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Conventional And Non-Conventional Techniques In Milk Adulteration: Ensuring Authenticity And Food Saftety

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Abstract:

Food fraud has become a global concern, impelled by innovation within the food industry and shifting buyer preferences. Milk adulteration is a widespread issue, with various substances being added to alter its composition. Common adulterants include unwanted water content, non-native proteins, whey derived proteins, melamine, and urea compounds. Additionally,

fats originating from both plant and animal source, along with other minor constituents of milk fat, have been intentionally added undermining the authenticity of milk and its processed to derivatives. These practices not only diminish the quality of dairy products but also pose potential health potential hazards to end users. As a result, the need for reliable authentication processes has grown, leading to the transition from protein-based techniques to more sensitive and reproducible genetic analysis techniques utilize to authenticate dairy items. Among the commonly employed molecular techniques are PCR, Quantitative real time PCR, Multiplex PCR, PCR - RFLP. Despite the availability of various molecular tools for identifying species and breeds in dairy products, there is still a necessity for improved and more reliable methodologies. This review examines both conventional and modern genomic verification techniques applied to diary authentication. Additionally, it highlights the increasing significance of computational biology for managing extensive data collection and in the discovery of DNA markers that can improve authentication accuracy. One of the primary challenges in molecular-based authentication of dairy products is the quality of DNA, which can be affected by factors such as processing techniques, extraction methods, the chemical properties of the food matrix. PCR-based techniques remain the most widely utilized and successful in ensuring dairy product authenticity. The selection of DNA markers plays a critical role in the success of species and breed detection. While various

molecular approaches exist for identifying adulterant species, there is a notable gap in methods for detecting adulterant species. Public databases and advancements in bioinformatics have revolutionized data examination and will be essential in developing effective DNA markers to improve dairy authentication processes.

Keywords: Milk adulteration, species differentiation, authentication methods, molecular techniques.

Introduction

Milk is a vital source of nutrition, offering a high-quality proteins, essential fats, carbohydrates, vitamins, and minerals that contribute to overall health and development. It has a vital role in the diets of infants, nursing women, children, and the elderly due to its digestibility and ease of absorption. Despite its benefits, adulteration significantly compromises milk quality, creating economic burdens and potential health risks (Givens, 2020). Individuals with allergies to cow's milk may experience severe reactions if unknowingly exposed to adulterated dairy products that contain bovine milk or whey. Milk adulteration can occur through intentional or unintentional means. Intentional adulteration is motivated by economic benefits and includes the addition of substances such as excess amount of water, non-dairy proteins, melamine, urea, animal fat, and artificial milk (Giglioti, et al. 2022). Meanwhile, unintentional adulteration may result from antibiotics administered to cattle for treatment or environmental contaminants introduced during milk processing. Economically motivated adulteration (EMA) has been increasingly acknowledged as a public health concern, prompting agencies such as the Food and Drug Administration (FDA) to establish guidelines and preventive measures (Anagaw, et al. 2024).

Research on milk adulteration, including the National Survey on Milk Adulteration organised by the Food Safety and Standards Authority of India (FSSAI) in 2011, has shown that water is the widely used adulterant, which lowers milk's nutritional value and creates health risks for consumers (Yadav et al. 2022). A study in Hyderabad, India, also revealed widespread adulteration in milk samples, where different adulterant levels varied significantly—skim milk powder was identified in 80% of the samples, while sucrose was present in 22% (Singuluri, 2014). Globally, cases such as the melamine contamination in China in 2008 resulted in infant deaths, illustrating the severity of milk fraud (Gossner, et al. 2009). Similarly, melamine contamination in pet food and human food supplies in the United States was detected in 2007 (Rumbeiha & Morrison, 2011). The increasing presence of harmful substances in milk highlights the urgent need for efficient detection methods to safeguard consumers.

Food fraud, particularly EMA, is a growing global concern in the food industry. Despite its categorization by the FDA, a universal definition of food fraud has not yet established in the United States or Europe. Due to evolving consumer demands and advancements in the food sector, the extent of food fraud remains unclear, as many cases go undetected (Visciano, et al. 2021). To address these risks, the FDA introduced a Food Protection Plan in 2007 that prioritizes prevention over intervention. Milk and the dairy products are highly susceptible to fraud because of their nutritional value, global consumption, short shelf life, and insufficient authentication techniques. Fraudulent activities often involve replacing premium dairy products such as buffalo, sheep, and goat milk with cheaper alternatives like cow's milk or lower-quality breeds. Adulteration may also occur with addition of reconstituted milk powder, urea, rennet, sugar, salt, and skim milk powder, among other substances. Some instances of fraud involve supplementing milk with water or toxic compounds like melamine, which not only reduces nutritional benefits but also poses severe health hazards (Stadler et al. 2016).

Dairy products such as cheese, yogurt, cottage cheese, and cream cheese have long held cultural and economic significance in Europe. In 2017, fresh cheese represented the largest portion of the EU's total cheese production. Many varieties of European cheese, such as Greek Feta, French Roquefort, and Italian Mozzarella di Bufala Campana, are closely binded with their geographic origins. To protect the authenticity of these regional dairy products, the European Union introduced labels such as Protected Designation of Origin (PDO), Protected Geographical Indication (PGI), and Traditional Specialties Guaranteed (TSG). PDO ensures that a food product is manufactured in a specific location using local expertise, while PGI requires that at least one production step occurs in the designated region. TSG protects food products made using traditional methods,

even if production is not limited to a single geographic area. These labels help maintain product integrity, yet they also make such items more vulnerable to fraud due to their high market value (Barron et al. 2017).

Despite stringent food safety regulations set by organizations such as the British Retail Consortium (BRC), the International Organization for Standardization (ISO), globalization has contributed to an increase in food fraud cases (Banati, 2014). While not all cases pose health risks, some incidents have had severe consequences, such as the 2008 melamine contamination in China, which resulted in multiple fatalities. Given the complexity of global food supply chains, there is a growing demand for improved traceability methods. Food traceability, which allows businesses to track product information throughout its lifecycle, has become an essential tool for ensuring compliance with European food laws governing food and feed quality.

Traditional protein-based authentication methods for dairy products, such as ELISA and the chromatographic methods, have gradually been changed by DNA-based techniques that offer greater sensitivity and reproducibility. DNA is much more chemically and thermally stable in comparison to proteins, making it more resistant to food processing. DNA-based methods provide advantages in terms of efficiency, cost, sample processing, and accuracy, with detection limits comparable to protein-based techniques. The polymerase chain reaction (PCR) remains the mostly used molecular tool for dairy authentication due to its effectiveness in analyzing DNA (Downey, 2016). While various molecular methods exist for differentiating dairy species and breeds, technical challenges persist when applying these methods to traditional dairy products due to complex processing conditions.

Given increasing consumer awareness of food fraud, researchers are working to refine molecular detection techniques for dairy authentication. The primary objectives include compiling existing the molecular techniques for milk breed and species differentiation, such as endpoint PCR, multiplex PCR, real-time PCR, high-resolution melting (HRM), DNA hybridization assays, PCR-single strand conformation polymorphisms (PCR-SSCP), and restriction fragment length polymorphism (RFLP). Additionally, technical aspects related to DNA extraction and authentication in processed dairy products must be examined. Lastly, advances in next-generation sequencing and bioinformatics are expected to play a critical role in developing novel DNA-based authentication tools, strengthening food integrity, and ensuring the safety and quality of dairy products.

