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**Authentication of selected game species in food products
based on nuclear markers and qPCR**

Uwierzytelnianie wybranych gatunków zwierząt łownych w produktach
spożywczych w oparciu o markery jądrowe i qPCR

Doctoral dissertation in the field of agricultural sciences
in the discipline of Food Technology and Nutrition

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Poznań, 2025

Dedication

This work is dedicated to the glory of the Almighty Allah who in His infinite blessings made it possible to begin and complete this project.

To my mum, Mrs. Mojisola Durojaye Adenuga for being my constant rock and refuge.

A special mention to my dear friend, sister, and supporter Joy Emezuo for her unflinching and unwavering support on this journey – you are the best!

To my wonderful community of friends and colleagues, Adeola Eletu, Srutisudha Mohanty, Cynthia Aniekwena, Daniel Anyebe, Mar Albert, Marcellus Arnold, Yolanda Rajagukguk, Marcin Sadowski, Natalia Czarna, Dr. Anita Spychaj, Dr. Mirosława Krzywdzińska-Bartkowiak and the entire Department of Meat Technology, I say a very big thank you.

To my advisor, Professor Magdalena Montowska, for her patience, support, belief in me, and most of all, for opening the doors of knowledge to me and allowing me to learn and grow under her gentle tutelage.

And to my siblings, Wale, Tayo, and Gbenga Adenuga, I did not forget you. You are the best siblings in the world!

Acknowledgement

The doctoral thesis was carried out as part of the project entitled "Comparative peptidomic and genetic analysis of the authenticity of food of animal origin" financed by the National Science Centre, Poland, project number 2020/37/B/NZ9/00082.

List of Publications

- P1. Adenuga, B. M., Montowska, M. (2023). The Nigerian meat industry: An overview of products' market, fraud situations, and potential ways out. *Acta Scientiarum Polonorum Technologia Alimentaria*, 22(3), 305-329. <http://dx.doi.org/10.17306/J.AFS.2023.1157>
MNiSW = 40 points, IF = 1.30
- P2. Adenuga, B. M., Montowska, M. (2023). A systematic review of DNA-based methods in authentication of game and less common meat species. *Comprehensive Reviews in Food Science and Food Safety*, 22, 2112-2160. <https://doi.org/10.1111/1541-4337.13142>
MNiSW = 200 points, IF = 12.00
- P3. Adenuga, B. M., Biltres, R., Villa, C., Costa, J., Spychaj, A., Montowska, M., Mafra, I. (2025). Unravelling red deer (*Cervus elaphus*) meat adulteration in gourmet foods by quantitative real-time PCR. *Food Control*, 168, 110872. <https://doi.org/10.1016/j.foodcont.2024.110872>
MNiSW = 140 points, IF = 5.60
- P4. Adenuga, B. M., Biltres, R., Villa, C., Costa, J., Spychaj, A., Montowska, M., Mafra, I. (2024). A novel normalized quantitative real-time PCR approach for ensuring roe deer (*Capreolus capreolus*) meat authenticity in game meat foods. *Foods*, 13(23), 3728. <https://doi.org/10.3390/foods13233728>
MNiSW = 100 points, IF = 4.70
- P5. Adenuga, B. M., Spychaj, A., Montowska, M. (2025). A new nuclear marker for quantitative analysis of wild boar and domestic pig meat in game meat products using PLAG1 zinc finger gene. *Scientific Reports*, 15, 20454. <https://doi.org/10.1038/s41598-025-05167-x>
MNiSW = 140 points, IF = 3.80

Total points according to the MNiSW: **620**

Total Impact Factor: **27.40**

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Summary

Authentication of selected game species in food products based on nuclear markers and qPCR

The global food industry faces increasing challenges in ensuring the authenticity of high-quality products, particularly within the niche market for game meat. As consumer demand for exotic and sustainably sourced proteins grows, so does the risk of fraudulent practices such as species substitution, mislabelling, and illegal trade. Therefore, this doctoral thesis aimed to establish and validate sensitive, specific, and reproducible quantitative real-time PCR (qPCR) methods for the identification and quantification of three common game species in Poland (red deer, roe deer, and wild boar/pig) which are also popular in the European game meat market.

The study focused on the design of three novel species-specific nuclear DNA markers, carefully selected for their high interspecies variability and short amplicon length (76–127 bp) to ensure stability in processed meat products. The assays targeted the following marker genes: the troponin I gene (TNNT2), the agouti signalling protein gene (ASIP), and the PLAG1 zinc finger gene. These TaqMan probe-based quantitative PCR assays enabled the accurate detection and quantification of red deer, roe deer, and wild boar or pig meat in processed food products.

The methodology encompassed primer design, *in silico* and *in vitro* specificity screening, and optimization of qPCR conditions. All assays demonstrated high analytical sensitivity, with limits of detection (LOD) and quantification (LOQ) as low as 0.01 ng for the species studied. The matrix-adapted quantification approach allowed for relative detection limits as low as 0.5% red deer, 0.1% wild boar, and 0.05% roe deer in simulated laboratory meat mixtures. Cross-reactivity tests using 16 animal species, including uncommon and domestic meats, along with various herbs and spices, confirmed high assay specificity. Validation experiments using laboratory-prepared meat mixtures subjected to various processing conditions (raw meat, oven-cooking, and autoclaving) highlighted the method's robustness.

Application of the validated method to commercially available game meat products from four European countries revealed widespread species misrepresentation. Overall, 64% of roe deer-labelled products and 54% of red deer-labelled products contained undeclared species or lacked the declared species entirely, while four out of 38 products contained undeclared wild boar meat or pork. These findings raise significant concerns regarding regulatory compliance, consumer deception, and food safety risks. The study necessitates the urgent need for effective routine monitoring of game meat authenticity using DNA-based tools. The developed qPCR assays provide a practical solution for industry stakeholders and regulatory authorities, enabling improved enforcement of labelling laws, halal monitoring, protection of consumer rights, and mitigation of economic losses due to fraud. Furthermore, the findings contribute valuable insights into market integrity and highlight the importance of transparent, traceable supply chains in maintaining consumer trust in high-value niche products like game meat.

Keywords: food authentication, meat products, game species quantification, nuclear markers, qPCR.

Streszczenie

Uwierzytelnianie wybranych gatunków zwierząt łownych w produktach spożywczych w oparciu o markery jądrowe i qPCR

Globalny przemysł spożywczy stoi przed rosnącymi wyzwaniami w zakresie zapewnienia autentyczności produktów wysokiej jakości, zwłaszcza na niszowym rynku mięsa z dziczyzny. Wraz ze wzrostem popytu konsumentów na białka egzotyczne i pozyskiwane w sposób zrównoważony, rośnie również ryzyko oszustw, takich jak zamiana gatunków, błędne etykietowanie i nielegalny handel chronioną fauną. Dlatego celem niniejszej rozprawy doktorskiej było opracowanie i walidacja czułych, specyficznych i powtarzalnych ilościowych metod PCR w czasie rzeczywistym (qPCR) do identyfikacji i kwantyfikacji trzech pospolitych w Polsce gatunków zwierząt łownych (jelenia szlachetnego, sarny i dzika/świni domowej), które cieszą się również popularnością na europejskim rynku dziczyzny.

Praca koncentrowała się na zaprojektowaniu trzech nowych, specyficznych dla gatunku markerów DNA jądrowego, starannie wyselekcjonowanych ze względu na ich wysoką zmienność międzygatunkową i krótką długość amplikonu (76–127 pz), aby zapewnić stabilność w przetworzonych produktach mięsnych. Analizy ukierunkowano na następujące geny markerowe: gen troponiny I (TNNI2), gen białka sygnałowego Agouti (ASIP) oraz gen palca cynkowego PLAG1. Te testy qPCR oparte na sondach TaqMan umożliwiły dokładne wykrywanie i analizę ilościową mięsa jelenia szlachetnego, sarny, dzika lub wieprzowiny w przetworzonych produktach spożywczych.

Metodologia obejmowała projektowanie starterów, weryfikację specyficzności *in silico* i *in vitro* oraz optymalizację warunków qPCR. Wszystkie testy wykazały wysoką czułość analityczną, z granicami wykrywalności (LOD) i kwantyfikacji (LOQ) wynoszącymi zaledwie 0,01 ng dla badanych gatunków. Metoda ilościowa z wykorzystaniem matrycy pozwoliła na uzyskanie względnych granic detekcji na poziomie zaledwie 0,5% mięsa jelenia szlachetnego, 0,1% dzika i 0,05% sarny w symulowanych laboratoryjnych mieszankach mięsnych. Testy reaktywności krzyżowej z użyciem 16 gatunków zwierząt, w tym rzadkich i hodowlanych, a także różnych ziół i przypraw, potwierdziły wysoką specyficzność testów. Eksperymenty walidacyjne z użyciem laboratoryjnie przygotowanych mieszanek mięsnych poddanych różnym warunkom przetwarzania (mięso surowe, pieczenie i autoklawowanie), podkreśliły siłę metody.

Zastosowanie zwalidowanej metody do dostępnych na rynku produktów z dziczyzny z czterech krajów europejskich ujawniło powszechne błędne przekazywanie informacji o gatunkach. Ogółem 64% produktów oznaczonych jako mięso sarny i 54% produktów oznaczonych jako mięso jelenia szlachetnego zawierało gatunki niezadeklarowane lub w ogóle ich brakowało w deklaracji, natomiast cztery z 38 produktów zawierały niezadeklarowane mięso dzika lub wieprzowinę. Ustalenia te budzą poważne obawy dotyczące zgodności z przepisami, wprowadzania konsumentów w błąd oraz zagrożeń dla bezpieczeństwa żywności. Praca wskazuje na pilną potrzebę skutecznego, rutynowego monitorowania autentyczności mięsa z dziczyzny za pomocą narzędzi opartych na DNA. Opracowane testy qPCR stanowią praktyczne rozwiązanie dla interesariuszy branży i organów regulacyjnych, umożliwiając skuteczniejsze egzekwowanie przepisów dotyczących etykietowania, monitorowanie halal, ochronę praw konsumentów i ograniczanie strat ekonomicznych spowodowanych oszustwami.

Słowa kluczowe: uwierzytelnianie żywności, produkty mięsne, kwantyfikacja gatunków łownych, markery jądrowe, qPCR

1 Introduction

The term ‘authenticity’ originates from the word "authentic," with a rich etymology tracing back through various ancient languages. These include Middle English ("authentik"), Old French ("authentique"), Latin ("authenticus"), and ultimately, Ancient Greek (αὐθεντικός, αὐθέντης). Throughout this linguistic journey, the core meanings of genuineness, authority, and originality have consistently been preserved. Within the food industry, authenticity is a multifaceted concept. It signifies food in its purest, most unaltered state – free from intentional or unintentional tampering. Essentially, food authenticity represents the fundamental truth of a food product within the consumer-producer or buyer-seller relationship. Food authenticity has been attributed to product originality in terms of location, origin, production process, product quality, packaging, branding and certifications (Chousou & Mattas, 2019). It acts as a shield, protecting consumers from food fraud (Figure 1) and serving as a cornerstone for maintaining consumer trust (P1).

