refers to a product obtained by any processing of milk, which may contain food additives, and other ingredients functionally necessary for the processing.

Milk and milk products encompass a wide range of products consumed worldwide including liquid milk, fermented milks and products thereof, cheeses, butter, ghee and dairy fat spreads, condensed milk, evaporated milk, cream, milk and cream powders, whey products and casein. Liquid milk, including raw milk and products such as pasteurised, skimmed, ultra-high-temperature (UHT) and fortified milk, is the most consumed, processed and marketed dairy product [4]. Fermented milk, obtained using suitable microorganisms, is generally used to produce dairy products such as yoghurt and kefir, among others. Cheese is the ripened or unripened soft, semi-hard, hard, or extra-hard product, obtained through the coagulation of milk protein by rennet, other suitable coagulating agents or processing technologies, and in which the whey protein/casein ratio does not exceed that of milk [5]. During this process, whey is also obtained, corresponding to the liquid part that remains after the separation of the curd. Whey can be used for several purposes such as the preparation of whey cheese, whey powder, whey drinks and for different industrial purposes [4]. Butter, ghee and dairy spreads are fatty milk products in the form of a water-in-oil emulsion. Cream is the fluid milk product comparatively rich in fat, in the form of an emulsion of fat-in-skimmed milk, obtained by physical separation from milk [4,6], and can give rise to a wide range of products such as whipping cream, whipped cream, acidified cream, among others. Condensed and evaporated milks are both obtained from the partial removal of water from whole or skimmed milk, with the first being frequently used in the form of sweetened condensed milk. Milk powders are obtained from the dehydration of milk and include several products such as whole milk powder, partly skimmed milk powder, skimmed milk powder and cream powder.

## 1.2. Current standards of identity or related legislation

### 1.2.1. Codex Alimentarius

Codex has developed several specific standards for milk and milk products. A compilation containing all Codex standards and related texts adopted by the CAC up to 2011 has been carried out [7]. It includes the standards of milk, milk powders, condensed milks, creams, butter and all sorts of cheeses. It also includes other general texts for milk and milk products such as the General standard for the use of dairy terms (CODEX STAN 206-1999) [8]; the Code of hygienic practice for milk and milk products (CAC/RCP 57-2004) [9]; the Guidelines for the preservation of raw milk by use of the lactoperoxidase system (CAC/GL 13-1991) [10] and the Model export certificate for milk and milk products (CAC/GL 67-2008) [11].

Codex has also developed several texts on food labelling, methods of analysis and sampling, food import and export, and certification systems that apply horizontally to all food products (including milk and milk products), such as:

- General standard for contaminants and toxins in food and feed,
- General standard for food additives,
- Guidelines for design and implementation of national regulatory food safety assurance programmes associated with the use of veterinary drugs in food producing animals,
- Maximum Residue Limits (MRLs) and Risk Management Recommendations (RMRs) for residues of veterinary drugs in foods,
- Maximum residues limits for pesticides.

### 1.2.2. EU Legislation

General principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety have been stated in Regulation (EC) No 178/2002 [12]. The general rules for food business operators on the hygiene of foodstuffs have been laid down in Regulation (EC) No 852/2004 [13]. Specific hygiene rules for food of animal origin are covered by Regulation (EC) No 853/2004 where a section is specifically dedicated to raw milk and dairy products (Annex III, Section IX). Finally, Commission Regulation (EC) No 1664/2006 deals with measures for certain products of animal origin intended for human consumption, in particular the testing for raw and heat-treated milk [14].

Rules on the common organization of the market in milk and milk products for drinking milk (Council Regulation (EC) No 1153/2007) are now considered in Regulation (EU) No 1308/2013 of the European Parliament and of the Council of 17 December 2013 [15].

Milk can easily be contaminated by micro-organisms that are naturally present in the environment or which originate from diverse human activities. Therefore milk and dairy products have been extensively covered in EU legislation. The European Parliament and Council have established specific rules for the organisation of official controls on products of animal origin intended for human consumption (Regulation (EC) No 854/2004) [16].

Regarding quality evaluation, the Commission has published methods for the analysis and quality evaluation of milk and milk products eligible for public intervention and aid for private storage (Commission Implementing Regulation (EU) 2018/150 of 30 January 2018) [17]. More specifically, the Commission has issued new rules on caseins and caseinates intended for human consumption with EU Directive 2015/2203 [18].

Requirements on microbiological criteria have been amended by Commission Regulation (EC) No 1441/2007 concerning microbiological criteria for foodstuffs with regards to milk and dairy products and Commission Regulation (EU) No 365/2010 on microbiological criteria for foodstuffs as regards *Enterobacteriaceae* in pasteurized milk and other pasteurized liquid dairy products and *Listeria monocytogenes* in food grade salt [19, 20].

The Commission has also ruled on maximum residue levels for several pesticides in or on certain products (Commission Regulation (EU) 2018/686 and 2018/687 of 4 May 2018) [21,22]. Very recently, a Commission Implementing Regulation (EU) 2018/555 concerning a coordinated multiannual control programme of the Union for 2019, 2020 and 2021 has been established to ensure compliance with maximum residue levels and to assess consumer exposure to pesticide residues in and on food of plant and animal origin; the date of entry into force is however unknown at the date of writing this text (pending notification) [23].

### **1.2.3. European Dairy Association (EDA)**

The European Dairy Association (EDA) is the European milk processors' platform for exchange throughout all parts of Europe and across all types of dairy companies, cooperatives and privately-owned dairies, world dairy leaders and enterprises. Recently, the EDA has issued its sectorial Guidelines for the voluntary indication of the origin of dairy products as an industry reference in the implementation of the new rules laid down in the EU Commission Implementing Regulation on voluntary origin labelling of foods. EDA also edited in June 2018 Guidelines on the principles and enforcement of the Protection of Dairy Terms. It takes place one year after the “Tofu Town” judgement by the European Court of Justice, in which the EU Court ruled that purely plant-based products cannot be marketed with designations such as ‘milk’, ‘cream’, ‘butter’, ‘cheese’ or ‘yoghurt’. The new dairy industry guidelines intend to address the use and misuse of protected definitions, designations and sales descriptions of milk and milk products within the European Single Market and to serve as a tool to facilitate their enforcement at national level.