Milk adulteration with different species

Milk adulteration with different species is a widespread issue in the dairy industry, affecting both consumers and producers. This fraudulent practice involves mixing milk from various animal sources—such as cow, buffalo, goat, and sheep—without proper disclosure. The primary motivation behind this practice is economic gain, as producers substitute high-value dairy products with cheaper alternatives to maximize profit. However, this deception undermines the integrity and authenticity of dairy products, leading to ethical concerns, regulatory challenges, and potential health risks (Azad & Ahmad, 2016). However, the increasing global demand for dairy products has created opportunities for fraudulent practices. Shortages, price fluctuations, and the high cost of premium-quality milk drive many producers to engage in adulteration.

Certain types of milk, such as the buffalo milk and goat milk, are often valued for their nutritional superiority, distinctive flavors, and cultural significance. These varieties tend to be more expensive than conventional cow's milk. Consequently, some unscrupulous producers dilute them with cow's milk or other lower-cost alternatives (Marcelo, et al. 2025). Additionally, dairy processors may mix undeclared milk species into the processed milk products such as cheese, yogurt, and butter, violating food labelling regulations. The adulteration of milk carries several implications, ranging from economic fraud to severe health risks. One of the most concerning aspects is the potential harm to individuals with milk allergies. Similarly, lactose-intolerant individuals could suffer adverse effects if the milk they consume contains undisclosed sources of lactose. The nutritional composition of different milk species also varies, affecting the dietary intake of vulnerable groups (Abedini, et al. 2023). Goat and sheep milk, for instance, contain higher levels of certain beneficial nutrients such as calcium, vitamins, and medium-chain fatty acids. When these high-value milk sources are replaced with lower-quality alternatives, consumers may receive fewer nutritional benefits than expected (Marcelo, et al. 2025).

Economic Implications

Milk adulteration with different species has significant economic consequences. Fraudulent substitution of high-value milk varieties (such as buffalo, goat, or sheep milk) with cheaper alternatives (such as cow milk or low-quality breeds) leads to financial losses for both consumers and ethical producers (Azad & Ahmad, 2016). Genuine dairy farmers who invest in high-quality milk production struggle to compete with fraudulent manufacturers who undercut prices through adulteration. This disrupts fair trade, distorts market pricing, and diminishes the value of premium dairy products. Additionally, food fraud erodes consumer trust, potentially reducing demand for dairy products and affecting industry profitability.

Religious Implications

Different cultures and religions have dietary guidelines that dictate the consumption of certain animal products. Many individuals follow strict religious practices that require milk from specific species, such as kosher or halal-certified dairy products. The undisclosed mixing of milk from different animals can lead to religious violations, making the adulterated product unacceptable for consumption among certain groups. This deception undermines consumer rights, as people unknowingly ingest products that go against their religious beliefs. Transparent labeling and rigorous authentication measures are essential to ensure adherence to religious dietary laws (Mccormick, 2012).

Health Implications

Milk adulteration poses serious health risks. Individuals with allergies or intolerances to specific milk proteins—such as those allergic to cow's milk—may experience adverse reactions if exposed to adulterated dairy containing bovine milk. These reactions can range from mild gastrointestinal discomfort to severe anaphylactic responses. Additionally, the adulteration process may introduce contaminants, such as antibiotics, synthetic additives, or toxic substances like melamine, compromising food safety. Fraudulent practices also dilute the nutritional benefits of milk, depriving consumers of essential nutrients required for healthy development. Reliable detection methods and strict food safety regulations are necessary to prevent health hazards and protect public well-being (Edwards & Younus 2024).

Strengthening food authenticity measures, implementing transparent labeling policies, and enhancing detection technologies are crucial in mitigating the economic, religious, and health risks associated with milk adulteration. Ensuring consumer protection and maintaining industry integrity will help promote ethical dairy practices and safeguard public health (Duan et al. 2024).

Challenges in Detection and Prevention

Detecting milk adulteration is complex because fraudulent practices often involve subtle modifications to milk composition. Traditional detection methods, such as protein-based techniques (e.g., ELISA, chromatographic analysis), are effective to some extent but may lack the precision required to identify adulteration at the species level. As a result, newer DNA-based molecular techniques have gained prominence for milk authentication (Azad & Ahmad, 2016).

Polymerase chain reaction (PCR) is the mostly used molecular tool for detecting milk adulteration. PCR can analyze DNA from milk samples to determine the presence of different species. High-resolution melting (HRM) and restriction fragment length polymorphism (RFLP) analysis are additional molecular approaches that help differentiate milk sources with high accuracy. These techniques offer advantages in terms of sensitivity, reliability, and cost-effectiveness compared to protein-based methods. Advances in bioinformatics have also improved fraud detection and prevention. By analyzing genetic markers associated with different milk species, bioinformatic tools enable researchers to develop standardized testing protocols for identifying adulterated products (Abdelfatah, et al. 2015). Public databases containing genetic profiles of various milk species contribute to the refinement of authentication procedures, ensuring transparency in the dairy supply chain.

Despite these technological advancements, challenges persist in enforcing milk quality regulations. Strengthening food safety regulations, improving surveillance mechanisms, and fostering collaboration between regulatory agencies and dairy producers are essential steps in addressing milk adulteration effectively. Government authorities and food safety organizations play a critical role in safeguarding dairy quality. Regulatory bodies such as the Food and Drug Administration (FDA) and the Food Safety and Standards Authority of India (FSSAI) implement testing standards to identify adulterants and penalize violators. Consumer awareness campaigns also help educate the public on identifying potential signs of fraud and choosing certified dairy products (Gopalan, et al. 2025).

DNA based Authentication methods

DNA-based techniques, particularly PCR, have emerged as valuable tools for authenticating dairy products by identifying specific genetic markers. Milk somatic cells serve as a reliable DNA source, as they withstand processing treatments and accumulate during cheese production, ripening, and other dairy manufacturing processes. Several factors influence somatic cell concentration, including breed, environmental conditions, lactation stage, and overall animal health. This variability affects the ability to detect fraudulent species or breeds and complicates the quantitative analysis of mixed milk products. Additionally, dairy product composition—such as varying fat content in butter (80–84%), cream (25%), and cheese (30%)—can hinder DNA extraction efficiency. Microbial DNA from dairy fermentation further impacts target DNA yield (Baptista et al. 2021).

Common DNA extraction methods in dairy include centrifugation for milk and the homogenization for cheese, followed by lysis and the purification. Traditional approaches like phenol-chloroform and salting-out remain effective, but newer, faster methods such as silica-based column extraction and magnetic bead-based techniques offer improved usability and reliability for food authentication.