The global food industry faces increasing challenges in ensuring the authenticity of high-quality products, particularly within the niche market for game meat. The 2013 European horsemeat scandal severely eroded consumer trust in the meat industry, underscoring the critical importance of food authenticity. As consumer demand for exotic and sustainably sourced proteins grows, so does the risk of fraudulent practices such as species substitution, mislabelling, and illegal trade in protected wildlife. This vulnerability is particularly pronounced in the game meat market, which is prized for its unique flavour, cultural significance, and perceived health benefits. Driven by a growing emphasis on sustainable agriculture, healthy eating, and food tourism, game meat has witnessed a surge in consumer interest in recent times (Hoffman & Wiklund, 2006). Characterized by lower levels of fatty acids and cholesterol, game meat is also highly regarded for its distinctive flavour.

In Europe, popular game species include wild boar, roe deer, red deer, hare, mouflon, Alpine chamois, Alpine ibex, and wild ducks, mostly sourced primarily from wild or free-ranging populations (Needham et al., 2023). This aligns with a strong hunting tradition across the continent, with over 20 European countries actively practicing hunting as a popular sport. France, Spain, Italy, Poland, and Germany boast the highest participation rates. Currently, an estimated 7 million registered hunters across the EU (European Union) contribute to the game industry sector (FACE, 2025). This active hunting community plays

a significant role in the European game meat industry, which boasts an estimated annual market value exceeding €1.1 billion (Thomas et al., 2020).

Authenticity challenges in the game meat market are primarily driven by economic incentives. Game meat commands a higher price compared to common meat animals. For instance, in Poland, the price of deer loin (~34 EUR) is nearly five times higher than that of pork loin (~7 EUR). This significant price disparity creates a strong incentive for game meat adulteration, often involving the substitution of high-value game meat with cheaper alternatives (Fajardo et al., 2010), mislabelling, or even illegal sourcing (Quinto et al., 2016). The lack of transparency and traceability within the meat supply chain further exacerbates these authenticity challenges (Szemethy et al., 2021). While fresh or raw meat can be authenticated using morphological or traditional species identification techniques, determining the biological origin becomes increasingly difficult when meat undergoes technological, thermal, or chemical processing (Danezis et al., 2016).



Figure 1. Categories of food fraud (Source: European Commission, 2021; P1).

1.1 Justification of the study

Growing consumer awareness and increased media scrutiny have intensified the focus on the integrity of game meat supply chains. In regions such as the European Union and North America, regulators are increasingly prioritizing the combatting of food fraud (Europol, 2022; Hanner & Kelly, n.d.). However, effective enforcement remains challenging due to technological and logistical hurdles.

Studies on meat product authentication worldwide constantly reveal discrepancies between declared and actual product content. A Canadian study analysing 100 raw meat sausages from grocery stores detected undeclared species mixtures, including the presence of horse meat in one sample (Naaum et al., 2018). Similarly, a Chinese study uncovered the presence of fur-bearing animals such as raccoon, mink, and fox in processed meat products like sausages and kebabs (Yu et al., 2021). The close genetic relatedness between some game species and common meat animals facilitates fraudulent species substitution. For example, a study verifying yak meat authenticity discovered that one beef product was falsely labelled as yak (Zhao et al., 2019). Similar substitution cases have been observed in Brazil, where buffalo meat was mislabelled as beef (Dantas et al., 2019), and in Switzerland, where undeclared roe deer meat was detected in one product (Köppel et al., 2020). These discrepancies are not limited to mammals. In the game bird industry, a study in China found that chicken was mislabelled as quail or pigeon (He et al., 2018). In Africa, where many meat products are often sold unpackaged and unregulated, similar irregularities are prevalent (P1). In Tanzania, molecular analysis revealed that 46 out of 151 game meat samples were misrepresented (Schilling et al., 2020). Another study in Uganda found that 25 out of 229 samples were actually from domestic species (Dell et al., 2021).

These discrepancies have significant repercussions for both consumers and the game meat industry. Consumers adhering to specific dietary restrictions, such as Kosher or Halal diets, may unknowingly consume forbidden products, violating their religious or cultural beliefs. Furthermore, mislabelling leads to financial losses, as consumers pay premium prices for counterfeit products, ultimately eroding trust in the market. The game meat industry also suffers. Food fraud enforcement measures have led to major seizures. For example, during Operation OPSON XI, a Europol-led initiative targeting food fraud between December 2021 and May 2022, authorities seized 26,800 tonnes of illegal products and dismantled several criminal networks (Europol, 2022). Fraudulent practices create unfair competition, undermine legitimate producers, and reduce overall productivity and profit

margins. Exposed cases of fraud can lead to product recalls, resulting in significant financial losses and potential legal consequences. Moreover, a country's reputation in the global meat trade can be damaged, leading to export bans and a decline in national revenue (P1).

1.2 DNA-based methods for game meat authentication

Since all living organisms possess a unique set of genetic blueprints, known as deoxyribonucleic acid (DNA), this has facilitated the development of DNA-based authentication approaches for meat verification. By targeting, extracting, and identifying specific molecular markers, species in food products can be reliably identified. DNA-based methods offer a highly accurate and sensitive approach, capable of detecting species even in extensively processed or degraded meat products. The most widely used DNA-based technique for species detection is the polymerase chain reaction (PCR), which amplifies specific DNA sequences in vitro, ensuring that even a small sample portion is sufficient for analysis. Molecular methods for game meat detection can be classified as PCR-based or non-PCR-based. Among the latter including techniques such as DNA barcoding, next-generation sequencing (NGS), and nanopore sequencing (P2).

Current methods for game meat authentication primarily rely on molecular markers, such as DNA or peptides. Protein-based assays offer several advantages, including minimal sample preparation, the ability to detect heat-stable peptide markers, and the potential for simultaneous identification of multiple proteins and peptides (Montowska et al., 2015; Montowska & Fornal, 2017). However, these methods can be limited by close relationships between species, higher detection limits, and also susceptibility to extreme processing conditions (Montowska et al., 2024). DNA-based methods offer high specificity due to the unique nature of DNA sequences within individual species. These methods can target highly conserved or species-specific regions of the genome, enabling precise species identification also in complex mixtures or highly processed samples. DNA-based techniques are renowned for their high sensitivity, allowing for the detection of trace amounts of DNA from even minor components in a mixture.

The polymerase chain reaction (PCR) remains a cornerstone of DNA-based authentication for game meat products (P2). Numerous PCR-based methods have been developed, including conventional PCR, multiplex PCR, digital droplet PCR (ddPCR), PCR-sequencing, PCR-restriction fragment length polymorphism (PCR-RFLP), PCR-single nucleotide polymorphism (PCR-SNP), immunoassay-based PCR, and real-time PCR

(qPCR). These techniques have proven effective in verifying various game species (Ahamad et al., 2017; Fajardo et al., 2006, 2008; Galal-Khallaf et al., 2021; Thanakiatkrai et al., 2019; Yu et al., 2021; Zhao et al., 2021).

Among these, real-time PCR (qPCR) is particularly well-suited for game meat authentication due to several key advantages. qPCR utilizes fluorescent dye reporters that emit fluorescence upon hybridization, enabling both detection and quantification of DNA (Merck, 2025). This dual capability allows for species identification and precise quantification within food products. Highly sensitive, qPCR can detect species at concentrations as low as 0.003 ng of DNA (Ahamad et al., 2019). Its power and accuracy are evident in its ability to discriminate between closely related species. For example, a sika deer-specific qPCR assay developed by Kaltenbrunner et al. (2018c) demonstrated minimal cross-reactivity with other deer species, ensuring high specificity. Furthermore, qPCR is robust and reproducible, making it applicable to processed and thermally treated products where traditional species identification methods may be ineffective (P2).

Across several European countries, including Czech Republic (Nesvadbová et al., 2019), Austria (Druml et al., 2016; Kaltenbrunner et al., 2018a, 2018d, 2018c, 2019, 2020), Portugal (Amaral et al., 2014), and Germany (Dolch et al., 2020), studies have assessed label compliance in game meat products to ensure accurate labeling for consumers. In Poland, the Statistical Yearbook of Forestry reports hundreds of thousands of wild boar, roe deer, and red deer, hunted in 2023, fueling a substantial game meat market (Statistics Poland, 2023). However, existing Polish studies on game meat authentication have primarily employed qualitative PCR methods (Koseniuk et al., 2021; Rak et al., 2014; Spychaj et al., 2021), highlighting the need for more advanced, quantitative approaches for precise species verification. Furthermore, the low price and high consumption of chicken and pork in Poland make them potential adulterants in game meat products. Past inspections, e.g. one by the Inspectorate for Commercial Quality of Agricultural and Food Products (IJHARS) have revealed irregularities in kebab meat, demonstrating vulnerabilities within the food supply chain (Szyłak et al., 2023). A previous report by the Supreme Audit Office (NIK) had noted deficiencies in Poland's food safety control system, including insufficient continuous inspections of manufacturers, inadequate penalties for non-compliant producers, and weak monitoring of online food sales (Whitworth, 2022). Addressing these challenges, this study aims to develop a robust method for the reliable detection and quantification of red deer, roe deer and wild boar meat in highly processed products. By improving authentication

techniques, this research attempted to enhance consumer protection and support regulatory authorities in strengthening the food safety framework.

2 Research hypothesis and objectives

The following research hypothesis was formulated: “Red deer, roe deer and wild boar contain specific DNA sequences that can be considered as nuclear markers for testing authenticity in food products”. In order to verify this hypothesis, the primary objective of the work and its specific objectives were established.

The primary objective of this doctoral thesis was to develop and validate real-time, nuclear-marker-based qPCR methods for the detection and quantification of specific DNA sequences from red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), and wild boar (*Sus scrofa scrofa*) in food products.

In addition, the following specific objectives were established in the work:

1. Identification of unique, small-sized DNA sequences (50–150 bp) that are highly conserved within species but variable between species to serve as target markers.
2. Method optimization to ensure repeatability and adaptability for use in both laboratory and field settings.
3. Assessment of specificity by performing cross-reactivity tests using DNA from closely related species and common contaminants.
4. Assessment of sensitivity by conducting serial DNA dilutions to determine the lowest detectable concentration.
5. Method validation based on differentially processed blind meat mixtures.
6. Evaluation of the application of the validated method to market meat products.