### **1.2.4. ISO Standards**

The Technical Committee 34 of ISO (International Organization for Standardization) (ISO/TC 34) is responsible for the development of International Standards on topics connected to food and feed products. ISO/TC34/SC 5, created in 1970, focuses especially on standards for milk and milk products. It has published 184 ISO Standards. Their scope is the standardisation of methods of analysis and sampling, covering the dairy chain from primary production to consumption. The standards are used to determine, for example, the nitrogen content (ISO 8968-4: 2016) [24]. With reference to the melamine crisis in 2008, ISO and the International Dairy Federation (IDF) worked together on the edition of ISO/TC 15495 (ISO/TS 15495: 2010), which gives guidance for the quantitative determination of melamine and cyanuric acid content in milk, powdered milk products, and infant formulae by electrospray ionization liquid chromatography tandem mass spectrometry (LC-MS/MS) [25]. In 2013, ISO/TC 34 published guidelines for the application of mid-infrared spectroscopy in milk and liquid milk products (ISO 9622: 2013). It gives guidelines for the quantitative compositional analysis of milk and liquid milk products, such as raw milk, processed milk, cream and whey, by measurement of the absorption of mid-infrared radiation. The guidelines specified are applicable to the analysis of cow's milk and to the analysis of milk of other species (goat, ewe, buffalo, etc.) and derived liquid milk products, provided adequate calibrations are generated for each application and adequate control procedures are in place [26].

### **1.2.5. US Regulation**

In the United States (US), the Department of Agriculture (USDA) and the Food and Drug Administration (FDA) regulate milk production and its guidelines are some of the strictest in the industrialised world. Farmers, processors and government agencies all work together to ensure the milk is safe and of the highest quality. The US FDA edits Guidance Documents and Regulatory Information including Coded Memoranda Issued by the Milk Safety Branch, Interstate Milk Shipments and Dairy HACCP.

The Pasteurized Milk Ordinance (PMO) of the US FDA serves as the basic milk sanitation standard for National Conference on Interstate Milk Shipments (NCIMS) members (all 50 states and Puerto Rico). Pasteurized Milk Ordinance, revised in 2015, presents the most current developments in milk sanitation for ‘Grade A’ milk and milk products [27].

## 2. Authenticity issues

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### 2.1. Identification of current authenticity issues

Milk has been considered one of the seven foods most vulnerable to economically motivated adulteration. Due to the high demand for milk and the value of some dairy products, fraud in the dairy industry has become a widespread problem and a real concern for many consumers and authorities since adulteration invariably reduces product quality and may introduce hazards that can jeopardise health. Over the last decades, there has been an increasing interest in the quality evaluation and authentication of milk and milk products, in order to ensure consumer protection, avoid unfair competition among producers and improve general confidence in the sector.

#### 2.1.1. Undeclared addition of certain ingredients

The practice of milk adulteration invariably reduces its quality and may introduce hazardous substances into the dairy supply chain threatening consumers' health [28]. Milk adulteration typically involves dilution and/or addition of inexpensive, low-quality and sometimes dangerous products to increase volume, mask inferior quality or replace the natural substances in milk for economic gain [29].

##### 2.1.1.1. *Adulteration with water*

Water is the most commonly and simplest case of adulteration in milk. Addition of water not only reduces the nutritional value of milk, but also poses a health risk to the consumer [28]. However this is closely monitored by dairy companies when they purchase their milk. In addition, since many dairy companies pay for milk on the basis of its compositional quality any water addition would, to some extent, be self-defeating [30].

##### 2.1.1.2. *Adulteration of nitrogen content*

Nitrogen-rich adulterants constitute also a well-known issue in milk adulteration which has received much attention in recent years owing to a series of food safety incidents [28]. These include the addition of nitrogenous compounds to increase the apparent protein content. This type of adulteration is very usual because the non-protein nitrogen cannot be distinguished by the Kjeldahl and Dumas methods that are commonly used for determining total protein content in dairy products. Melamine, urea and whey are the main adulterants for this purpose due to their high nitrogen content and low cost. Melamine (2,4,6-triamino-1,3,5-triazine) is a nitrogen-rich organic compound commonly used to increase the apparent protein content of liquid and powdered milk and thus their economic value. Whey/whey protein is a very cheap by-product of cheese manufacturing that somehow resembles skimmed milk as it retains some milky aspect and flavour and is added to liquid milk not only to increase volume but also protein content. Urea is also extensively used in frauds because of its low cost [29]. Urea is added to milk to provide whiteness, increase the consistency of milk and standardise the solid-not-fat content to the value expected for the natural milk. Soya constitutes also a common source of nitrogen-rich adulterants. Low grade soya powder is a common vegetable protein used to increase the protein content of the adulterated milk, due to its lower price and easy availability in the market. Soya protein has good water holding and binding capacity and therefore can improve the texture of a product (e.g. cheese) [31]. Soya milk is also added to bovine milk either for sale as fluid milk or in the preparation of skimmed milk powder (SMP) and cheese for revenue maximisation. This is because of its similar properties to cow's milk [28].