Traditional methods

The phenol-chloroform method, also known as organic solvent extraction, is one of the traditional approaches for DNA extraction. Due to the need for improved DNA recovery and the removal of inhibitory substances, various modifications have been introduced, including changes to cell separation and enrichment in milk, as well as adjustments to cell lysis techniques (Liu et al 2014). Additionally, researchers have combined this method with purification kits like the Wizard DNA cleanup system to enhance efficiency. The extraction process begins with cell lysis, during which sodium dodecyl sulfate (SDS) and proteinase K used to release DNA. A mixture of phenol, chloroform, and isoamyl alcohol is then added to separate the proteins from the DNA (Psifidi et al. 2010). Centrifugation follows, ensuring that unwanted proteins and molecular debris settle into the organic phase, while the DNA remains in the aqueous phase. This aqueous phase is carefully collected to prevent contamination. Thereafter, DNA is precipitated and suspended again in Tris-ethylenediaminetetraacetic acid (EDTA) buffer or the ultra-pure water. This method is highly effective in obtaining the DNA with good integrity, purity, and yield. However, it has notable drawbacks, including the use of toxic solvents substance, a time-intensive protocols, and the need for multiple sample transfers. Moreover, large-scale implementation can be inconsistent due to variations in DNA yield (Liao, et al. 2017).

Researchers such as Liao et al. have developed alternative methods for DNA extraction from the milk powder, which involve centrifugation to separate milk cells, followed by a washing step using phosphate-buffered saline (PBS). The sediment is then combined with an extraction buffer containing the SDS, EDTA, and proteinase K. The supernatant is subjected to phenol-chloroform-isoamyl alcohol extraction, yielding approximately 5.3 µg of DNA per gram of milk powder, albeit with reduced purity. In another study, Liao and colleagues compared the efficiency of organic solvent extraction with silica-based purification kits, such as Star Spin Blood DNA and DNeasy Blood and Tissue. Their findings revealed that organic solvent extraction produced higher DNA yields per millilitre of milk compared to silica-based methods, with purity levels comparable to those achieved using DNeasy Blood and Tissue and superior to Star Spin Blood DNA. Some researchers have successfully combined organic solvent extraction with silica-based column loading, resulting in effective DNA extraction from both milk and cheese samples. This approach enhances DNA

quantity and quality, making it a viable alternative for improved authentication processes (Liao and Liu, 2018).

The salting-out method has emerged as a cost-effective alternative for DNA extraction. It relies on the selective precipitation of the proteins and the contaminants by adding a saturated salt solution, typically 5 or 6 M sodium chloride (NaCl). Following centrifugation, the DNA-containing supernatant is separated from the insoluble proteins, and DNA is subsequently precipitated using the ethanol or the isopropanol. It can then be resuspended in ultra-pure water or an appropriate storage buffer. Compared to the phenol-chloroform extractions, salting-out is less labour-intensive, faster, and safe, as it eliminates the need for toxic solvents. However, its major limitation lies in the reduced purity of the extracted DNA (D'Angelo et al, 2007). On the positive side, DNA integrity is better preserved and compared to silica-based methods. D'Angelo et al. developed a salting-out method specifically for extracting DNA from caprine milk samples, achieving yields ranging from 2.12 to 612.12 µg per 40 mL of raw milk. While this method is simple and rapid, challenges remain, including the large sample volume required and inconsistencies in downstream applications (Liao et al. 2017).

Overall, DNA extraction methods continue to evolve, with researchers striving to balance efficiency, purity, and scalability. While organic solvent extraction remains a preferred method, advancements in salting-out and silica-based purification techniques offer promising alternatives. Enhancing DNA authentication processes is crucial in ensuring food safety and detecting fraudulent practices in dairy products.

Silica-based DNA extraction methods utilize chaotropic agents, like the guanidinium chloride, into lysis buffer to promote selective whereas reversible DNA binding to a silica matrix within the column. Once the DNA binds to the silica, it undergoes a washing process with an alcohol solution, which eliminates contaminants like proteins, RNA, and fats, commonly present in dairy samples. The purified DNA is then eluted with either water or the Tris-EDTA buffer. The efficiency of DNA yield depends on factors such as types of dairy samples, sample volume, and buffer composition. Several commercially available silica-based kits have been evaluated for their effectiveness in extracting DNA from milk and cheese samples. One study by Díaz et al. utilized the Wizard DNA clean-up kit (Promega, Madison, WI) following overnight cell lysis, successfully detecting cow milk in goat cheeses by amplifying the 12S rRNA gene of goats. Another study compared six DNA extraction protocols for milk samples, incorporating two commercial kits—NucleoSpin Blood and NucleoSpin Tissue (Macherey-Nagel GmbH & Co. KG, Germany)—as well as modifications to these kits. These modifications aimed to eliminate PCR inhibitors, involving the addition of Tris-EDTA to dissolve casein and chloroform to remove lipids after cell lysis. An in-house protocol using guanidinium hydrochloride instead of proteinase K was also assessed. Spectrophotometric analyses indicated that the modified commercial kits achieved superior DNA purity, and real-time PCR results confirmed their effectiveness in obtaining sufficient DNA quantities (Kovacevic , 2016).

Silica-based column extraction has also been successfully used for the isolation 0f DNA from goat and cow cheeses. Maudet et al. (2001) demonstrated that PCR amplification of the D-loop region was achievable even from pasteurized cheese using the DNeasy Blood and Tissue kit. Similarly, Bottero et al. (2003) applied the same kit for DNA extraction from cheese samples, utilizing multiplex PCR to identify bovine, caprine, and ovine species in milk mixtures. Compared to phenol-chloroform extraction, silica-based spin-column methods offer greater consistency, efficiency, and reduced processing time. They also circumvent challenges associated with incomplete phase separation in phenol-chloroform techniques. However, silica-based methods have some limitations, such as increased genomic DNA shearing, higher costs, and lower DNA yield. Another approach, magnetic bead-based extraction, relies on attachment of DNA to charged paramagnetic particles within a chaotropic buffer. DNA separation occurs in the presence of a magnetic field, effectively isolating it from other impurities. Despite the advantages of automation for high-throughput sample processing, this method has drawbacks, including DNA shearing and lower purity. A comparative study by Di Pinto et al. (2007) examined a paramagnetic-based extraction kit (Wizard Magnetic DNA Purification for Food, Promega Italia) and a column-based kit (DNeasy Tissue Kit, QIAGEN). Results indicated that the DNeasy Tissue Kit provided superior DNA yield and quality.

Overall, silica-based extraction techniques offer a reliable approach to DNA isolation, particularly for milk and cheese samples, but require improvements in DNA recovery and purity. While magnetic bead-based methods facilitate large-scale automation, further refinement is needed to enhance extraction efficiency and DNA integrity across different applications.