The scope of the work included: a review of literature data from the area of food analysis regarding the latest molecular methods as well as the design of primers and probes which were then tested experimentally. Comparative qualitative PCR analyses, primers' performance evaluation and real-time PCR analyses were performed for quantification of the selected species in market samples to identify instances of mislabelling, adulteration, or substitution of game meat species in commercial products and address conservation and public health concerns while providing a framework for global application of the method.

3 Materials and methods

3.1 Samples

Study samples were collected from multiple sources, including hunting circuit No. 57, Sarbia Forest District, Greater Poland, specialty game meat shops, local shops, and supermarkets in Poznań, Poland, local shops in Portugal, and online retailers in France and Spain. This study included a diverse range of samples, categorized as follows:

- a) target species samples: fresh meat samples were collected from red deer (*Cervus elaphus*) (P3), roe deer (*Capreolus capreolus*) (P4), and wild boar (*Sus scrofa scrofa*) (P5);
- b) non-target species samples: to assess the specificity of the developed method, fresh meat samples were obtained from various non-target species of mammals and birds, selected based on their common presence in the food supply chain and potential for cross-reactivity with the target species;
- c) plant-based ingredients: A variety of plant-derived ingredients (cereals, seeds, herbs and spices) commonly found in processed meat products were included to evaluate potential matrix effects.
- d) commercial game meat products: fifty-seven processed meat products (loin, canned meat, ham, terrine, pâté, salami, and sausages, etc.) labelled as containing the target species were obtained from commercial sources in Poland, Portugal, Spain and France;
- e) laboratory-produced model products: to simulate adulteration scenarios, model products were prepared by mixing each target species (roe deer, red deer, wild boar) with one non-target species (pork or beef) at varying proportions. These mixtures were designed to mimic real-world adulteration cases and assess the method's performance in detecting species substitution.

3.2 Preparation of model mixtures

Laboratory model mixtures, including validation samples and reference mixtures, were prepared to simulate game meat pâtés for each target species: roe deer, red deer, and wild boar. For each species, a blank pâté matrix was first prepared using non-target meats and common condiments. Roe deer and red deer matrices were prepared using equal proportions of pork loin, pork neck, and pork liver, whereas wild boar matrices utilized beef loin instead of pork. Each blank matrix was seasoned with salt, onion, garlic, pepper, coriander, ginger, and rosemary. A reference mixture containing 50% (w/w) of the target species was then

prepared by adding an equal weight of minced game meat (300 g) to the respective blank matrix (300 g). Further serial dilutions were performed by incorporating the blank matrix to obtain final target species concentrations of 10%, 5%, 1%, 0.5%, 0.1%, 0.05%, and 0.01% (w/w).

To simulate industrial processing conditions, a portion of each mixture was subjected to thermal treatment. For red deer and roe deer samples, autoclaving was performed at 115 °C for 30 minutes, while wild boar samples were autoclaved at 120 °C for 20 minutes. Additionally, a separate set of wild boar samples was oven-cooked in a Rational Combi convection oven (model SCC 61, Landsberg am Lech, Germany) at 160 °C until a core temperature of 75 °C was achieved.

Blind samples were independently prepared to assess the accuracy and robustness of the method. At least two sets of validation mixtures were produced for each target species, containing 0.3% to 30% (w/w) of the respective game meat in its corresponding blank matrix.

3.3 Nuclear markers and primers and probes design

At the outset of this study, several markers and primers were designed, including troponin gene variant (TNNI6), lactoferrin (LTF), and endoglin (ENG) for red deer; haemoglobin subunit beta (HBB) for roe deer; and intergenic chromosome 9 (chr9wb) and nuclear receptor subfamily 6 group a member 1 (NR6A1) for wild boar, with the latter two adopted from (Kaltenbrunner et al., 2020). However, these primers were subsequently discarded following validation experiments due to issues such as lack of specificity, primer dimer formation, excessive artifacts in non-target samples, amplification in non-template controls (NTC), and low sensitivity.

The final species-specific nuclear markers selected for this study were TNNI2 for red deer (P3), ASIP for roe deer (P4), and PLAG1 for wild boar (P5). These markers underwent rigorous validation across multiple stages of method development. Primer specificity was initially assessed *in silico* using the NCBI Primer-BLAST tool, confirming 100% target specificity for TNNI2 and ASIP. However, PLAG1 showed 98% identity with pig. Given that wild boar and domestic pig are subspecies within the same species group, PLAG1 was retained to allow detection of both species. In addition to species-specific markers, universal markers were incorporated as internal controls. These universal markers, present in mammals (MSTN – myostatin) or eukaryotes (18S rRNA), were used to verify the presence

of amplifiable DNA (P3 and P5) and for the ΔC_q normalization approach (P4). Bioinformatics tools such as OligoCalc (<http://biotools.nubic.northwestern.edu/OligoCalc.html>) and PrimerStat (https://www.bioinformatics.org/sms2/pcr_primer_stats.html) were used to assess primer properties, including hairpin formation, primer dimerization, and GC content. Probes were designed using the Integrated DNA Technologies tool, PrimerQuest (<https://www.idtdna.com/PrimerQuest/Home/>). A compilation of all study primers and probes is presented in Table 1.

Table 1. Primers and probes used in this study.

Target species	Target gene	Primer	Sequence (5'–3')	Amplicon	NCBI accession or reference
<i>Cervus elaphus</i>	Troponin I	TNNI2-F	AGCAGAACTCTTCCGGGTTG	76 bp	OU343091.1
		TNNI2-R	TGTCACGGCTTATGGTCCAC		
		TNNI2-P	FAM-AGAGTCTGTCTGTGCTGGTGAAATG G-BHQ1		
<i>Capreolus capreolus</i>	Agouti signaling protein	ASIP-F	GAGTCCATTTTCCAGGGCCG	120 bp	NC_057828.1
		ASIP-R	GGGACTTAGGCCTGCAGTTT		
		ASIP-P	FAM-ACTCTCTGCTCTCTGTCTCTGGCT- BHQ1		
<i>Phacochoerus africanus</i>	PLAG1 zinc finger	PLAG1-F	ACGCCATCTCTATTCCCGAG	127 bp	XM_047783901.1 (LOC125129313)
		PLAG1-R	AGCCCCGAGCTTAGATGACGA		
		PLAG1-P	5'-FAM-TCGAGAGCTATCTGATGGAGCTGCA- BHQ1-3'		
Mammals	Myostatin	MSTN-F	TTGTGCAAATCCTGAGACTCAT	70bp	Druml et al., 2016
		MSTN-R	TTCAGAGATCGGATTCCAGTATA		
		MSTN -P	5'FAM-CCCATGAAAGACGGTACAAG- BHQ1-3'		
Eukaryotes	18S rRNA	18SRG-F	CTGCCCTATCAACTTTCGATGGTA	113 bp	Costa et al., 2013
		18SRG-R	TTGGATGTGGTAGCCGTTTCTCA		
		18SRG-P	FAM-ACGGGTAACGGGGAATCAGGGTT CGATT-BHQ1		

3.4 DNA extraction

The commercial DNA isolation kits, NucleoSpin Food and NucleoSpin Plant II, manufactured by Macherey-Nagel (Düren, Germany), were used for DNA extraction from animal and plant tissues, respectively. For extraction, 200 mg of animal tissue and 100 mg of plant material were weighed and processed according to the manufacturers' protocols, with a slight modification—the addition of 2 μ L of RNase (2 mg/mL) after the lysis step. At least, duplicate extractions were performed for each sample to ensure reproducibility.

3.5 Conventional PCR

Conventional PCR was utilized to determine optimal primer annealing temperatures via gradient PCR and to assess primer specificity and sensitivity. PCR cycling parameters varied depending on the marker. For ASIP and TNNI2, amplification consisted of an initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 65 °C (ASIP) or 59 °C (TNNI2) for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min. The 18S rRNA marker was amplified under similar conditions but with 33 cycles and an annealing temperature of 65 °C. PLAG1 and MSTN primer annealing temperatures were optimized using gradient PCR across a range of 50 °C to 70 °C. The final cycling parameters for PLAG1 were 30 cycles of 95 °C for 1 min, 60 °C for 50 s, and 72 °C for 30 s, while MSTN was amplified using 35 cycles of 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s.

Amplified fragments were analysed via agarose gel electrophoresis. Visualization was performed using a Gel Doc™ EZ System or Gel Doc™ XR+ (Bio-Rad Laboratories, Hercules, CA, USA), with digital images processed via Image Lab software (version 5.2.1).

3.6 Quantitative real-time PCR (qPCR)

TaqMan probe-based quantitative PCR (qPCR) assays were developed for the specific detection and quantification of target game meat species (red deer, roe deer, and wild boar). Amplifications were carried out using either the Azure Cielo™ 6 Real-Time PCR System (Azure Biosystems, Dublin, CA, USA) or the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA), with No Template Controls (NTCs) included in all experiments. The thermal cycling conditions varied depending on the target gene (see P3-P5 for details). Data analysis was performed using CFX Manager 3.1 (Bio-Rad Laboratories, Hercules, CA, USA), with Cycle of Quantification (Cq) values determined at user-defined threshold settings. Each real-time PCR assay was conducted in three independent runs, with $n = 4$ replicates per run to ensure reproducibility.

3.7 Calibration curve preparation

To quantify the proportion of target species in meat products, calibration curves were generated using DNA extracted from reference samples containing 0.01% to 50% (w/w) of wild boar, roe deer, or red deer meat in beef patties or simulated pâté, prepared both raw and thermally processed. DNA extracts were adjusted to a final concentration of 10 ng/μL for real-time TaqMan probe-based amplification. Each reference sample was analysed in four

technical replicates and three assay replicates to ensure reproducibility. The resulting cycle threshold (Cq) values were averaged for each standard and plotted against the logarithmic (w/w) percentage concentration of the target species.

To accept the generated calibration curves, strong linearity across the tested concentration ranges must be exhibited, with R^2 values exceeding 0.98, PCR efficiencies ranging from 90% to 110%, and slopes between -3.1 and -3.6 . These values must consistently meet the acceptance criteria outlined by Bustin et al. (2009), ENGL (2015) and FAO CAC/GL (2010) across all 12 replicates, encompassing both raw and thermally processed samples.

However, the efficiency of the autoclaved roe deer pâté calibration curves fell slightly below the recommended range (P4). To address this, the quantitative performance of the real-time PCR assays was improved by employing parallel amplification of the endogenous reference gene (18S rRNA) for normalization. This normalization method is based on the principle of relative quantification, which accounts for variations in PCR efficiency and sample input. It involves constructing a calibration curve using the cycle threshold (Cq) values of two carefully selected DNA sequences with comparable amplification efficiencies: the universal eukaryotic reference gene (nuclear 18S rRNA) and the species-specific target gene (ASIP). The normalization is achieved by calculating the delta Cq (ΔCq), which is the difference between the Cq value of the target gene and the Cq value of the reference gene ($\Delta Cq = Cq(\text{target}) - Cq(\text{reference})$).