### **2.1.1.3. Adulteration of the fat content**

Fat is one of the major components of milk and generally constitutes 3-5 % (m/m) of cow's milk. Triacylglycerols constitute about 97-98 % of the fat in milk and are important components that provide the characteristic flavour and texture. Major adulterants are vegetable oils (e.g. soybean, sunflower, groundnut, coconut, palm and peanut oil) and animal fat (e.g. cow tallow and pork lard). Detecting adulteration with vegetable oils is often difficult because of the variation in the chemical composition of these oils. Detecting adulteration with animal fat is also difficult because its chemical composition is similar to milk fat [29]. Moreover, it must be considered that lipid composition can naturally vary according to the different seasons and feeding regimes. On the other hand, a characteristic lipid profile can be associated with a particular product, produced in a certain period and geographical region with a specific feeding regime. In this sense, it can be challenging for the counterfeiter to imitate that specific composition, which can facilitate the detection of fraud in the case of suspect samples that can be matched against specific samples retained at the dairy industries [32].

### **2.1.1.4. Synthetic or reconstituted milk**

Synthetic milk is an excellent imitation of natural milk containing vegetable oil, urea and emulsifier. It has the fat, nitrogen content and frothiness as well as similar specific gravity to natural buffalo milk. When mixed with natural milk in varying proportions, it becomes identical in milky aroma [28]. Synthetic milk is reported to be used for the adulteration of dairy milk at 5-10 %. In addition to this fraud, because detergents are essential components for the emulsification of fat added to the preparation of synthetic milk, they are considered a new class of milk adulterants. The presence of detergents in infant milk formula can sometimes be detected by means of colour and smell. Long-term consumption can cause serious deleterious health effects such as heart and digestive problems [29]. Adulteration of fresh milk with reconstituted milk containing cheap powdered milk is also a malpractice in common use [28].

### **2.1.1.5. Adulteration with preservatives**

This practice involves the addition of substances to decrease microbiological growth and thus increase the product shelf life. This group includes several substances such as hydrogen peroxide, formaldehyde, hypochlorite, salicylic acid, and even potassium dichromate. These substances are toxic for humans and their monitoring is required for quality control [29,33].

## **2.1.2. Species substitution**

Among the several possible adulterations in milk and milk products, one of the most frequent regards the species of origin, namely the substitution of high valued milks (such as sheep, goat or buffalo) by less expensive cow's milk, to reduce production costs and increase profits [34,35]. This is explained by seasonal oscillations and lower production yields of ovine, caprine and bubaline (or more exotic species such as camel or donkey), which raises the economic values of these types of milk and products thereof. Species substitution, besides having a negative economic impact, is also a problem for several groups of consumers because of other reasons such as religious, ethical or cultural objections.

In several EU countries, in particular those from the Mediterranean area, and other parts of the globe, namely the Middle East, a variety of valued cheeses are traditionally produced from goat's, sheep's, the mixture of both or buffalo milk. Traditionally produced cheeses are regarded as specialities and generally attain higher market prices and therefore are more prone to adulteration. Moreover, recently, in some countries, there has been a growth in the market for

milk species other than cow, in particular for goat's milk, due to its superior nutritional features and other aspects such as its attractive odour and taste, and superior digestibility when compared to cow's milk [36]. Additionally, according to some authors, goat's milk can be a possible alternative to cow's milk because it is considered less allergenic [37]. In this case, the undeclared presence of cow's milk could be a potential health risk for allergic consumers. Nevertheless, due to protein similarity, people allergic to cow's milk proteins can be affected by milk from any species, which demonstrates the importance for correct labelling.

### **2.1.3. Geographical origin (PDO, PGI products)**

To recognise and support the potential of certain foods, in 1992 the EU created different labels, including the Protected Designation of Origin (PDO) and the Protected Geographical Indication (PGI), to promote and protect the names of quality foods from misuse and imitation. The PDO label covers agricultural products or foods that are produced, processed and prepared in a specific geographical area, using recognised know-how, therefore ensuring a strong link to the territory. Besides PDO, the PGI label also has a specific link to the region where the product comes from, however it only requires that at least one of the stages of production, processing or preparation occurs in that area, allowing the ingredients used in production to come from another region. In 2010, products with geographic indication (GI), namely PDO or PGI, had an estimated wholesale of EUR 54.3 billion, with agricultural products and foodstuffs corresponding to 29 % of this amount (EUR 15.8 billion). Among PDO products, cheeses account for a third of total turnover [38]. Presently, there are 189 PDO cheeses registered on the EU Database of Origin & Registration (DOOR), from a total of 14 EU countries, with Italy, France, Spain and Greece being the ones with higher number of products each (50, 45, 26 and 21 PDO cheeses, respectively). A PDO label has also been attributed to other dairy products, such as butter (e.g. Beurre d'Isigny and Beurre de Bresse (France), Mantequilla de Soria (Spain), Beurre rose (Luxembourg), Beurre d'Ardenne (Belgium)) and cream (Crème de Bresse and Crème d'Isigny, France).

Currently, consumers are increasingly interested in traditional, local and higher quality products, which in turn encourages agricultural producers to use geographical indications to differentiate and capitalise on the value of their products, thereby improving competitiveness and profitability. Thus, premium foods, frequently face competition with fraudulent products, which discourages producers, disappoints consumers and severely affects the agri-food industry and market. This is the case of PDO cheeses for which consumers frequently pay 1.5 times as much for GI products than for non-GI products [39]. In fact, the high market value of the PDO cheeses and their reputation worldwide make these products very prone to adulteration. Cheese is considered the 3rd GI food with higher infringing rates (10.6 %) corresponding to losses estimated in EUR 644.7 million [39]. The avoidance of economic losses due to mislabelling/fraud related to geographical origin is therefore a driving force behind the authentication of dairy products.