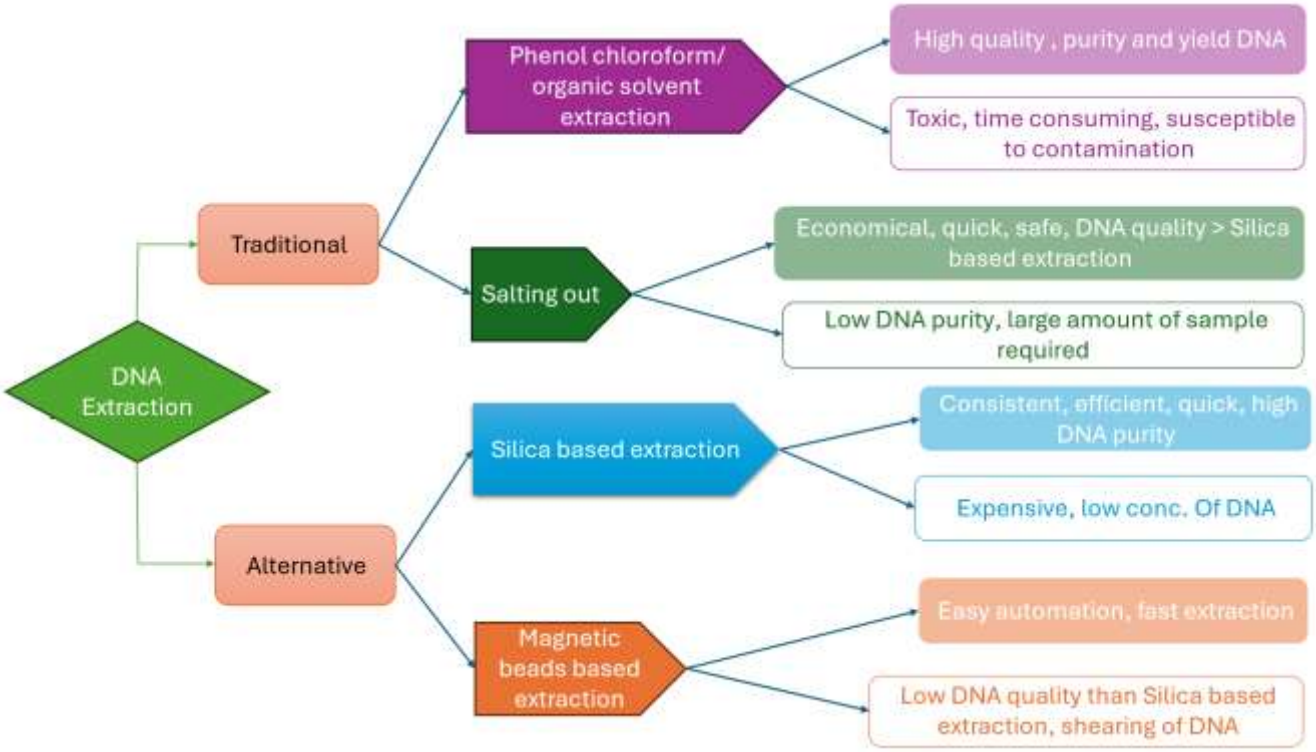


Figure 1: DNA extraction methods: Advantages and Disadvantages.

PCR-Based Authentication of Dairy Products

Polymerase Chain Reaction (PCR) has emerged as the most widely used method to detect the animal origin of dairy products. Nuclear as well as mitochondrial DNA (mtDNA) can be utilized for this validation process. However, mtDNA is the preferred choice due to several advantages, including its highly conserved sequence within a species along with easy accessibility in public databases (Pereira et al. 2013).

Early Applications of PCR for Dairy Authentication

PCR was first employed in dairy authentication by Plath et al. (1997) who utilized Restriction Fragment Length Polymorphism (RFLP) analysis alongside polyacrylamide gel electrophoresis. The PCR-RFLP method involves two major steps: magnification of a target DNA sequence and enzymatic digestion with restriction enzymes. The fragments are then separated through electrophoresis, producing distinct banding patterns for species differentiation. This technique is cost-effective and simple, making it a viable choice for milk authentication.

The utility of PCR-RFLP was further demonstrated by Abdel-Rahman et al. (2007) who applied it to detect buffalo, cattle, and sheep milk in dairy samples. Their study focused on the cytochrome b gene, to differentiate between buffalo and cattle milk. This underscores the significance of mtDNA markers in dairy authentication due to their stability and specificity.

Agarose Gel Electrophoresis for PCR Products

Commonly adopted dairy authentication methods involve PCR amplification and agarose gel electrophoresis separation of species-specific amplicons. This approach significantly reduces labor time while

maintaining simplicity. López-Calleja et al. (2005) implemented this method to identify goat milk in sheep milk with a remarkable sensitivity of 0.1%. They aligned the 12S rRNA gene sequences of multiple species, to design primers for specific fragment amplification.

Later, Masková and Paulícková (2006) refined this method to detect cow's milk in goat and sheep cheeses across different European countries. The results demonstrated distinct amplicon sizes corresponding to different species, specifically a 274 bp fragment for cow DNA, a 157 bp fragment for goat DNA, and a 331 bp fragment for sheep DNA.

High-Sensitivity PCR Detection of Milk Adulteration

Maudet et al. (2001) contributed significantly to PCR-based dairy authentication by designing a highly sensitive technique capable of detecting as little as 0.1%. in cheese samples from French markets. To prevent cross-amplification, cow-specific primers were meticulously premeditated, ensuring that goat mtDNA remained unamplified. By selecting primer positions within regions exhibiting multiple deletions between cow and sheep sequences, researchers successfully amplified a cow-specific fragment of 413 bp, further improving authentication accuracy.

In 2009, Bai et al. advanced PCR methodologies for dairy authentication by developing a system capable of detecting cow milk in yak milk mixtures, even at concentrations as low as 0.1%. Their study relied on mtDNA sequence alignment between bovine and yak species to design primers targeting the ND1 gene, a highly conserved mitochondrial marker.

Advancements in PCR-Based Dairy Authentication

Over the years, researchers have refined various PCR techniques, including multiplex PCR, duplex PCR, and real-time quantitative PCR, making dairy authentication more precise, efficient, and reliable.

Multiplex PCR

One of the major advancements in dairy authentication was developed by Bottero et al. (2003) who introduced multiplex PCR for the instantaneous identification of cow, goat, and sheep milk in laboratory-prepared cheese and commercially available Italian cheese. Their method, which provided a detection sensitivity of 0.5%. By using multiple primers targeting different species, their multiplex PCR system was capable to classify animal origins in dairy products accurately.

Duplex PCR

Deng et al. (2020) further refined PCR-based authentication by developing a duplex PCR method. Their method demonstrated a sensitivity of 0.1%. Duplex PCR was utilized to analyze two-fold mixtures of raw milk samples at fixed percentages, as well as processed dairy products. By amplifying 16S rRNA genes and D-LOOP genes, researchers ensured accurate differentiation of milk sources.

Real-Time Quantitative PCR

Real-time PCR offers significant advantages, including reduced analysis time and the ability to detect multiple DNA fragments simultaneously using fluorescent dyes. This method is particularly valuable in identifying species and determining the composition of milk used in cheese production.

Pereira et al. (2013) emphasized the benefits of real-time PCR in dairy authentication, particularly its ability to enhance species detection in processed dairy products. Real-time PCR is especially effective in testing UHT milk and high lipid content dairy products. In another study, Pirondini et al. (2010) demonstrated that real-time PCR surpasses conventional PCR methods in sensitivity when analyzing processed dairy samples. Lopparelli et al. (2007) further contributed to PCR advancements by developing a TaqMan minor groove binding (MGB) probes-based RT-PCR method to quantify cow milk in buffalo cheese. This development significantly origin improved the precision of dairy authentication with high detection sensitivity with low concentrations.