Finally, target quantities in the blind samples were interpolated using the established calibration curves.

3.8 Statistical analysis

Statistical analyses were conducted using GraphPad Prism version 8.0.2 (GraphPad Software, San Diego, CA, USA) for P3 and P4 and Statistica 13.1 (StatSoft Inc., Tulsa, OK, USA) for P5. The Shapiro–Wilk test was applied to assess the normality of the data. Data were evaluated with the non-parametric Mann–Whitney test (P3 and P4) or Tukey’s multiple comparison test (P5). The significance of differences ($p < 0.05$) was assessed for the cycle threshold (Cq) values of raw and thermally processed reference mixtures at the same concentration level, particularly in the normalized calibration models. The results were expressed as mean $\pm$ standard deviation, with statistically significant differences considered at $p < 0.05$.

4 Results and interpretation

Article P1 showed the vulnerability of Nigerian meat products to fraudulent practices, stemming in part from consumer preferences for cost-effective items and the limited coordination within the meat sector due to the absence of robust standards enforcement in areas such as product authenticity, safety, sales, and packaging. Whereas, various DNA-based methods have been reviewed in P2 that focused exclusively on the detection and quantification of game, uncommon meat animals, and wildlife species. To maintain consumer confidence, many researchers have developed a variety of methods, ranging from complex, high-throughput procedures that require professional input to simple, inexpensive, on-site methods for verifying wild meat products. The DNA-based methods discussed in P2 have demonstrated their effectiveness in combating the problem of some fraudulent meat producers and in detecting cases of unintentional contamination.

The research presented in this study expands the possibilities of game authentication by introducing robust and specific quantification methods for red deer (P3), roe deer (P4) and wild boar meat (P5). These species were selected due to their economic importance in the European game meat market and their potential for fraudulent substitution. The aim was to develop and validate three novel nuclear markers, each specifically designed for roe deer, red deer, and wild boar, to enhance the precision and applicability of game meat authentication, particularly in processed food products.

4.1 DNA extraction and quality assessment

DNA extraction was a critical step for species detection and quantification, with its efficiency being fundamental to method development and study progression. Over the course of the study, more than 350 independent DNA extractions were performed on a diverse range of samples, including raw muscle tissues, plant materials, complex-matrix raw meat, thermally processed meat, and various commercially processed products. The successful extraction of high-quality DNA from these matrices was essential for ensuring reliable PCR and qPCR analyses.

Raw meat samples, laboratory-prepared products with complex matrices, and commercial samples exhibited high DNA yields and purity, confirming the effectiveness of the extraction method for processed food samples. However, thermally processed samples required modifications to the extraction protocol to improve yield. Similarly, plant-based

samples yielded lower DNA concentrations, with reduced A260/A230 ratios, suggesting potential interference from polysaccharides or phenolic compounds.

To optimize extraction efficiency and mitigate potential inhibitors, particularly in thermally processed and plant-based samples, additional processing steps were implemented, i.e. the surface area of thermally processed samples was increased by homogenizing them into a fine paste along with an extended incubation period of 24 hours. Despite these efforts, DNA degradation was more pronounced in highly processed samples, particularly those subjected to autoclaving, leading to fragmented DNA and lower extraction yields.

High-purity DNA from raw and minimally processed samples contributed to robust amplification, whereas lower-quality DNA from extensively processed samples sometimes resulted in reduced amplification efficiency. However, while reduced amplification efficiency could pose challenges, it did not limit the applicability of the qPCR assay. This limitation was effectively addressed using the ΔC_q normalization calibration method, as described in P4, ensuring the reliability of the assay even for highly processed samples.

4.2 Species-specific nuclear marker selection

While mitochondrial markers, such as cytochrome b (Cyt b) and cytochrome c oxidase subunit I (COI), have been widely employed due to their high copy number per cell and maternal inheritance (Aina et al., 2020; Ngadi, 2019; Szychaj et al., 2016; Thomas et al., 2021), their utility for quantitative detection is limited due to cell content variability (Iwobi et al., 2015). In contrast, nuclear markers, with their fixed copy number per cell, offer greater reliability for accurate species quantification in food authentication studies (P2).

In this study, the selected nuclear markers included PLAG1, a protein-coding gene associated with the regulation of several growth factors in wild boar and pigs; TNNT2, a gene coding troponin I specifically expressed in skeletal muscle, serving as a marker for red deer; and ASIP, which plays a role in agouti coat colour variation, making it a suitable marker for roe deer. All selected markers were novel and had not been previously used for species identification or authentication. Their validation was a central focus of this study. In addition to species-specific markers, universal markers were incorporated as internal controls (Table 1). These universal markers, present in mammals (MSTN – myostatin) or eukaryotes (18S rRNA), were used to verify the presence of amplifiable DNA (P3-P5).

To optimize primer performance, gradient PCR was performed to determine the optimal annealing temperature, followed by further PCR condition refinements to enhance primer efficiency. The validated markers were then cross-tested against 16 non-target species, including domestic livestock, other game species, and plant species. No cross-reactivity was observed, except within closely related wild boar and pig. The initial cross-species validation results were visualized via gel electrophoresis, confirming the expected amplicon sizes for red deer TNNI2 76 bp, roe deer ASIP 120 bp, and wild boar/pig PLAG1 127 bp. Further reproducibility tests, including duplicate analyses, independent replication by another operator, and verification using different PCR machines at another institution, confirmed the markers' reliability and suitability.

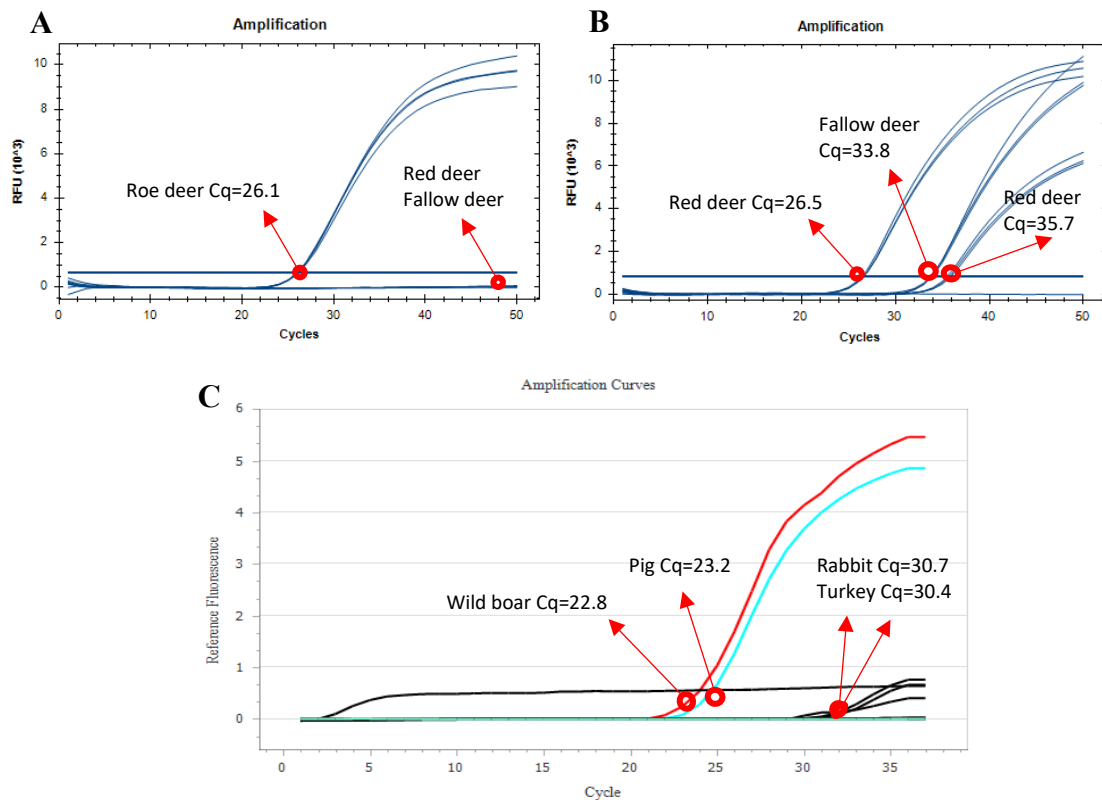


Figure 2. TaqMan probe-based real-time PCR amplification curves for target and non-target species; (A) ASIP probe targeting the ASIP gene using DNA extracts of roe deer, red deer and fallow deer; (B) TNNI2-P TaqMan probe targeting the troponin I gene using DNA extracts of red deer, fallow deer and roe deer; (C) PLAG1 probe amplifying the PLAG1 gene in both pig and wild boar.

4.3 Specificity, sensitivity and efficiency of qPCR assays

In addition to conventional PCR specificity assays, TaqMan probe-based real-time PCR was employed to further assess assay specificity (Figure 2). As detailed in P3 and P4, this approach reinforced the specificity of the red deer- and roe deer-targeted assays. Specificity testing across three closely related species (roe deer, red deer, and fallow deer) confirmed amplification exclusively for the intended target in roe deer. For red deer, amplification was observed in all three species; however, the cycle threshold (C_q) values for roe and fallow deer were delayed by 7.2 and 9.3 cycles, respectively, indicating minimal cross-reactivity. Similarly, P5 reported specificity analysis for wild boar, where non-target species exhibited only minimal fluorescence signals around cycle 30, approximately 7–8 C_q values beyond the target, suggesting negligible non-specific amplification.

The sensitivity of the TaqMan qPCR assays for wild boar, roe deer, and red deer was evaluated using 10-fold serial DNA dilutions to determine the absolute limit of detection (LOD), limit of quantification (LOQ), and overall assay performance. The PLAG1-targeted wild boar assay, tested with DNA dilutions from 100 ng to 0.01 ng, achieved an LOD and LOQ of 0.1 ng at 95% confidence. The standard curve demonstrated strong linearity ($R^2 = 0.992$), a slope of -3.198, and a PCR efficiency of 105.4%, indicating high amplification fidelity (Table 2). A similar sensitivity was observed for porcine DNA. For roe deer, the ASIP-targeted assay covered four orders of magnitude in dynamic range, with an LOD and LOQ of 0.04 ng, as 0.004 ng was not consistently amplified across 12 replicates. The calibration curve exhibited a PCR efficiency of 95.5%, a slope of -3.424, and a correlation coefficient ($R^2 = 0.998$), all within accepted qPCR performance criteria. The red deer assay, tested across seven DNA concentration levels (50 ng/μL to 0.00005 ng/μL), reached an LOD and LOQ of 0.005 ng/μL (0.01 ng), with successful amplification in all eight replicates across two runs. The dynamic range covered four orders of magnitude, and the assay parameters (slope = -3.424, efficiency = 103.8%, $R^2 = 0.995$) met established real-time PCR standards (Table 2).