### **2.1.4. Rennet origin**

During cheese production, the conversion of milk to cheese curd is usually made through an enzymatic coagulation process, either using animal, vegetable or microbial coagulants. Among those, animal rennet, which corresponds to enzymes (mainly chymosin and pepsin) secreted in the fourth stomach of unweaned ruminants (calves, lambs or kids), is frequently used in traditional cheese production [40, 41]. The use of rennet generally has a significant role in the sensory output of the produced cheese because it also contains lipolytic enzymes that release free fatty acids during ripening, therefore affecting the final characteristics of the product [40]. According to the specifications of several added-value cheeses, in particular various PDO labelled cheeses from



Southern European countries, specific animal or vegetable rennet should be used [41]. In general, lamb or kid rennet is preferred in the case of some sheep and/or goat PDO cheeses, such as Roncal cheese in Spain, Pecorino Romano and Fiore Sardo cheeses in Italy and Feta cheese in Greece, among others. In the case of the Italian PDO cheese Pecorino Romano, the specifications mention that, besides using exclusively lamb rennet paste, the fourth-stomachs used to produce this rennet should also come from animals raised in the PDO geographical area [42]. On the other hand, other PDO cheeses such as Azeitão, Serpa and Évora cheeses in Portugal, prefer the use of specific vegetable rennet, namely that from *Cynara cardunculus*. Portuguese sheep milk PDO cheeses, when compared to other cheeses from the same species, generally present a creamy semi-soft texture and exquisite flavour, these characteristics being attributed in part to the vegetable coagulant used, which is very proteolytic [43]. Thus, when specifications of PDO cheeses stipulate the origin of rennet used for manufacturing, the use of another type of rennet, such as those from microbial origin, constitutes an adulteration and the characteristics of the final product may even be different, since the use of a specific rennet is frequently associated to particular characteristics of the cheese.

### 2.1.5. Technological processes (heat processing, freezing) and maturation

Heat processing is frequently used in the dairy industry because it provides a guarantee of the microbiological safety of raw milk as well as enhancing its stability, being also used in the production of some milk products such as SMP. Different technological processes are currently available, ranging from the use of mild temperatures, such as pasteurisation, to more severe heat treatments, such as UHT. Depending on the temperature or heat processing technology applied, natural milk components, such as vitamins, can be degraded or novel substances formed. The extent of chemical changes that milk and milk products undergo during processing and storage depends on the intensity of the heat treatment applied to milk [44]. Therefore, higher concentrations of Maillard compounds than those lawfully expected can be due to either excessive or repeated heat treatments, thus indicating milk of inferior microbiological quality, or fraudulent use of milk powders [45]. Thermal processing of milk is also an important parameter to check in the case of cheeses traditionally prepared from raw milk since pasteurisation of milk can alter the indigenous milk microflora, affecting the final organoleptic characteristics of the product. Additionally, milk freshness is also a concern as regards high-quality milk products such as some PDO cheeses that must be produced from fresh milk. An example is Mozzarella di Bufala Campana cheese, for which the use of frozen material is prohibited. However, due to the seasonality of water buffalo milk production (which reaches a peak during winter, while mozzarella consumption is higher in the summer) as well as a rapid decrease in product quality, adulteration can occur by the use of frozen curd or frozen milk [46].

Another important aspect in some cheeses is the ripening period, during which several biochemical processes occur. Among those, proteolysis is one of the most important for the development of flavour and texture. Therefore, to guarantee the organoleptic characteristics of some cheeses, a minimum ripening period may be established, such as with the Spanish Manchego PDO cheese produced from sheep's milk, that requires a ripening period for at least two months, although the most prized cheeses are aged longer [47]. Thus, accelerated cheese ripening or mislabelling of the ripening period is also an authenticity issue to consider.

## 2.2. Potential threat to public health

Melamine (2,4,6-triamino-1,3,5-triazine) is a nitrogenous heterocyclic compound with several industrial uses, since it is a chemical intermediate in the manufacture of amino resins, laminates, coatings and plastics [48]. Melamine is not approved for direct addition to human food nor to animal feeds. However, since it is a nitrogen-rich compound (about 66 %), it has been fraudulently added to milk and infant formula to increase the apparent protein content. In 2008, melamine was detected in the infant formula of 22 dairy companies in China, resulting in 294 000 affected babies, more than 50 000 hospitalisations and 6 confirmed deaths [49,50]. Melamine itself has a low acute toxicity because it is absorbed from the gastrointestinal tract and rapidly excreted from the body [51]. However, in the presence of cyanuric acid impurities, melamine precipitates in the kidneys in the form of crystals, which can lead to kidney failure and even to death [52]. In the 2008 scandal, the melamine used contained only traces of cyanuric acid, however this compound was able to form complexes with uric acid, which is present in larger amounts in the urine of infants than adults, affecting kidney function [49]. In response to this scandal, a tolerable daily intake (TDI) of 0.2 mg/kg body weight was established by EFSA according to that established by the World Health Organization [49].

The use of other compounds to increase nitrogen content, such as urea, also presents a public health problem particularly for pregnant women, children and sick individuals. Urea in milk overburdens the kidneys as they have to filter out more urea content from the body and this can cause problems such as indigestion, acidity, ulcers and osteoporosis [31].

The use of preservatives to extend the shelf-life of milk also presents a significant risk to human health. Preservatives such as hydrogen peroxide and formaldehyde are probably the most frequent ones, but substances such as hypochlorite, salicylic acid and dichromate have also been reported [29]. The addition of low amounts of hydrogen peroxide, although permitted in some countries, is forbidden in others because of its toxic effects, such as irritation of mucous membranes, gastro-intestinal complications, which can lead to gastritis and inflammation of the intestine [32]. Hydrogen peroxide activates the natural enzyme lactoperoxidase, which has antimicrobial activity. The Codex Recommended Code of Practice CAC/GL 13-1991 allows its addition in small quantities, but only in countries that do not have dairy industries with a suitable refrigeration infrastructure [32]. Addition of formaldehyde or dichromate to milk is critical because of the associated toxicity and carcinogenicity [29].

The consumption of adulterated milk and milk products with cheap food materials, such as whey or soya proteins, can also impose serious health problems in particular in vulnerable groups such as infants and children. In these groups, where milk products are frequently the entire or major source of nutrition (e.g. milk formula such as infant milk powders), severe or even fatal effects can occur if the nutritional balance of the food is compromised due to adulteration of the product [53]. Additionally, adulterants such as soya that belong to one of allergen groups whose presence must always be declared in processed foods (Directive 2007/68/EC; Regulation (EU) No. 1169/2011) can also be a problem to sensitised individuals [54, 55].