DNA-Based Authentication of Milk Powder

In recent studies, researchers have focused on the authentication of milk powders, ensuring that powdered dairy products maintain integrity in terms of species. Liao et al. (2017) devised an efficient DNA extraction method for milk powder, including conventional and RT-PCR-based detection. The method exhibited an impressive detection sensitivity of 0.1%, providing an effective tool for assessing dairy product purity.

Advancements in DNA-Based Authentication of Dairy Products

DNA-based verification has revolutionized the detection of milk adulteration, providing efficient and highly sensitive methods for identifying different species in dairy products. Over the years, several molecular techniques—including high-resolution melting analysis, DNA hybridization, biosensors, and various forms of PCR—have been developed to improve authentication accuracy, detect fraudulent practices, and maintain dairy industry integrity.

High-Resolution Melting (HRM)

HRM is a powerful molecular technique that enhances the sensitivity of DNA-based identification methods. It involves the amplification by PCR in the presence of a saturation dye, followed by controlled melting using a high-resolution instrument. Saturation dyes ensure all PCR products are labeled, allowing detailed analysis of the melting domains. Compared to conventional real-time PCR, high-resolution equipment provides enhanced data acquisition with finer temperature increments, improving the ability to differentiate species-specific DNA sequences.

Ganopoulos et al. (2013) devised a strong HRM-RT-PCR method enabling authentication of Greek PDO Feta cheese. Initially, bovine species were subjected to RT-PCR with species-specific primers. Then HRM analysis was done targeting the mitochondrial D-loop and tRNA^Lys regions. This approach successfully detected adulteration levels as low as 0.1%. Similarly, Agrimonti et al. (2019) introduced a quadruplex RT-PCR method utilizing SYBR GreenER fluorescent dye and HRM analysis. With a detection sensitivity of 0.1%, this method provided a valuable tool for quality control in dairy manufacturing.

DNA Hybridization for Rapid Species Detection

Kounelli et al. (2017) developed a fluorometric DNA hybridization assay using polystyrene microspheres with carboxyl functional groups. Species-specific probes were designed to target bovine, sheep, and goat DNA sequences. The method demonstrated impressive identification limits—0.01% and 0.05% bovine milk in goat and sheep yogurt mixtures.

Biosensor Technology

Recent innovations have introduced biosensor technology as an effective molecular tool for species identification. A paper based DNA biosensor technique used Species-specific primers to amplify distinct DNA fragments (83 bp for cows, 88 bp for sheep, and 150 bp for goats). The biotinylated PCR products were hybridized to complementary DNA probes, followed by interaction with streptavidin-coated gold nanoparticles for visible species detection. The assay proved capable of detecting cow milk adulteration at a threshold as low as 0.01%. Bougadi et al. (2020)

PCR-Single-Strand Conformation Polymorphism (SSCP)

PCR-SSCP is a simple and rapid method for identifying DNA polymorphisms and mutations across short sequences. The fundamental principle of SSCP relies on how DNA conformations vary with sequence length and composition under non-denaturing electrophoresis conditions, resulting in different migration patterns. Csikos et al. (2016) applied SSCP-PCR to analyze mitochondrial 12S rRNA gene sequences. The method achieved detection thresholds as low as 3% cattle DNA, demonstrating its efficacy for dairy product authentication.

Next-Generation Sequencing (NGS)

Ribani et al. (2018) pioneered the next-generation semiconductor-based sequencing (NGS) technology in dairy authentication. They employed Ion Torrent NGS technology to amplify mitochondrial 12S and 16S rRNA genes using universal species-specific primers. The resulting DNA sequences were analyzed to identify goat, sheep, cow, and buffalo DNA. Although refinement of detection sensitivity was still needed to reduce background signal interference, the method successfully detected 10% goat milk in mixtures containing 90% cow milk.

Isothermal Amplification

Alternative nucleic acid extension methods are developed to simplify dairy authentication without requiring conventional PCR equipment. These techniques provide rapid and low-cost molecular identification solutions.

- **Loop-Mediated Isothermal Amplification (LAMP)** - includes strand-displacing DNA polymerase to magnify multiple target regions using 4–6 primers. The reaction generates loop-like DNA structures that facilitate continuous amplification.
- **Strand Displacement Amplification (SDA)** utilizes a nicking endonuclease and strand-displacing polymerase to create nicks that regenerate amplification sites, leading to exponential amplification.
- **Recombinase Polymerase Amplification (RPA)** employs a recombinase enzyme to allow primers to invade and initiate amplification directly on ds-DNA, to bypass thermal cycling.

Kim et al. (2018) developed a rapid duplex LAMP method for on-site recognition of adulteration using a portable fluorescence device. The method amplified mitochondrial cytochrome b gene sequences, providing efficient detection at a minimum threshold of 2%.

Recombinase Polymerase Amplification (RPA)

Wang et al. (2020) introduced an innovative RPA method to authenticate yak milk, combining the technique with a lateral flow nucleic acid assay (LFNAA) for species detection. The method distinguished yak milk from potential fraudulent species such as cow, goat, camel, and donkey. LFNAA provided visual detection capabilities, confirming yak milk presence at concentrations as low as 5% in adulterated milk samples.

Although isothermal amplification methods offer rapid and cost-effective solutions, PCR-based techniques still demonstrate superior sensitivity. The results obtained using RPA for yak milk authentication exhibited higher detection limits than PCR-based approaches, suggesting that further optimization is needed.

Technique	Author	Adulterant	Target DNA	Detection ability (concentration)
PCR/agarose gel electrophoresis	Bai et al. (2009)	cows' milk	NADH- (ND1)	0.1%
	Díaz et al. (2007)	goats' milk	12S rRNA	1%
	Reale et al. (2008)	cows' milk	κ-casein	0.1%
	Abdel-Rahman & Ahmed (2007)	buffalo's, catttle's and sheep's milk	SSR and cytochrome-b	
RT-Taqman PCR	L´opez-Calleja et al. (2007)	goats' milk	12S rRNA	0.6%
	Di Domenico et al. (2017).	cattle, buffalo, sheep, and goat species	Cytochrome b	<1%
	Dalmasso, Civera, La Neve, and Bottero (2011)	cows' milk	Cytochrome b	2%
multiplex qPCR	Cottenet, Blancpain, and Golay (2011)	cow milk	Cytochrome b	1%
	Bottero et al. (2003)	goat, sheep and cow species	12s and 16s rRNA	0.5%
RT- PCR Intercalating Fluorescent Dye	Liao et al., 2017	cow milk	Cytochrome b ATP6	0.1%
Quadruplex PCR/ Capillary electrophoresis	Gonçalves et al. (2012)	cow, goat, sheep, and water buffalo	MtDNA	1%
	Rentsch et al. (2013)	cow milk	cytochrome b	0.2%
Duplex qPCR	Mafra et al. (2007)	cows' and goats' milk	12S rRNA	0.1%
Quadruplex RT-PCR/HRM analysis	Agrimonti et al. (2015)	cow, goat, sheep and buffalo species	Cytochrome b and 12S rRNA	0.1%
Triplex qPCR	Guo et al. (2018)	bovine and equine DNA	12S rRNA	1 pg to 5 pg of DNA
Duplex PCR	Deng et al. (2020)	cow milk	16S-RNA & D- LOOP	0.1%
	Golinelli et al. (2014)	cows' milk	12S rRNA	0.5%
PCR/DNA hybridization on fluorescent microspheres	Kounelli and Kalogianni (2017)	cow milk	Cytochrome b	0.01% to 0.05%
RT-PCR HRM analysis	Ganopoulos et al. (2013)	cow milk	MtDNA, D-loop	0.1%
PCR/DNA Hybridization assay on a biochip	Beltramo et al. (2017)	cattle, goat and Buffalo species	Cytochrome b	0.1%
LAMP	Kim and Kim (2018)	cow milk	Cytochrome b	2%