Table 2. Performance metrics of species-specific markers.

Article	Species	Marker	Sensitivity (LOD)	LOQ	Amplification efficiency (%)
P3	Red deer	TNNI2	0.01 ng	0.01 ng	103.8
P4	Roe deer	ASIP	0.04 ng	0.04 ng	95.5
P5	Wild boar	PLAG1	0.1 ng	0.1 ng	105.4

4.4 qPCR quantification of game meat in processed foods

DNA extracts from raw and thermally processed reference model mixtures containing red deer, roe deer, and wild boar meat at varying concentrations (0.01% to 50% w/w) were analysed using real-time PCR to establish calibration models and determine the relative limit of detection (LOD) and limit of quantification (LOQ). The calibration curves enabled the quantification of the relative abundance of target species in mixed samples, as described in the methods section.

For red deer (P3), although DNA amplification was successful down to 0.05% (w/w), the final calibration curve was constrained to a dynamic range of 50%–0.5% (w/w) due to cross-reactivity with fallow deer at concentrations below 0.5% (w/w). Consequently, the relative LOQ and LOD for the red deer qPCR assay in both raw and autoclaved samples were set at 0.5% (w/w). Sensitivity parameters for qPCR detection of selected target meat species in raw and processed samples are presented in Table 3.

Table 3. Sensitivity parameters for qPCR detection of game meat species in raw and processed samples.

Species	Processing method	Dynamic range (% w/w)	Relative LOD (% w/w)	Relative LOQ (% w/w)	Calibration method	Notes
Red deer	Raw	50-0.5	0.5	0.5	Standard curve	Cross-reactivity with fallow deer below 0.5%
	Autoclaved	50-0.5	0.5	0.5	Standard curve	The same as raw samples
Roe deer	Raw	50-0.05	0.05	0.05	ΔC_q normalization	Corrected for PCR inhibition using 18S rRNA
	Autoclaved	50-0.05	0.05	0.05	ΔC_q normalization	Initial efficiency = 86.0%
Wild boar	Raw	50-0.1	0.1	0.1	Standard curve	Binary mixtures with beef
	Oven-cooked	50-0.05	0.5	0.5	Standard curve	Intermediate heat treatment
	Autoclaved	50-0.5	5	5	Standard curve	Significant sensitivity reduction

For roe deer (P4), the ΔC_q normalization calibration technique was employed due to the initially low efficiency parameters observed for the autoclaved calibrator (slope = -3.712 , efficiency = 86.0%). To address PCR inhibition encountered during preliminary analysis, parallel amplification of the ASIP gene and 18S rRNA primers was conducted across all reference products. This approach allowed for the correction of C_q values, ensuring accurate quantification of roe deer DNA. The qPCR assay exhibited a relative LOD and LOQ of 0.05% (w/w) for both raw and thermally processed samples. The calibration

curve parameters, including PCR efficiency, slope, and R^2 values, demonstrated the reliability of the assay in detecting and quantifying roe deer DNA even in complex food matrices.

For wild boar (P5), binary reference mixtures with beef were analysed in three states: raw, oven-cooked, and autoclaved. The LOD was determined as 0.1% for raw samples, 0.5% for oven-cooked samples, and 5% for autoclaved samples, indicating a significant impact of autoclaving on DNA detectability. The LOQ followed the same trend, with values of 0.1%, 0.5%, and 5% (w/w) for the respective treatments. Despite the lower sensitivity observed for autoclaved samples, the assay remains applicable for detecting adulterants in game products, as commercial game meat products are primarily preserved by pasteurization.

4.5 Validation of the qPCR assays

The validation process was designed to assess the performance of the developed qPCR assays using samples with known compositions. These reference samples were meticulously prepared in the laboratory to simulate real-world food matrices, incorporating a complex mixture of multiple species in both raw and thermally processed states. The primary objective was to verify the effectiveness of the developed method in detecting and quantifying target species across a defined concentration range.

To achieve this, validation experiments were conducted using samples containing the target species at moderate (30%) to low (0.3%) concentrations. The assays were evaluated for their accuracy, reproducibility, and robustness under different processing conditions to ensure their reliability for routine species identification and quantification. The validation step enabled a comprehensive assessment of the method's precision, sensitivity, and overall performance. Validation parameters were considered acceptable when precision estimates were $\leq 25\%$ and bias (i.e., closeness to the true value) was within $\pm 25\%$. These criteria confirmed the suitability of the qPCR system for detecting and quantifying target DNA in both raw and heat-treated food products.

Across all studies, the method demonstrated acceptable precision, with CV values ranging from 4.6% to 23.0%, well within the $\leq 25\%$ acceptance criterion. Trueness was assessed by comparing actual and estimated species concentrations, with bias values generally falling within $\pm 25\%$, except for the lowest concentration levels in raw and heat-treated samples, where enzymatic activity and thermal degradation contributed to higher variability.

For wild boar validation in beef patties, raw and oven-cooked samples showed relatively accurate quantification, with CV values between 7.52% and 17.61% and bias ranging from -25% to 19%. However, at the lowest concentration (0.3%) in autoclaved samples, the estimated values fell below the detection and quantification limits (>5% w/w LOD/LOQ), highlighting DNA degradation due to intense thermal processing.

Despite minor deviations at low concentrations and in heavily processed samples, the validation results confirmed the robustness and applicability of the qPCR assays for species identification and quantification in complex food matrices. The method meets international guidelines (CAC-GL) for precision and accuracy, ensuring its suitability for routine food authenticity testing.

4.6 Detection of adulteration and mislabelling in commercial samples

Following the successful validation of species-specific qPCR methods, which confirmed the applicability of the developed assays, commercial game meat products were analysed to assess labelling accuracy and detect potential adulteration. A total of 58 products were tested across three studies focusing on red deer, roe deer, and wild boar/pork content. The samples originated from Poland, Spain, Portugal, and France, encompassing a diverse range of processed products, including sausages, pâtés, loins, terrines, and canned meats.

In the red deer study (P3), 46 products were analysed. Based on product labels, 28 were declared to contain red deer meat, 14 roe deer meat, and 2 fallow deer meat. This selection aimed to verify compliance in products both declaring and not declaring the target species, ensuring the detection of potential species substitutions. Among the 28 red deer-labelled products, 13 exhibited either a substantial reduction or complete absence of red deer meat. Some products contained red deer at levels more than ten times lower than declared. The lowest quantified red deer content was $0.49 \pm 0.05\%$ (w/w) in a *red deer salami*, which was supposed to contain 10% red deer meat. The highest quantified red deer content was $44.55 \pm 3.64\%$ (w/w) in a *roe deer sausage*, which was supposed to contain >50% roe deer meat and no red deer. Additionally, undeclared red deer was detected in seven out of 14 roe deer-labelled products and in both fallow deer-labelled samples. Geographic trends revealed that Poland had the highest number of mislabelled or adulterated products (15), likely due to the larger proportion of samples originating from Poland, followed by Spain (4) and Portugal (3). A summary of mislabelling and adulteration findings in commercial game meat products is presented in Table 4.

The roe deer study (P4) analysed 46 products of similar compositions and sources. Of the 14 samples labelled as containing roe deer, only nine tested positive for the species. However, all positive samples exhibited significantly lower roe deer content than declared, with some containing as little as 0.07%. The maximum roe deer content quantified in the study was 19.23%, found in a sample that was supposed to contain 70% roe deer meat. Six samples showed total substitution, while eight were significantly reduced in roe deer content. Roe deer DNA was also detected in eight red deer-labelled samples and one fallow deer-labelled product, indicating undeclared addition. The combined analysis of roe and red deer highlighted widespread mislabelling, with 61% of the analysed products being adulterated.

Table 4. Summary of mislabelling and adulteration findings in commercial game meat products.

Study parameter	Red deer (P3)	Roe deer (P4)	Wild boar/pork (P5)
Total samples analysed	46	46	38
Product types	Sausages, pâtés, loins, terrines, canned meats	Similar composition to red deer study	Raw-cured sausages, smoked, baked pâtés, pasteurized
Label compliance or content discrepancies			
Declared positive samples	15/28 (53.6%) red deer-labelled	9/14 (64.3%) roe deer-labelled	32/32 wild boar/pork products
Undeclared presence	7/14 roe deer-labelled, 2/2 fallow deer	8 red deer-labelled, 1 fallow deer	4/4 deer-labelled products
Worst under-declaration	0.49% vs declared 10%	0.07% vs declared 100%	1.91% vs declared >38%
Maximum detected	44.55% in roe deer sausage	19.23% vs declared 70%	TSG kabanos compliant (91.8%)
Key findings	13/28 red deer products adulterated	14/14 roe deer products non-compliant	Wild boar in deer products
Substitution patterns	Pork, fallow deer	Other deer species	Non-meat fillers, alternative substitutes

The wild boar/pork study (P5) analysed 38 products, including 32 declared to contain wild boar or pork and 4 containing deer species. This analysis revealed varying levels of species content accuracy. Some samples showed good agreement between estimated and declared content, particularly in smoked and cured products. However, raw-cured, baked, and pasteurized products often contained lower-than-declared pork content, likely due to the addition of non-meat fillers or alternative substitutes. The four products labelled as containing only deer species tested positive for wild boar/pork, with levels suggesting intentional adulteration in some cases. One Traditional Specialty Guaranteed (TSG) kabanos product met regulatory composition standards, confirming compliance with EU labelling regulations.

Across all studies, the analysis revealed significant levels of mislabelling and adulteration, affecting a majority of the tested products. Red and roe deer were commonly substituted or reduced, while undeclared wild boar/pork was detected in some samples. Discrepancies were observed across all examined countries. All three studies collectively highlight systemic issues in the game meat industry, where intentional mislabelling and substitution are prevalent. The findings emphasize the need for stricter regulatory enforcement, halal monitoring, improved traceability, and routine quantitative molecular testing to ensure product authenticity and protect consumer rights.

5 General discussion

This study aimed to develop, validate, and apply a sensitive and specific real-time qPCR assay capable of detecting and quantifying DNA from red deer, roe deer, and wild boar in processed meat products. The motivation for this work stemmed from growing concerns regarding species misrepresentation within the European game meat market, where economically motivated adulteration increasingly undermines consumer trust and food integrity. A systematic literature review on the molecular authentication of game meat and less common meat species, covering the period from 2016 to 2022 and published as Article P2, highlighted that numerous publications referenced the 2013 European horsemeat scandal as a key driver for authenticity research. Notably, one study within this period directly compared meat products obtained before and after the scandal, detecting horsemeat content of 0.10% in a sample of beef sausage (Meira et al., 2017). As further emphasized in Article P2, most existing meat authentication studies have primarily focused on domestic livestock species. This underscores the need to expand research efforts toward the authentication of game species, both within Europe and globally, as also advocated in Article P1.