## 3. Analytical methods used to test for authenticity

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### 3.1. Officially recognised methods

#### 3.1.1. Addition of water

The proportion of extraneous water added to milk can be estimated by cryoscopy to determine the freezing point of milk, according to standard ISO 5764|IDF 108:2009. This standard specifies a reference method by using a thermistor cryoscope for the determination of the freezing point of raw bovine milk, heat-treated whole, reduced fat and skimmed bovine milk, as well as raw ovine and caprine milk [56]. Calculation of the amount of extraneous water is subject to daily and seasonal variations but, generally, for pooled milk from the same farm the variation range is quite narrow [32].

#### 3.1.2. Species identification

According to EU legislation [17], isoelectric focusing of  $\gamma$ -caseins after plasminolysis should be used as the reference method to guarantee that cheese made exclusively from ewes' milk, goats' milk or buffalo milk or from a mixture of ewes', goats' and buffalo milk does not contain cows' milk casein. In this method, samples should be analysed together with reference standards containing 0 % and 1 % cows' milk, being considered positive if both bovine  $\gamma_2$ - and  $\gamma_3$ -caseins (obtained by plasminolysis), or the corresponding peak area ratios when applying densitometry, are equal to or greater than the level of the 1 % reference standard. 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 buffalo milk or their mixtures, though it is not suitable for the detection of milk and cheese adulteration by heat-treated bovine whey protein concentrates. It is not adequate for species quantification, especially in ternary mixtures due to the similarities between some species, such as ovine and caprine [28]. Also according to this legislation, routine methods for detecting cows' milk casein in ovine, caprine and water buffalo cheeses may be used provided that (i) the detection limit is a maximum of 0.5 %, (ii) there are no false-positive results and that (iii) cows' milk casein is detectable with the required sensitivity even after long ripening periods, as may occur in usual commercial conditions [17]. However, the reference method is considered laborious and requires specific equipment, not always available in small dairy industries. Therefore, other approaches based on immunochemical methods are frequently used for routine screening, namely lateral flow immunocromatographic tests and enzyme-linked immunosorbent assays (ELISA). Currently, both options are commercially available in kit format for detecting cow's milk in sheep's and goat's milk and cheese, based on the detection of bovine immunoglobulin G (IgG). These approaches are suited for rapid screening, however since IgGs denature with thermal processing, adulteration with UHT cow's milk will give false negative results. More recently, a commercial kit has become available which is based on a competitive ELISA using a mouse monoclonal antibody (Mab) raised against bovine  $\kappa$ -casein that allows screening both raw and heat treated cow's and buffalo milk in the milk and cheese of other species and sources.

#### 3.1.3. Lipid analysis

As mentioned previously, adulteration of milk and milk products can include milk fat substitution by vegetable or other animal fat, or even the addition of these to skimmed milk to sell this as full-fat milk. To detect such adulteration, a lipid profile analysis is generally performed, either by the determination of fatty acids based on standard ISO 15885|IDF 184:2002 after obtaining the methyl

ester derivatives (standard ISO 15884|IDF 182:2002) or by the determination of triacylglycerides (standard ISO 17678|IDF 202:2010). The principle of fatty acid analysis relies on the preparation of the methyl esters of milk fat fatty acids (FAME) by base-catalysed methanolysis of the glycerides and transesterification [57]; the obtained FAME are then separated and determined by capillary gas-liquid chromatography with flame ionisation detector (GC-FID) [58]. The purity of milk fat extracted from milk or milk products can also be determined based on triacylglycerides analysed by GC-FID [59]. The presence of vegetable or animal (beef tallow and lard) fat can be inferred using suitable equations to calculate S-values, which should comply with those established for pure milk fat. Nevertheless, some cases can result in false positives when applying this method, particularly when the animals are given exceptionally high feed of pure vegetable oils, such as rapeseed oil; milk products from individual cows; milk fat subjected to technological treatment (e.g. cholesterol removal) or obtained from skimmed milk or buttermilk; and some cases of fat extracted from cheese as the ripening process can affect fat composition.

#### **3.1.4. Adulteration of nitrogen content and addition of reconstituted milk**

Several milk products, by definition, should not contain proteins other than those naturally present in milk. However, as mentioned, some non-milk protein sources, such as soya, are attractive as potential adulterants due to their low price. The detection of vegetable proteins added to milk products, namely the addition of cheaper soya and pea protein isolates to low-heat milk powder, can be achieved using capillary electrophoresis in the presence of sodium dodecyl sulphate (SDS-CE) as described in ISO 17129|IDF 206:2006. However, the method is not suitable for detecting the presence of hydrolysed plant proteins in milk powder. An alternative option uses ELISA kits to screen for the presence of soya proteins [60].

The addition of non-food proteins to milk, powdered milk products and infant formulae, can be achieved based on the quantitative determination of melamine and cyanuric acid (mg/kg of product) by electrospray ionization liquid chromatography tandem mass spectrometry (LC-MS/MS), according to standard ISO/TS 15495|IDF/RM 230:2010 [61].

According to EU legislation [17], the detection of rennet whey in skimmed-milk powder can be performed by using the reference method based on the determination of the caseinomacropetides by high performance liquid chromatography with ultraviolet detector (HPLC-UV). As well as for species identification purposes, there are available on the market as competitive enzyme immunoassay kits to screen for the presence of bovine rennet whey in bovine milk and milk products. As an example, one of such ELISA kits is based on the detection of an epitope located on the glycomacropetide (CMP) part of  $\kappa$ -casein, which is released during cheese production, and therefore indicates fraud when detected in milk powder.