PCR-SSCP	Csikos et al. (2016)	cattle, buffalo, sheep, and goat	12S rRNA	3%
RPA-LFNAA	Wang et al. (2020)	cow, goat, camel and donkey milk	Mitochondrial genome complete sequence	5%
RAPD-SCAR	Cunha et al. (2016)	adulterant breeds	Random	
Paper-based DNA biosensor	Bougadi and Kalogianni (2020)	cow species	cytochrome b	0.01%

Table: DNA based Authentication techniques for milk adulteration

Species Differentiation Methods for Dairy Authentication

The authentication of dairy products, particularly milk and cheese, has historically focused on identifying species-specific markers. However, species differentiation in these products has received relatively less attention. Species identification is critical for traceability, especially in the dairy sector, where certain products, hold substantial commercial and nutritional value. These cheeses are produced from specific species that thrive in specific geographic regions and are recognized for their unique milk composition and quality. However, various challenges, including climatic variations affecting animal feed availability, low milk yields associated with specific species, and seasonal vegetation changes, can lead to adulteration of PDO products. This adulteration often results in defects in nutrition, flavor, and texture due to the introduction of milk from non-authentic species (Dias & Mendes 2018).

Species differentiation techniques provide essential benefits in the dairy industry, ensuring transparency, quality control, and fair trade. Many high-value dairy products rely on milk from specific breeds that contribute to distinct flavor profiles and nutritional compositions. Adulteration of PDO products with milk from non-authentic species can lead to economic losses, consumer deception, and compromised product quality. Therefore, implementing reliable DNA-based authentication methods is essential for protecting species-specific dairy products from fraud.

Challenges in Species Identification for Dairy Products

One of the main difficulties in breed differentiation stems from the limited availability of breed-specific DNA databases. The lack of comprehensive reference sequences restricts the use of various DNA-based methods. Although sequencing gives most accurate genetic identification, it is not always viable due to cost constraints and technological limitations.

Several PCR-based fingerprinting methods, have been developed to address breed differentiation challenges (Chuna et al 2016). These methods offer significant advantages in detecting fraudulent dairy products by identifying species-specific genetic markers .

RAPD-PCR and SCAR Markers

RAPD-PCR utilizes a single arbitrary primer (8–15 nucleotides long) to hybridize randomly with the template DNA, generating breed-specific fingerprints. This technique has been employed for dairy product authentication, particularly in verifying milk authenticity. By comparing the fragment patterns obtained from adulterant breeds against those of the legitimate breed, researchers can identify discriminatory genetic fragments that indicate fraud. Once identified, these are isolated using agarose gel electrophoresis, cloned, and sequenced. The resulting DNA sequences, known as Sequenced Characterized Amplified Region (SCAR) markers, allow researchers to design longer and more specific primers tailored to breed differentiation. The combination of RAPD and SCAR techniques provides a powerful tool for detecting milk origins in dairy products.

PCR-SSCP

PCR-SSCP analysis is another DNA-based fingerprinting method that can facilitate breed authentication. This technique identifies genetic polymorphisms by observing how single-stranded DNA molecules fold into specific conformations under non-denaturing conditions. Different conformations produce unique migration patterns in gel electrophoresis, allowing researchers to distinguish genetic variations between breeds. Alex et al. (2017) utilized PCR-SSCP analysis which uncovered important genetic polymorphisms that could be used for breed differentiation and reproductive trait selection. This approach has significant potential for breed-specific identification in dairy products, offering insights into genetic diversity and traceability.

PCR-RFLP

Another effective technique for breed differentiation is PCR-RFLP, which counts on enzymatic digestion of amplified DNA sequences to generate distinct fragment patterns. This method has proven valuable in authenticating Egyptian goat dairy products. Abdel-Aziem et al. (2018) developed a PCR-RFLP method capable of distinguishing between three Egyptian goat breeds based on **myostatin (MSTN)** and **prolactin (PRL)** genes polymorphism. Since these genes play critical roles in milk yield and composition, their genetic variation can serve as a key marker for breed authentication. By analyzing restriction fragment patterns obtained from different breeds, researchers can confirm the authenticity of goat milk used in dairy production.

DNA Markers and Bioinformatics in Dairy Products Traceability and Authentication

The authentication and traceability of dairy products have become increasingly vital due to global concerns about food fraud, mislabeling, and biodiversity conservation. DNA-based techniques play a crucial role in addressing these issues, providing methods to distinguish species, breeds, and geographical origins dairy products. In spite of the efficiency of these methods, there remains a lack of standardized protocols and universal procedures for DNA-based food authentication (Wilson et al. 2019).

DNA Barcoding

DNA barcoding is a molecular method used to detect species, breeds, or individuals based on standardized genomic regions known as DNA barcodes (Nair, et al. 2024). DNA barcoding in food authentication is attributed to several factors:

1. **Species Variability Studies** – DNA barcoding enables the study of genetic differences among taxa.
2. **Standardized Protocols** – From sample collection to data analysis, the method follows a structured approach.
3. **Bioinformatics Integration** – Advanced computational tools allow data processing and publication in public databases, improving accessibility.

While DNA barcoding has been widely implemented in seafood authentication through projects such as the Fish Barcode of Life Initiative (FISH-BOL), its application in dairy products remains limited. FISH-BOL forms part of the Barcode of Life Data (BOLD) system, a robust DNA reference library that aids in global food traceability. For dairy products, particularly those with **Protected Designation of Origin (PDO)** labels, breed traceability is an essential authentication factor (Ward et al. 2009). Dairy producers and brands are advocating for conservation efforts to protect breeds associated with PDO-certified products, which are often marketed as symbols of quality and regional heritage.

Despite its potential, DNA barcoding remains underutilized in dairy product authentication, with no dedicated studies solely focused on milk traceability through barcoding. However, a notable example is the study by Ponzoni et al. (2009) which employed plant DNA barcoding markers which could be linked to pasture locations, potentially aiding the traceability of PDO cheeses.

Bioinformatics Tools for DNA Barcode Analysis

DNA barcoding relies on advanced computational tools to process sequencing data efficiently. Traditionally, **Sanger sequencing** has been the preferred method for barcode generation, followed by data analysis using various open-source platforms such as:

- **SPIDER** – Quality control and format conversion for barcode sequences.
- **ClinQC** – Filtering and overlapping sequence reads.
- **SeqTrace** – Streamlining barcode sequence alignment.