The key findings of this thesis unequivocally demonstrate a disturbingly high prevalence of species misrepresentation in commercially available game meat products in the European market. Specifically, mislabeling was identified in up to 54% of red deer products and 64% of roe deer products, with the additional detection of undeclared porcine content in several samples. These results are strikingly consistent with previous investigations into meat authenticity. For instance, Druml et al., (2015) reported similar discrepancies, revealing about a 40% shortfall between the declared and actual deer content in a game meat product. More recent evidence from a Bulgarian study (Stefanova et al., 2024), further corroborates this trend, indicating that 45% of analyzed commercial meat products were non-compliant with their labeling regarding beef and pork content. Similarly, a Greek study (Minoudi et al., 2025) found undeclared swine, bovine, or chicken DNA in a staggering 90% of surveyed meat products. These converging lines of evidence, including the significant levels of mislabeling observed in this study, strongly suggest a persistent issue of meat adulteration within the European food supply chain, extending beyond domestic livestock to the game meat sector. While the levels of adulteration detected were in line with findings from other European surveys, this study suggests that misrepresentation may stem from species misidentification due to close evolutionary relationships within the *Cervidae*

family, price differences among species, and seasonal availability. Across all three studies, red deer and roe deer were often under-represented or nearly absent, while wild boar or domestic pork were commonly detected instead, suggesting potential substitution with pork or pork-derived offal, even in products labeled as wild boar, likely due to the relative abundance and lower cost of these raw materials.

In the current study, the quantification of adulteration levels as low as <2% in technologically processed, complex commercial food matrices was made possible through the development of novel species-specific TaqMan probe-based assays. These assays, targeting nuclear DNA markers (TNNI2, ASIP, and PLAG1), were validated under real food matrix conditions. This quantitative approach was designed using laboratory-prepared model products as assay calibrators and validators and it represents a significant advancement over previous methods based on DNA mixtures or meat extracts (Druml et al., 2016; Druml et al., 2015; Kaltenbrunner et al., 2018d). The method demonstrated excellent analytical performance, achieving high specificity, with no significant cross-reactivity observed among non-target species, even within complex matrices. The nuclear markers were specifically selected for their single-copy nature, which supports more consistent quantification across species, tissues, and cells compared to multi-copy mitochondrial markers. While mitochondrial markers often provide greater sensitivity due to their higher copy number per cell, nuclear markers offer superior specificity and discriminatory power between closely related game species (Iwobi et al., 2015; Wang et al., 2021; Yu et al., 2021). The advantages of using nuclear DNA, alongside the limitations of mitochondrial targets, were further discussed in Article P2.

This study also demonstrated high sensitivity in detecting red deer, roe deer, and wild boar DNA in complex meat matrices, including those subjected to thermal processing. For red deer (Article P3), the assays achieved a sensitivity level of 0.01% w/w in both raw and cooked pâté mixtures, surpassing earlier methods such as those by Amaral et al. (2014), who reported a 0.1% w/w detection limit using 12S rRNA mtDNA, and Rak et al. (2014), who observed reduced sensitivity (0.5% w/w) in thermally processed samples targeting the mitochondrial D-loop region. Although these studies benefited from the high copy number of mitochondrial DNA, their sensitivity diminished significantly under realistic processing conditions. For roe deer (Article P4), the current assays also outperformed previously reported detection limits, which ranged from 0.1% to 0.64% w/w (Kaltenbrunner et al., 2018d; Köppel et al., 2020), and were often based on diluted DNA rather than food matrices. Although Druml et al. (2015) reported a lower LOD (limit of detection) of 0.03% w/w, it

was achieved using DNA extracts and not actual processed food. Similarly, for wild boar (Article P5), detection was more reliable than in previous assays targeting mitochondrial *cyt-b* or nuclear SNPs, which exhibited variable sensitivity and occasional cross-reactivity (Aina et al., 2020; Kaltenbrunner et al., 2019). Some studies, such as Amaral et al. (2017), reported LODs as low as 0.0001% w/w in raw mixtures, but these values decreased substantially after thermal processing.

The relative quantification limits (LOQ), defined as the lowest sample concentration that can be reliably quantified, ranged from 0.05% to 5% (w/w) in this study. These values were either lower than or comparable to those previously reported, for example, earlier LOQs for roe deer ranged from 0.125% to 0.5% (w/w) (Druml et al., 2016; Druml et al., 2015; Kaltenbrunner et al., 2018d). In comparison with other deer species, previous studies reported LOQs of 0.4% and 0.1% (w/w) for fallow, sika, and red deer (Kaltenbrunner et al., 2018a, 2018b; Liu et al., 2023). Although the current LOQ of 0.5% (w/w) for red deer is slightly higher, the difference is not substantial, and the lower LOQ of 0.1% w/w for sika and red deer in the other study was obtained using mtDNA, which generally enables higher sensitivity. Other studies using droplet digital PCR (ddPCR) to quantify pork in mixtures with horse, cattle, chicken, or beef reported LOQs of 0.01% (Floren et al., 2015), 0.1% (Xu et al., 2022), and 1% (w/w) (Temisak et al., 2021), values that are comparable to those achieved in the present study for the *Suide* assay. The notably low LOQ reported by Floren et al. (2015) was made possible through the use of DNA mixtures.

The precision of the assays was assessed through intra- and inter-assay replicates, with most yielding coefficient of variation (CV) values below 25% across 12 independent runs. This demonstrates strong reproducibility and robustness, particularly important when quantifying DNA in thermally processed or fat-rich food matrices (Minoudi et al., 2025). The use of separate calibrators for raw and processed meat products further enhanced accuracy by accounting for matrix-related amplification shifts. High linearity was consistently observed in all standard curves ($R^2 > 0.98$), reinforcing the quantitative reliability of the assays across various product types. Precision was further validated through application to 58 commercial game meat products, where all but two samples exhibited CV values below 25%. These findings confirm the suitability of the TaqMan real-time PCR assays for precise species quantification in commercial food matrices.

5.1 Implications for the consumers, food industry and regulations

In Poland, recent studies have shown that consumers' perceptions of game meat are largely shaped by its taste, nutritional value, and cultural familiarity (Czarniecka-Skubina et al., 2022; Niewiadomska et al., 2020). While taste remains the most decisive factor influencing consumption, other key attributes such as origin, fat content, and perceived health benefits significantly increase the likelihood of regular intake. Therefore, consumption is likely to decline when product irregularities compromise species origin claims, thereby undermining perceived health benefits and overall appeal.

The impact of such irregularities on consumers cannot be overstated. Game meat commands a premium price, and when consumers unknowingly pay more for adulterated or mislabelled products, it results in economic losses, potential health risks, and possible ethical or cultural violations (Du et al., 2023; Li et al., 2020). These concerns are compounded by a broader erosion of trust and increase consumers' willingness to independently verify food label information using technology (Charlebois et al., 2016). This is especially relevant for high-value niche products like game meat, where mislabelling not only breaches consumer trust but may also discourage future purchases. Therefore, building transparent, verifiable supply chains and promoting affordable, effective, and consumer-accessible authentication tools are critical steps toward restoring and maintaining consumer confidence.

One of the dire consequences of fraud for the industry is loss of integrity and brand authenticity (Visciano & Schirone, 2021). This present study revealed several product irregularities, including the presence of undeclared species, the absence or low quantity of the primary game meat ingredient, and species substitution. These findings mirror global reports of similar fraudulent practices (Li et al., 2023), and suggest widespread non-compliance with standard operational practices (Premanandh, 2013). Producers who adhere to ethical sourcing and accurate labelling face unfair competition and financial disadvantage compared to unscrupulous operators.

The complexity of meat supply chains further increases the risk of fraudulent product introduction. A recent global review found that 43% of fraud cases in the beef supply chain originated from unlicensed operations (Elliott et al., 2019). For processed products such as sausages and pâtés, where visual inspection is insufficient to detect anomalies, DNA-based methods have become essential. Previous scandals, including the Chinese rat meat fraud (Dai & Jiang, 2013), the European horse meat incident (Smith & McElwee, 2021), and the Brazilian rotten meat scandal (Analytica, 2017), have shown that such breaches can cause

widespread disruption across the meat industry, shaking consumer confidence and triggering regulatory overhauls.

Moreover, as game meat is typically wild-sourced, the inclusion of unverified or unauthorized species may pose serious food safety risks. This includes the potential introduction of zoonotic pathogens, allergens, or chemical residues, especially in the absence of appropriate veterinary inspection.

The findings of this study carry serious implications for both regulatory enforcement and consumer protection. Since a series of high-profile food-related incidents in the 1990s, the European Union has adopted a proactive stance on food safety, implementing comprehensive legislation and establishing regulatory bodies such as the European Food Safety Authority (EFSA) to ensure that “European citizens have access to safe and wholesome food of the highest standard” (European Commission, 2025). However, the high frequency of mislabelling and species misrepresentation uncovered in this study challenges the effectiveness of existing regulatory frameworks.

Specifically, these findings suggest widespread violations of the EU Food Information to Consumers (FIC) Regulation (No. 1169/2011), which mandates that food information must be clear, accurate, and not misleading. A substantial proportion of products examined in this study failed to meet these requirements, showing discrepancies between labelled species and actual content. Notably, the Traditional Specialty Guaranteed (TSG) kabanos (P17), which adheres to the EU’s Geographical Indications and Quality Schemes, was fully compliant. Its accuracy in labelling highlights the disparity in industry practices and raises important questions about broader compliance and regulatory oversight.

5.2 Limitations of the study

That said, it is important to acknowledge several limitations of the current study. While the efficacy of the qPCR methods developed here has been validated through robust performance across different matrices, the technique is not user-friendly for end consumers. It requires specialized knowledge, costly equipment, proprietary reagents, and technical expertise. Some researchers have proposed portable, on-site real-time PCR systems capable of detecting target species within 20 minutes (Furutani et al., 2017), while others have explored the potential of DNA-based lateral flow strips for rapid field monitoring (Denyinghot et al., 2022). However, despite their promises, these methods remain limited by the need for trained personnel and controlled testing conditions.

Additional technical limitations were identified during this study. First, due to the genetic similarity among certain *Cervidae* subspecies, which was particularly evident in P3, the assay's sensitivity and quantification limit were reduced to 0.5% (w/w). While lower limits would have been desirable, this still supports the assay's practical applicability. Another challenge involved differentiating between *Suidae* subspecies within complex food matrices. In P5, interspecies variation was clearly established, but the high genetic similarity between wild boar and domestic pig hindered precise species-level resolution.