HPLC-UV is also the technique proposed by the Chinese Ministry of Agriculture (Chinese standard NY/T 939-2016) for the detection of furosine and lactulose, used to detect the addition of reconstituted milk in pasteurized or UHT milk [62]. A method based on the determination of lactulose content by HPLC-UV is also proposed by ISO and IDF but to distinguish milk sterilized by UHT from in-bottle sterilised milk (ISO 11868|IDF 147:2006) [63]. Lactulose is not present in raw milk, being formed by epimerisation of lactose due to heat treatment. The extent of the isomerisation is related to time and temperature, and can therefore be used to evaluate the severity of the heat treatment [64]. Determination of alkaline phosphatase and lactoperoxidase, two naturally occurring enzymes in raw milk, is also used to evaluate the use of thermal processing [65, 66].

### 3.1.5. Spectroscopy (MIR)

In the particular case of milk and milk products, FT-MIR spectrometry is the worldwide method of choice for composition and quality controls during routine liquid milk testing. In 1961, a patent application for a FT-MIR method determining fat, protein and lactose in milk was introduced [67]. The first apparatus, an IRMA (Infrared Milk Analyzer, Grubb Parsons, Newcastle upon Tyne, UK) using a monochromator, was based on the principle of measuring direct absorption of the infrared energy at specific frequencies by carbonyl groups in the ester linkages of the fat molecules, by peptide linkages between amino acids of protein molecules, and by the O-H groups in lactose molecules. A second generation of infrared instrumentation has adopted the change from wavenumber selection by diffraction grating to optical filters [68] and was largely used by central milk laboratory testing, where samples of milk from both tanks and individual cows were tested. FT-MIR supplies complementary chemical information and allows a high throughput with high sensitivity in a short response time from a very small quantity of sample [69]. In 1993, the first purpose-built FT-MIR instrument based on Fourier Transform Infrared (FT-MIR) technology was marketed (Anadis MI-200) [70]. With the introduction of FT-MIR, new applications have been developed because of the use of the full spectrum of the sample. In this way, FT-MIR has been applied for the determination of more and more milk components such as free fatty acids [71], protein composition [72], minerals [73], ketone bodies [74], lactoferrin [75] and fatty acid profile [76,77]. Recent studies have been performed using these milk components predicted by FT-MIR in order to predict physiological indicators of the animal [78-80].

More recently, the FOSS company (Foss, Hillerød, Denmark) has developed an Abnormal Spectrum Screening Module (ASM) where new milk samples are automatically compared to the spectra of the natural (not contaminated) historical dataset obtained with the MilkoScan™ FT120 ([www.foss.fr/industry-solution/products/milkoscan-ft1/](http://www.foss.fr/industry-solution/products/milkoscan-ft1/)), then outliers are detected by a combination of the residuals from the PCA on natural samples and the Mahalanobis distance.

## 3.2. Alternative methods

### 3.2.1. DNA-based methods

During recent years, analytical methods relying on DNA analysis have rapidly progressed as alternatives to overcome the limitations of protein analysis and have been successfully applied for milk authenticity testing. DNA-based methods present several advantages, specifically the ubiquity of nucleic acids in every type of cells and their superior stability to proteins. Most DNA-based methods rely on the polymerase chain reaction (PCR) technique due to its high specificity, sensitivity, simplicity and rapidity, allowing the identification of species of origin even in complex and processed foods, such as dairy products. Although both nuclear and mitochondrial genes can be targeted as species-specific DNA markers, the latter has been preferred because of the high number of copies per cell and sequences are highly conserved within different animal species.

Several PCR-based methods have been developed and applied to the authentication of milk and milk products, such as heat-treated dairy products, cheeses, yogurts, butter and milk-based sweets [81]. The methods include mainly PCR with restriction fragment length polymorphism (RFLP), species-specific PCR, multiplex PCR and real-time PCR. The detection of cow's milk in milk mixtures [82], in goat's and sheep's cheeses [83,84] and in buffalo cheeses [85,86] was successfully achieved with species-specific PCR with sensitivities down to 0.1 %. The use of two or more sets of primers in the same reaction allows multi-species detection based on multiplex PCR. Bottero et al.

(2003) and Gonçalves et al. (2012) proposed multiplex PCR assays to detect cow, goat, sheep and buffalo species in dairy products [87,88]. The development of duplex PCR assays enabled the detection and quantification of cow's milk in sheep's [34] and goat's [35] cheeses.

Real-time PCR has been the technique of choice in many laboratories for species identification and food authentication, including for dairy products. The combination of high sensitivity, specificity, reproducibility and quantitative analysis are major advantages of real-time PCR. Additionally, the amplification of short DNA fragments (100-200 bp) is a major benefit when analysing highly processed foods [89]. Several authors have proposed real-time PCR assays with TaqMan probes to detect cow's milk in dairy products [90-94]. The simultaneous detection of several species in dairy products has also been succeeded by multiplex real-time PCR assays [95,96].

### **3.2.2. Other protein-based methods (chromatography/mass spectrometry)**

The evaluation of proteins and/or the sequence of peptides by mass spectrometry (MS) or liquid chromatography (LC) coupled to MS, is increasingly being used as reliable biomarkers for dairy product authentication. This has been possible due to several technological advances that allow for accurate analysis of proteins and peptides, namely the use of soft-ionisation techniques, such as electrospray ionisation (ESI) and matrix-assisted laser desorption ionisation (MALDI). MALDI time-of-flight mass spectrometry (MALDI-TOF-MS) provides informative fingerprints of milk proteins for dairy authentication, and is also a simple, fast, sensitive and highly reproducible technique. LC-MS techniques are advantageous in terms of high selectivity and sensitivity, which makes them useful as confirmatory techniques. However, the development of specific LC-MS methodologies is laborious and requires skilled technicians and costly equipment. Besides the already mentioned use of LC-MS as a reference method in the analysis of nitrogen-based adulterants, namely melamine, in the last years, LC-MS and MALDI-TOF-MS have demonstrated their usefulness in the detection of other types of fraud in dairy products, such as species identification, accessing freshness, addition of rennet whey, etc. based on the analysis of specific peptides as biomarkers [97-101]. For instance, the use of specific peptides as biomarkers for milk species identification presents advantages over other protein-methods whose results are affected by thermal processes since the sequence of peptides is related to the genetically determined primary structure of proteins, which is generally resistant to processing.