Recently, **PIPEBAR**, an automated DNA barcode pipeline, was developed by Oliveira et al. (2018) to enhance Sanger sequence processing, improving efficiency in food authentication research.

Mitochondrial DNA for Food Authentication

Mitochondrial DNA (mtDNA) serves as the foundation for DNA barcoding in food authentication (Rooney, et al. 2015). Compared to nuclear DNA, mtDNA offers several advantages:

- **High Copy Number** – Ensures sufficient quantity for amplification.
- **Lack of Introns** – Simplifies sequencing and data interpretation.
- **Low Recombination Rate** – Minimizes genetic variability within species.
- **Stability in Processed Food** – mtDNA remains intact even after heat treatments.

Among mitochondrial genes, **cytochrome c oxidase subunit 1 (COI)** is the standard barcode for animal species (Hebert, et al. 2003). Other alternatives include:

- **Cytochrome b (cob)** – Encodes apocytochrome b.
- **16S rDNA and 12S rDNA** – Frequently used for phylogenetic analysis.

The Control Region (D-Loop)

The mitochondrial **D-loop**, is responsible for mtDNA transcription and replication, has been utilized in breed authentication. Liu et al. (2016) conducted a study on fifteen Tibetan sheep breeds using the **mtDNA control region**, employing a four-step approach:

1. **DNA Extraction** – Blood samples were collected and processed for genomic analysis.
2. **PCR Amplification** – Primers targeting the D-loop sequence were used.
3. **Sequencing** – Amplified fragments were analyzed through genomic sequencing.
4. **Bioinformatics Analysis** – Computational tools processed data to evaluate genetic diversity.

Multiple bioinformatics platforms (Daugelaite, et al. 2013) facilitated the analysis, including:

- **Clustal Omega** – Multiple sequence alignment.
- **DnaSP** – Sequence polymorphism assessment.
- **Arlequin** – Genetic differentiation coefficient estimation (GST).
- **MEGA** – Phylogenetic and evolutionary analysis.
- **NETWORK** – Median-joining network construction for genetic relationships.

These studies demonstrate the effectiveness of DNA barcoding in breed authentication and traceability, presenting opportunities for its broader adoption in dairy product verification.

Applications in Dairy Authentication

The integration of DNA markers and bioinformatics into dairy product authentication holds promising potential for improving food safety and preventing fraud. While DNA barcoding has successfully established itself in seafood authentication through FISH-BOL and BOLD, its application in dairy remains underdeveloped. The prospect of using DNA fragments from pasture plants to enhance PDO cheese traceability suggests innovative pathways for authentication.

Despite its advantages, DNA barcoding requires greater standardization to ensure universal applicability in dairy authentication. Establishing protocols for sample collection, amplification, sequencing, and bioinformatics processing will be crucial for its adoption in food traceability systems. Future studies could focus on refining DNA barcode markers for species and breed authentication, further developing mtDNA-based traceability methods for PDO-certified dairy products (Dawan & Ahn 2022).

Advancements in sequencing technologies, including **next-generation sequencing (NGS)**, could also enhance DNA barcode applications by increasing throughput and reducing costs. As food authentication research continues to evolve, the synergy between **DNA markers, barcoding methodologies, and bioinformatics** may contribute to ensure transparency and consumer trust in dairy products.

DNA Markers for Livestock Traceability and Species Differentiation in Dairy Products

The authentication and traceability of livestock breeds used in dairy production have become critical areas of research, particularly with the increasing importance of protecting **Protected Designation of Origin (PDO)** products. Various DNA markers have been studied to ensure that dairy products originate from the correct breeds and geographic regions. The most widely utilized molecular markers for breed differentiation and traceability include **microsatellites (STRs)** and **single nucleotide polymorphisms (SNPs)**. These markers have played a significant role in genetic diversity studies, breed identification, parentage assessment, and population structure evaluation (Vishnuraj, et al. 2023).

Microsatellites (STRs) in Livestock Traceability

Microsatellites or **short tandem repeats (STRs)**, are polymorphic, 1–6 base pair long DNA motifs arranged in repeated sequences. Their high variability and polymorphism have made them valuable tools for:

- Genetic diversity studies (Ozmen, et al. 2020)
- Breed characterization (Silva et al., 2017)
- Parentage verification (Pei et al., 2018)

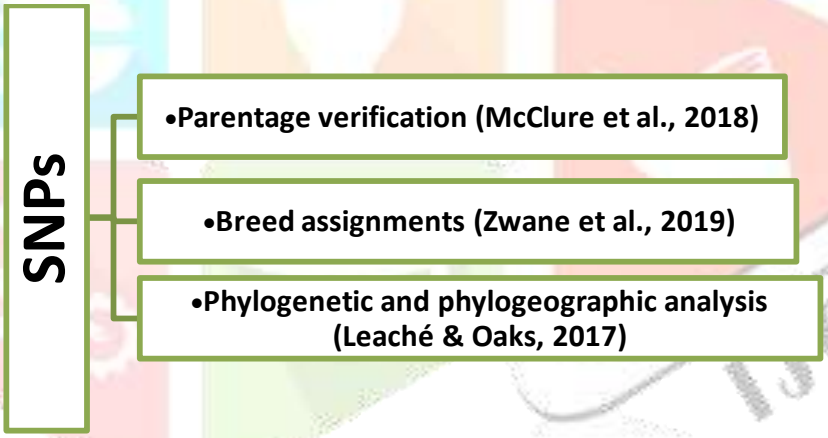
Despite their usefulness, STRs have not yet been widely implemented for breed differentiation or dairy product authentication. However, studies suggest that specific STRs could serve as powerful tools for dairy breed identification in the future. Sardina et al. (2015) investigated microsatellite markers to detect adulteration in Girgentana dairy products from Sicilia, Italy. Their methodology involved:

1. **DNA Extraction** – Collecting DNA from blood samples and cheese products.
2. **Pooling of DNA Samples** – Mixing DNA from various breeds in controlled proportions.
3. **Microsatellite Amplification** – Using primers recommended by the **International Society for Animal Genetics (ISAG)** and **Food and Agricultural Organization (FAO)**.
4. **Statistical Analysis** – Evaluating genetic variability through specialized software such as **CERVUS, FSTAT, ARLEQUIN, and GENEPOP**.

These microsatellite-based approaches demonstrated potential for breed traceability in dairy products, although further refinement is needed for large-scale implementation.

SNPs: The Preferred Marker for Species Identification

While STRs have played an important role in genetic studies, **single nucleotide polymorphisms (SNPs)** have become the preferred markers for breed assignment due to their abundance, discriminatory power, and cost-effectiveness. SNPs represent single nucleotide substitutions occurring at specific positions within the genome, allowing precise identification of genetic differences among breeds.



Pecka-Kiełb et al. (2020) identified four SNPs, with the TT genotype at **SNP4** being associated with superior milk quality. Similarly, Moradi et al. (2017) examined SNPs within the **mtDNA D-loop region** to classify **indigenous Iranian sheep breeds** into unique mitochondrial haplotypes.