5.3 Future Research Directions

Finally, while this study included products sourced from several European countries, a broader geographic scope would provide a more comprehensive picture of food fraud's transnational impact. Future research should also expand the assay panel to include additional game species such as fallow deer, mouflon, and hare, and apply the method in larger-scale market surveillance to further validate its utility in real-world regulatory and industrial contexts.

6 Conclusions

This research has shown that new nuclear markers can be developed as effective tools in authentication of meat and meat products in selected game species using real-time quantitative polymerase chain reaction technique. In this study, several nuclear marker-based qPCR assays for authenticating game meat species were developed and validated. Therefore major conclusions from this study are outlined thus:

1. Three species-specific nuclear DNA markers – troponin I (TNNT2), Agouti signaling protein (ASIP), and PLAG1 zinc finger – were identified. These markers are highly conserved within red deer, roe deer, and wild boar/pig, respectively. With short amplicon lengths ranging from 76 bp to 127 bp, these nuclear markers proved to be effective targets for accurate species identification and quantification in both raw and processed meat products.
2. Cross-reactivity tests confirmed that the developed primers and probes did not amplify DNA from closely related non-target species or common meat contaminants. This specificity assessment ensured reliable identification and eliminates the risk of false positives due to background DNA.
3. The method demonstrated high sensitivity, with limits of detection (LOD) and quantification (LOQ) effectively set at 0.01 ng, 0.04 ng, and 0.1 ng, respectively. These levels are sufficient to detect trace amounts of red deer, roe deer, wild boar, and pig DNA.
4. Serial dilution experiments further confirmed the method's ability to accurately quantify species-specific DNA even at low concentrations, specifically down to 0.5% for red deer, 0.1% for wild boar, and 0.05% for roe deer meat. Such performance characteristics make this method highly suitable for detecting adulteration, even in complex or highly processed meat products.
5. Blind sample tests containing various proportions of game and non-game meats confirmed the method's quantitative accuracy and precision. The assays reliably quantified the target species' DNA content across different processing conditions with precision ranging from 4.6% to 23.0% across all three studies and well within the $\leq 25\%$ acceptance criterion, demonstrating their practical reliability.

6. All qPCR assays showcased acceptable performance metrics that remained consistent across different reaction conditions, making them suitable for both controlled laboratory environments and potential adaptation to field-level diagnostics.
7. The application of the validated method to market-sourced game meat products revealed widespread species misrepresentation. The findings exposed cases of undeclared species, species substitution, and incorrect quantitative labelling, highlighting serious authenticity and compliance failures in the game meat supply chain.

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8 Authorship contribution statements

AUTHORSHIP CONTRIBUTION STATEMENT

Article:

Bukola M. Adenuga, Magdalena Montowska. 2023. The Nigerian meat industry: An overview of products' market, fraud situations, and potential ways out. *Acta Scientiarum Polonorum. Technologia Alimentaria*, 22(3), 305-329. <http://dx.doi.org/10.17306/J.AFS.2023.1157>

Author contributions:

I hereby declare the author's contribution. I consent to the use of the mentioned article in the doctoral dissertation of MSc. Bukola M. Adenuga.

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Magdalena Montowska	Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition.	09.10.2023. 

AUTHORSHIP CONTRIBUTION STATEMENT

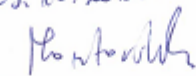
Article:

Bukola M. Adenuga, Magdalena Montowska. 2023. A systematic review of DNA-based methods in authentication of game and less common meat species. *Comprehensive Reviews in Food Science and Food Safety*, 1–49.

<https://doi.org/10.1111/1541-4337.13142>

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Author	Contribution	Date and Signature
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Magdalena Montowska	Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.	09.10.2025 

AUTHORSHIP CONTRIBUTION STATEMENT

Article:

Bukola M. Adenuga, Rita Biltres, Caterina Villa, Joana Costa, Anita Szychaj, Magdalena Montowska, Isabel Mafra. 2025. Unravelling red deer (*Cervus elaphus*) meat adulteration in gourmet foods by quantitative real-time PCR. *Food Control*, 168, 110872.

<https://doi.org/10.1016/j.foodcont.2024.110872>

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AUTHORSHIP CONTRIBUTION STATEMENT

Article:

Bukola M. Adenuga, Rita Biltres, Caterina Villa, Joana Costa, Anita Spychaj, Magdalena Montowska, Isabel Mafra. 2024. A Novel Normalized Quantitative Real-Time PCR Approach for Ensuring Roe Deer (*Capreolus capreolus*) Meat Authenticity in Game Meat Foods. *Foods*, 13, 3728.

<https://doi.org/10.3390/foods13233728>

Author contributions:

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AUTHORSHIP CONTRIBUTION STATEMENT

Article:

Bukola M. Adenuga, Anita Spychaj, Magdalena Montowska. 2025. A new nuclear marker for quantitative analysis of wild boar and domestic pig meat in game meat products using PLAG1 zinc finger gene. *Scientific Reports*, 15, 20454. <https://doi.org/10.1038/s41598-025-05167-x>

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Magdalena Montowska	Conceptualisation, Methodology, Writing - Review & Editing, Funding acquisition, Project administration, Supervision.	09-10-2025 Magdalena Montowska

9 Copies of published papers comprising the series of publications with supplementary materials

- P1. Adenuga, B. M., Montowska, M. (2023). The Nigerian meat industry: An overview of products' market, fraud situations, and potential ways out. *Acta Scientiarum Polonorum Technologia Alimentaria*, 22(3), 305-329. <http://dx.doi.org/10.17306/J.AFS.2023.1157>
- P2. Adenuga, B. M., Montowska, M. (2023). A systematic review of DNA-based methods in authentication of game and less common meat species. *Comprehensive Reviews in Food Science and Food Safety*, 22, 2112-2160. <https://doi.org/10.1111/1541-4337.13142>
- P3. Adenuga, B. M., Biltres, R., Villa, C., Costa, J., Szychaj, A., Montowska, M., Mafra, I. (2025). Unravelling red deer (*Cervus elaphus*) meat adulteration in gourmet foods by quantitative real-time PCR. *Food Control*, 168, 110872. <https://doi.org/10.1016/j.foodcont.2024.110872>
- P4. Adenuga, B. M., Biltres, R., Villa, C., Costa, J., Szychaj, A., Montowska, M., Mafra, I. (2024). A novel normalized quantitative real-time PCR approach for ensuring roe deer (*Capreolus capreolus*) meat authenticity in game meat foods. *Foods*, 13(23), 3728. <https://doi.org/10.3390/foods13233728>
- P5. Adenuga, B. M., Szychaj, A., Montowska, M. (2025). A new nuclear marker for quantitative analysis of wild boar and domestic pig meat in game meat products using PLAG1 zinc finger gene. *Scientific Reports*, 15, 20454. <https://doi.org/10.1038/s41598-025-05167-x>

Publication 1 (P1)

Adenuga, B. M., Montowska, M. (2023). The Nigerian meat industry: An overview of products' market, fraud situations, and potential ways out. *Acta Scientiarum Polonorum Technologia Alimentaria*, 22(3), 305-329. <http://dx.doi.org/10.17306/J.AFS.2023.1157>

THE NIGERIAN MEAT INDUSTRY: AN OVERVIEW OF PRODUCTS' MARKET, FRAUD SITUATIONS, AND POTENTIAL WAYS OUT

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ABSTRACT

Meat is an integral part of the human diet worldwide and plays a central role in the world's culinary traditions. Meat serves as a vital source of essential nutrients, including amino acids, vitamins, and minerals. In Nigeria, it occupies a unique place in the social fabric of the nation and promotes unity and tradition. The meat industry in Nigeria encompasses a range of sources, with beef taking precedence, yet also including options like chicken, chevon, mutton, pork, and more. However, despite its importance, the industry faces multiple challenges, encompassing environmental, socio-economic, and governance issues. The Nigerian government has actively contributed to enhancing meat production through various policies, investments, and modernization efforts. Nevertheless, challenges such as disease outbreaks and inefficient value chains persist. Surprisingly, meat fraud and adulteration have received limited attention despite these issues significantly impacting consumer welfare, public health, and the economy. This article provides an overview of the Nigerian meat industry, explores specific cases of meat fraud, and discusses authentication methods used in neighboring African countries, offering insights for Nigerian researchers and the government.

Keywords: meat authentication, meat industry, meat processing, meat market, food frauds, analytical methods

INTRODUCTION

Meat has been a fundamental part of the human diet for millions of years, occupying a prominent place in many global culinary traditions, with annual consumption and production worldwide reaching up to 350 metric million tons (Hannah et al., 2017). In addition to its cultural importance, meat serves as a vital source of essential amino acids, vitamins, and minerals such as iron, zinc, and selenium (Klurfeld, 2018). These nutrients are essential for growth, development, and overall health. In Nigeria, meat is an integral part of most meals, and its significance extends beyond its role as a source of nutrients: it plays a central role in the social fabric of the country, fostering a sense of unity and tradition among the people. One such tradition is

the custom of eating meat only after finishing a meal – a ritual with which many Nigerians can identify. The various reasons attributed to this custom were tradition/upbringing, rewarding the completion of a meal, budget, or financial limitations (Vanguard, 2017).

In Nigeria, a wide spectrum of meat sources plays a crucial role in influencing the nation's preferences for animal protein, with beef reigning as the predominant choice. Complementing this preference are options such as chicken, chevon (goat meat), mutton, pork, venison, and an array of other animal meats. The prevalence of beef can be attributed to the substantial cattle population in Nigeria, surpassing that of other animal species. Meat production predominantly occurs within

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modest households and small-scale farms, particularly concentrated in the northern regions. As reported by Flanders Investment & Trade Market Survey (2020), approximately 60% of Nigeria's cattle population thrives in the semi-arid zones, under the care of nomadic herders. However, it is essential to acknowledge that domestic livestock production significantly falls short of the country's overall demand, a fact underscored by the Food and Agriculture Organization (2023). On average, per capita meat consumption is estimated at approximately 6.4 kilograms annually per person (Akinsulu et al., 2019).

The meat industry is an important economic factor in Nigeria. But despite its contributions to one-third of the nation's agricultural GDP (Kwaghe et al., 2016), the industry remains relatively modest and is growing slowly, compared to the burgeoning population that depends on it for sustenance (Ogbeide, 2015). The sector faces a variety of challenges that include environmental, socioeconomic, and regulatory issues, which will be explored in depth in this article. The Nigerian government has taken a central role in promoting meat production in the country. One notable policy implemented by the government was a strict ban on the importation of meat and meat products. This strategic move was aimed at curtailing the influence of foreign companies in the Nigerian meat market while providing incentives for domestic production. In addition, governments have made significant investments in livestock production, established research institutes dedicated to the advancement of livestock production, and promoted modern practices in the sector. However, the meat industry faces some unique challenges, including disease outbreaks and inefficient value chains. Addressing these complex issues is urgently needed to ensure the longevity of the industry, meet the growing demand for meat, and improve the livelihoods of those deeply involved in the meat production process.