### **3.2.3. Spectroscopy (NIR, Raman)**

There are various types of analytical methods applied to food authentication that can provide information concerning its physical and chemical characteristics, including major and minor constituents. However, most of these methods are often tedious, time consuming and use reagents that may be harmful for the environment. In the food sector, and especially in the milk and milk products area, with the increasing demands being made by consumers and legislators, there is a general need for methods that are suitable for process and quality control and are simple, rapid and reach the required accuracy, repeatability and sensitivity. Fingerprint methods are the ideal candidates to replace these analytical procedures. The term "fingerprinting" can be defined as a variety of techniques that can measure the composition of foodstuffs in a non-selective way. Among these methods, vibrational spectroscopy methods based on infrared and Raman spectroscopic techniques, use the information from major compounds present in food products [102-104]. Organic compounds absorb radiation at specific wavelengths or frequencies, thus giving rise to spectral signatures which are characteristic of the food composition and may be considered as «fingerprints» of the food. However, these signatures also include interference due

to variation occurring as a result of natural events (e.g. weather, climate, disease etc.) during growth or the production of primary foods or to batch-to-batch variations in processed foods or food ingredients. Interrogation of signals from sufficiently large and characteristic sample collections by mathematical techniques can detect primary foods which are not what they claim to be or processed foods that do not comply with a declared specification. Vibrational spectroscopy methods are suitable for implementation in factories and milk parlours as they allow on-line control and the screening of a high number of samples by unit of time. Fingerprinting methods are also of interest to regulatory bodies because they enable rapid preventative action to be taken. It should be noted that, despite the many studies demonstrating their potential, the application of fingerprinting methods in routine analysis and food authenticity surveillance still remains limited [105].

Until now, untargeted analysis has been associated mainly with direct analysis techniques, such as mass spectrometric-based metabolomics or isotope-assisted methods. Only a few studies have linked untargeted analysis with vibrational spectroscopic methods [106]. Moore et al. [107] developed non-targeted screening tools to detect adulteration in skimmed milk powder using NIR spectroscopy and Xu et al. developed a method for the untargeted detection of protein adulteration in yogurt by removing unwanted variations in pure yogurt [108]. In all these cases, the approach involved building statistical models based on the measured fingerprints of a large representative set of normal and abnormal samples, and then applying these models to unknown samples in order to characterise them. More recently, Fernández Pierna et al. have developed a moving window based PCA method using vibrational spectroscopic data. The PCA spectral score residuals are evaluated and used to define thresholds to be applied to the spectral score residuals of unknown samples [109]. The method was applied to study milk contaminated with melamine. Since the discovery of melamine contamination in infant milk formula in China, strict regulations have been enforced throughout the world and many papers have been published on the use of such methods as wet chemistry, chromatography, mass spectrometry and vibrational spectroscopy to detect melamine in both raw and powdered milk. In this study, liquid UHT milk was contaminated with melamine at various levels ranging from 0.01 % to 1 % (100 – 10 000 ppm) and measured using Fourier Transform Mid-Infrared (FT-MIR) spectrometry. Samples spiked at levels higher than 100 ppm were easily detected using this method, which would not have been possible using classical techniques such as Mahalanobis distance, usually applied as an outlier detection method.

#### **3.2.4. Isotope-ratio mass spectrometry (IRMS) and elemental analysis**

The authentication of the geographical origin of milk and dairy products is difficult to achieve because it needs to consider not only the variability inherent to a product of animal origin but also that of environmental conditions [110]. So far, the techniques employed for geographical authentication and/or differentiation of PDO dairy products are mainly based on stable isotope fingerprinting determined by IRMS or its combination with elemental composition most frequently determined by inductively coupled plasma-mass spectrometry (ICP-MS) or inductively coupled plasma atomic emission spectroscopy (ICP-AES). Stable isotopes mostly depend on climatic or geographical conditions, being affected by biological/environmental interactions in addition to hydrological and climatic variations, while the elemental composition is mainly affected by geology and pedological characteristics of the soil [111]. Isotopic analysis has been applied to the discrimination of several different cheeses with distinct geographical origin [112] and was officially adopted in 2011 as a reference method for verifying the authenticity of PDO Grana Padano cheese [113]. Isotopic analysis has also been proposed as a useful parameter to access the addition of

maize in the animal's diet and corresponding mislabelling of dairy products declared as being produced by pastured animals or PDO cheeses for which the diet of the animal has an established maximum amount of maize in the diet [114]. The analysis of mineral and trace elements coupled with the development of classification models based on chemometrics have also been applied for the differentiation of the type of milk production, namely organic versus conventional [115].

## 4. Overview of methods for authenticity testing

The following table provides a summary of the methods and the authenticity issues they address.

Analytical technique	Indicative data or analyte	Authenticity issue / information
Cryoscopy	Freezing point	Addition of water
Spectroscopy	Spectroscopic profile	Addition of water; melamine; addition of vegetable lipid or proteins; adulteration (non-targeted approach)
Isoelectric focusing	bovine $\gamma$ 2- and $\gamma$ 3-caseins	Bovine milk in sheep's, goat's and buffalo cheese
Lateral flow immunocromatographic tests	Bovine immunoglobulin G	Raw bovine milk in sheep's and goat's milk and cheese
ELISA	Bovine immunoglobulin G	Raw bovine milk in sheep's and goat's milk and cheese
ELISA	Soybean proteins	Vegetable proteins (soybean)
Competitive ELISA	Mouse monoclonal antibody raised against bovine $\kappa$ -casein	Raw and heat treated bovine and buffalo's milk in the milk and cheese of other species
Competitive ELISA	Glycomacropeptide of $\kappa$ -casein	Bovine rennet whey in bovine milk and milk products
GC-FID	Fatty acids, Triacylglycerols	Vegetable or other animal fat
SDS-CE		Vegetable proteins
LC-MS/MS	Melamine, cyanuric acid	Addition of non-food nitrogenous compound
HPLC-UV	Caseinomacropeptides	Addition of rennet whey
HPLC-UV	Furosine and lactulose	Addition of reconstituted milk
Species-specific PCR, real-time PCR, PCR-RFLP, LAMP, NGS	Molecular markers	Adulteration regarding species origin
Mass spectrometry (LC-MS and MALDI-TOF-MS)	Specific peptides	Adulteration regarding species origin; assessing freshness; addition of foreign proteins
IRMS	Isotope fingerprinting	Geographical origin
ICP-AES, ICP-MS	Trace Metals	Geographical origin