Next-Generation Sequencing (NGS) for High-Throughput SNP Analysis

The advent of **next-generation sequencing (NGS)** has allowed simultaneous analysis of thousands of SNPs, significantly improving breed differentiation accuracy. However, NGS requires high-quality DNA samples for amplification, posing challenges in practical applications. Among the various sequencing platforms, **Illumina** has emerged as the most widely used for high-throughput DNA sequencing (Haynes et al., 2019).

Several open-source bioinformatics tools have facilitated SNP analysis, including:

- **GATK** – Genome analysis toolkit for SNP calling.
- **SAMtools** – Widely used for SNP identification.
- **MUMmer** – Assists in genome comparison and alignment.

Despite their utility, these tools require expertise in bioinformatics, limiting accessibility to non-specialist users. To address this, Leekitcharoenphon et al. (2012) developed **snpTree**, an automated online platform for SNP analysis and tree construction. This server integrates SNP analysis tools into a user-friendly interface, making genomic comparisons more accessible.

Microarray-Based Bead Chip Technology for Breed Authentication

Apart from sequencing-based approaches, **Bead Array (Bead Chip) technology** has been widely applied for **SNP genotyping** in livestock species. Developed by **Illumina**, Bead Chip technology enables rapid SNP analysis and has been used in multiple animal genomics projects, including:

- **BovineSNP50** (Cattle)
- **PorcineSNP60** (Pigs)
- **CanineSNP20** (Dogs)
- **OvineSNP50** (Sheep)

The **OvineSNP50 chip**, established in collaboration with the **International Sheep Genomics Consortium (ISGC)**, has successfully assigned parentage in sheep breeds.

Challenges in SNP-Based Dairy Product Authentication

While SNPs offer promising solutions for breed identification, no established protocol currently exists for their use in dairy product authentication. The complexity of breed evolution and population mixing poses challenges in defining distinctive SNP markers for traceability (Baptista, et al. 2021). Successful implementation of SNP-based authentication will require:

1. **Standardized Marker Sets** – A minimal number of SNPs capable of distinguishing purebred and crossbred populations.
2. **Comprehensive Databases** – Development of breed-specific genomic libraries.
3. **Refinement of Bioinformatics Tools** – Improving user accessibility in data analysis.
4. **Regulatory Approval** – Establishing guidelines for SNP-based food authentication.

Future Perspective: Identification of Novel Species-Specific Markers

Efforts to eliminate milk adulteration require a multifaceted approach, incorporating advanced authentication techniques, regulatory enforcement, consumer education, and industry collaboration. Strengthening molecular detection methods, establishing standardized testing protocols, and improving food labeling regulations are essential steps toward ensuring the authenticity and integrity of dairy products.

PCR has significantly improved detection of species adulteration in milk, cheese, and powdered dairy products. Multiplex PCR enables the identification of multiple species in a single reaction, while duplex PCR increases precision by isolating bovine milk adulteration in different dairy types. Real-time PCR enhances efficiency by offering high-throughput analysis, ensuring the rapid authentication of dairy products. As PCR sensitivity continues to improve—reaching detection limits as low as 0.1%—fraudulent practices can be effectively identified. SNP haplotype analysis and capillary electrophoresis provide advanced alternatives to conventional agarose gel electrophoresis, enhancing automation and reliability.

Advancements in PCR technology will further refine dairy authentication, integrating next-generation sequencing (NGS) and biosensor-based systems for superior species detection. As the global dairy market expands, ensuring authenticity will remain a priority for regulators and consumers alike. Species authentication plays a crucial role in safeguarding product integrity. While molecular markers such as microsatellites (STRs) and SNPs have improved livestock traceability, further innovation is required to enhance accuracy, efficiency, and accessibility. The incorporation of next-generation sequencing (NGS) will provide detailed genomic insights into breed identification, enabling the creation of comprehensive species-specific genetic databases. Additionally, molecular markers targeting milk composition genes can improve authentication processes. Integrating multiple techniques—such as RAPD-PCR, SSCP analysis, and PCR-RFLP—into a unified workflow may offer a robust approach to species verification.

DNA-based authentication methods will continue to evolve, integrating biosensors, artificial intelligence, and high-throughput sequencing to refine species detection accuracy. Hybridization techniques, biosensor integration, and multiplex PCR could further strengthen dairy authentication strategies. The continuous advancement of real-time PCR, HRM analysis, and DNA hybridization will enhance quality control measures, reinforcing consumer trust in dairy products.

Applications for Improved Dairy Traceability

- Development of SNP-based standards for verifying PDO-certified dairy products.
- Expansion of global genetic databases to establish breed identity linked to milk composition.
- Integration of blockchain technology for secure and transparent dairy traceability.

The increasing adoption of AI-driven genome analysis and machine learning will elevate the efficiency and accuracy of breed authentication, ensuring dairy product integrity across international markets. Continued innovation will be key to maintaining consumer trust and preserving authenticity in the dairy industry.

Conclusion

Ensuring the authenticity of dairy products remains a critical issue due to the adulteration of milk with different species, affecting nutrition, health, and consumer trust. Fraudulent practices, often driven by economic incentives, lead to misrepresentation, compromise product integrity, and pose potential health risks. While molecular detection techniques and bioinformatics have significantly improved authentication methods, continued regulatory enforcement and public awareness efforts are essential. Strengthening monitoring systems, ensuring accurate labeling standards, and promoting ethical dairy practices will help maintain consumer confidence and availability of genuine, high-quality milk products.

As technological advancements continue, integrating molecular genetics with high-throughput sequencing will refine breed authentication methods, enabling dairy producers to uphold product quality and safeguard breed-specific certifications. The emergence of PCR-based methods has transformed dairy product authentication by offering unmatched specificity, sensitivity, and precision. Initially, conventional PCR and RFLP techniques were employed for species identification, but subsequent advancements introduced highly sensitive approaches capable of detecting even minute traces of adulteration. The integration of agarose gel electrophoresis and mitochondrial DNA markers has further refined dairy authentication, preventing fraudulent adulteration and ensuring product integrity. As multiplex PCR enhances detection efficiency, the evolution of dairy authenticity testing continues to strengthen quality control within the industry.

DNA markers, such as microsatellites (STRs) and single nucleotide polymorphisms (SNPs), have played a crucial role in livestock traceability and breed verification. While STRs provide valuable genetic insights, SNPs offer greater precision in breed differentiation. Technological advancements, including next-generation sequencing (NGS) and microarray BeadChip platforms, have streamlined SNP analysis for genetic diversity studies. Despite existing challenges, ongoing research and innovation will further improve breed

authentication methods within the dairy sector. DNA barcoding and bioinformatics have emerged as valuable tools for dairy product verification and traceability. While widely adopted for seafood authentication, their application in dairy remains relatively new. The identification of mitochondrial barcode genes and bioinformatics-assisted sequence analysis presents promising opportunities for enhancing dairy authentication. Future food traceability efforts will depend on refining these techniques, establishing standardized protocols, and incorporating advanced technologies to improve accuracy, accessibility, and consumer trust.

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