## 5. Conclusion

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During the last decade, cultural and social shifts have occurred in developed societies with consumers becoming increasingly aware about subjects such as biodiversity, climate change and ecological footprint. Therefore, one can expect a growth in the number of consumers willing to spend more money on certain food products, such as specialties produced according to traditional processes and organic foods. In this sense, possible adulterations in the dairy industry that may occur in a near future include mislabelling of the organic origin of milk and milk products and of the breed origin of milk used in the production of some PDO products, such as cheeses. Several PDO milk products, mostly cheeses, besides requiring the use of milk from specific animal species also specify the animal breed. Among several other examples, 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 some traditional breeds specified in PDO products are less productive compared to others that are more frequently used, a possible fraud could imply the use of milk from the same animal species but from a different breed to that specified for a particular milk product.

Looking into the future, there are several trends in milk and milk products authentication, one of those being the use of untargeted approaches such as spectroscopic techniques. In recent years, food safety has become an increased concern for consumers due to several important crises related directly or indirectly to human health. Most of the studies published have attempted to develop analytical procedures based on spectroscopic techniques to characterise/authenticate milk or milk products and at the same time detect the presence of any possible known contaminant or adulterant before reaching the food chain. Until now, statistical tools have been used to interpret multivariate data obtained from the spectroscopic analysis of different products and this has led to the creation of some decision rules. These enable verification of compliance against specifications in order to decide whether to reject or accept the product. However, the challenge will be to exploit the huge amount of information contained in the data generated by such spectroscopic techniques but taking into account the concept of data-driven discovery or untargeted analysis. New crises of adulteration/contamination with illegal ingredients other than known ones continue to occur from time to time. By relying solely on targeted analysis methods, adulteration could get out of control and analysis would become trapped in a cycle of 'adulteration, targeted analysis, and new adulteration', and so on. In contrast to targeted analysis, which uses information from known possible unusual ingredients, an untargeted experiment registers all information within a certain correlation/similarity, including data from new products. Untargeted detection methods are therefore required for screening products for a range of known and unknown adulterants. Untargeted analysis will mean alerts can be given more rapidly and fraud detected more easily

Vibrational spectroscopic methods are based on measuring the amount of electromagnetic radiation absorbed by a sample according to the Beer-Lambert law and can be very useful when authenticity and quality controls need to be established at both the laboratory and the industry levels, as they can be applied at the point where products are delivered to factories or during the production process. They are rapid with almost no sample preparation; they do not use chemical reagents and do not require skilled staff. However, fingerprinting methods are not confirmatory techniques, and therefore are not used in official control and have no weight in a judicial court. Nevertheless, such methods could be interesting for regulatory bodies as they would enable preventative actions to be taken rapidly. Spectroscopic methods are increasingly presented as new approaches for at-line, on-line and in-line control of authentication of food products. As

mentioned, these techniques are already routinely used in the industry to control both raw materials and finished products for specific production standards as a common authenticity issue and it is expected that they will be increasingly used in a near future. The main limitation of the spectroscopic approach is the requirement for large datasets to calibrate any given instrument. Also, a main challenge facing the spectroscopists is to extract the information in such a way that it can be used in qualitative and quantitative analysis. NIR spectra can contain thousands of absorbance values at defined wavelengths (i.e. variables) and the challenge is to characterise the spectral data set and isolate the variables that can be correlated with the information of interest (i.e. authenticity issue) [116]. To achieve this goal, a wide range of chemometric tools are at the disposal of the analyst who has to select the most appropriate according to the specific aims of the method and the characteristics of the dataset. Among the many methods proposed for the authentication of food products, spectroscopic methods seem to be the preferred ones to flag suspicious samples before, during and after the production of a food product. The real future challenge for spectroscopic techniques will be the demonstration of their daily use in the industry and the marketplace for food product authentication.

More recently, other novel and advanced techniques, such as real-time PCR coupled with High Resolution Melting (HRM) analysis, Loop-Mediated Isothermal Amplification (LAMP), next generation sequencing (NGS) and biosensors have emerged and are being applied to milk authenticity testing [81]. By relying on isothermal amplification of DNA, LAMP presents several advantages, namely its simplicity, speed and the fact that it does not require specialised equipment such as thermocyclers [117]. This, as well as the possibility of being integrated on microfluidic devices, allows for its portability, giving this technology great potential for use as a screening tool. NGS technologies have changed the way in which DNA can be analysed by increasing sequencing throughput by several orders of magnitude. NGS combined with DNA barcoding has been termed metabarcoding, which relies on the use of universal PCR primers to amplify, massively, one or more taxonomically informative targets. Recently, Ion Torrent NGS technology was successfully applied for the identification of species in dairy products by sequencing targeted mtDNA fragments [118]. Although the cost of NGS platforms is still very high, this technology presents several advantages regarding species identification for food authentication, and its use is expected to increase in the near future.

Due to their small size and high integration, biosensors are simple to operate with and generally capable of fast measurements. Therefore one can also expect their increased use in the dairy industry with multiple applications [119].

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