Numerous authors have examined these problems in various studies on the Nigerian meat industry (Table 1). These studies have examined issues such as inadequate handling and packaging in slaughterhouses as discussed by Oluwafemi et al. (2013) and Nwanta et al. (2008), constraints in the poultry sector, and factors hindering commercial and economic development as explored by Anosike et al. (2018). Olaoye and Ntuen (2011) identified the role of infrastructure in meat

spoilage as a limiting factor for the industry, while Otu and Okon (2019) and Usman et al. (2015) research examined possible fraudulent practices by butchers, including soaking meat in water and neglecting proper measurement standards. It's worth noting, however, that intentional adulteration of meat and meat products in the Nigerian meat industry has received little attention in these discussions.

Meat product authenticity remains a relatively underexplored subject, primarily due to the fact that, in contrast to many other food products, meat is commonly retailed in a fresh state at open markets across the country, with limited availability of packaged meat in a select number of supermarkets (Osazuwa-Peters, 2021). This underdeveloped and disorganized meat sales system in Nigeria may account for the lack of attention given to product fraud. As Otu and Okon (2019) noted in their study, fraud is deeply ingrained in Nigerian society and has been rationalized through various arguments, including the belief that "everyone in Nigeria is a cheat" or that it represents a morally less deviant offense compared to other more heinous crimes. These factors, among others, emphasize the significance of shedding light on fraud within the Nigerian meat sector as a pivotal issue impacting consumer welfare, public health, the meat industry, and the broader Nigerian economy. In this article, we provide an overview of the Nigerian meat industry, highlight some specific cases of meat fraud, and discuss methods used in surrounding African countries to authenticate meat and how these can be adopted by Nigerian researchers.

SOME POPULAR MEAT PRODUCTS/DISHES IN NIGERIA

In terms of popularity, beef is the most consumed meat in Nigeria, followed by chicken, mutton, turkey, pork, chevon, etc. (Akinsulu et al., 2019; Ogbeide, 2015; Anyiro et al., 2014; Ogunwale and Adediji, 2014) but not necessarily in that order. As a result, the most popular meat products and meat dishes are made from beef. There are more meat products such as *tsire-suya*, *dambu*, *kilishi*, etc. in northern Nigeria than in the south. This is probably because of the larger populations of these animals there, whereas meat dishes such as *nkwobi* and *isi-ewu* are more common in the south.

Table 1. Some selected reviews and research on the Nigerian meat industry

Authors	Objectives	Findings	Conclusions
Nwanta et al., 2008	Assessment of the situations in abattoirs and meat processing plants in association with un-wholesome consumption.	Improper state of abattoirs across the country Lack of proper waste management system	A coordinated and sustained effort is essential to enhance the quality and safety of meat production in Nigeria.
Nwoga et al., n.d.	Exploration of alternative reproductive technology to improve assisted reproductive technologies (ARTs) to improve cattle production.	Low cattle production and breeding efficiency during dry season Insufficient technical knowledge, limited funding, and fund misuse limit ARTs adoption	ARTs have the potential to revolutionize the Nigerian meat industry by improving the productivity and quality of cattle despite high cost and need for special training.
Anosike et al., 2018	To analyze the challenges of poultry production in Nigeria and suggest possible solution for the purpose of increased production.	Small-scale poultry production offers opportunities for saving, investment, and risk security, constituting around 90% of total poultry production in Nigeria	Several significant challenges that have hindered its growth and productivity. proposed solutions, including veterinary intervention, knowledge dissemination, and financial support.
Usman et al., 2015	To estimate the effect of water-soaking on the minerals and nutritional compositions of beef.	Soaking was found to significantly reduce the nutritional quality of beef with great effects on its mineral element contents	Meat processing managers must ensure that all meat sold complies with health and food safety regulations through testing and quality assurance systems.
Oluwafemi et al., 2013	To highlight and identify areas that should be addressed to improve the quality of fresh beef at the retail point for human consumption.	Beef or meat products are not well processed and cared for in many parts of Nigeria	Government parastatal should use their good offices to enforce strict compliance to standard food safety regulations.
Kwaghe et al., 2016	To assess the current status of livestock production, management systems, and existing policies in Nigeria, with a focus on understanding the challenges.	Livestock production challenges such as inadequate grazing reserves, poor disease management, and substandard meat processing lead to low-quality meat and economic issues	Proper hygiene and effective management of livestock is the only way to achieve success in livestock production which could lead to agricultural development, better international trade, and an increase in the nation's GDP.
Olaoye & Ntuen, 2011	Evaluation of meat spoilage, potential of lactic acid bacteria as a bio-preservative and ways to manage wastage associated with meat due to spoilage.	Lack of standard facilities for handling and processing meat exposes the product to high risk of contamination and spoilage by pathogens and microorganisms	Lactic acid bacteria as biological preservatives could be used to supplement existing traditional preservation techniques.
Kubkomawa et al., 2018	To encourage entrepreneurship in animal agriculture by exploring the current systems and dynamics of beef production and marketing in the country.	Beef Production and Marketing Dynamics	The dominance of middlemen in the beef industry, with their disproportionate profits compared to producers and consumers, underscores the need for a holistic approach that incorporates socioeconomic factors to provide reliable data for effective interventions.
Ogbeide, 2015	To determine compositional quality preferences of consumers and to use the outcome of the objectives to advance improvement in the livestock industry.	A higher proportion of consumers preferred lean meat	Farmers and meat producers can utilize consumer preference as guide toward livestock and meat production.

Table 2. Some meat products and dishes in Nigeria

Meat product	Originating region	Key ingredients	Cooking method
<i>Suya/Tsire-suya</i>	Northern Nigeria but found in all Nigerian states	cow, goat, ram, or chicken meat, <i>yaji-kuli kuli</i> (defatted groundnut cake), dried herbs, chili pepper, spices, cloves, salt, ginger, etc.	grilled over an open charcoal fire
<i>Kilishi</i>	Northern Nigeria	beef and <i>yaji</i> slurry	oven-dried and slowly roasted
<i>Dambu</i>	Northern Nigeria	cow, goat, ram, chicken or camel meat, <i>yaji</i> slurry	pounded and fried in a shallow vegetable oil
<i>Balangu</i>	Northern Nigeria	beef, groundnut oil, spices, and salt	roasted over an open fire
<i>Kundi, Banda or Tinko</i>	Northern Nigeria	meat animals such as horses, donkeys, camels, or even elephants	boiled, sun-dried, or smoked
<i>Nkwobi</i>	Eastern Nigeria	cow foot, palm oil and potash, crayfish, pepper, spices, some herbs, <i>utazi</i> leaves and onions	slowly cooked
<i>Asun</i>	Western Nigeria	beef, chicken or mutton, peppers, onions, ginger, and various spices	grilled or roasted and marinated
Meat pepper soup	Western Nigeria	goat, ram, or cow intestines	boiled
Meat pie	throughout the country	minced or ground beef, pastry dough, diced potatoes, and carrots	oven-baked

Many of these products are regional and are traditionally produced, meaning that their production methods are based on ancient techniques and technologies. *Kilishi* for instance, is said to have been historically preserved for emirs and their soldiers as battle food supply. In the preparation of these meat products, care is taken to ensure a long shelf life, and preservation after production is minimal. Some of these products' information is summarized in Table 2.

THE NIGERIAN MEAT INDUSTRY

The meat industry is a segment of the food industry that includes all persons, enterprises, and activities involved in the production, processing, packaging, storage, and marketing of meat on a large scale. The meat industry in Nigeria is less structured and developed than the rest of the world and is plagued by many challenges such as insecurity, inadequate power supply, fuel shortage, etc., which limit its potential and growth. Despite this, the food industry in Nigeria is the largest in Africa with a national revenue of \$191.40 billion in 2022 and is expected to grow at an annual

rate of 8.68% by 2027 (Statista, 2022). The National Bureau of Statistics classified 122 food items into 15 major food groups to represent the main food items consumed in the Nigerian food market. In this classification, meat, poultry products, and seafood accounted for the largest expenditure on specific food items after cereals, roots, and starchy foods (Fig. 1) (National Bureau of Statistics, 2020). The Nigerian meat industry consists of the livestock industry, the meat processing sector, and the market.

Livestock industry

The meat industry is composed of several main players and the livestock industry is primarily at the base, which produces animals for the other production chains. The main livestock in Nigeria are cattle, goats, sheep, pigs, and poultry. Northern Nigeria has the highest number of cattle, goats, and sheep, most of which are nomadic or extensively raised. Based on October 2022 statistics from the Federal Ministry of Agriculture and Rural Development (Nkechi, 2022), the current livestock population in Nigeria is estimated at 22.4 million cattle, 53 million sheep, 99.9 million goats, 9 million pigs,

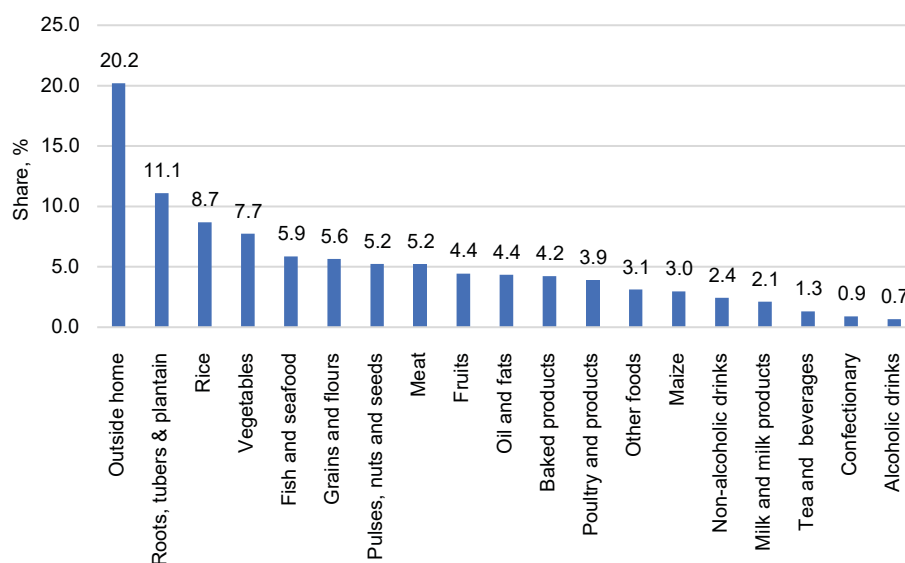


Fig. 1. Categories of food items consumed in Nigeria
Source: National Bureau of Statistics, 2020.

and over 425.8 million poultry. However, this number is still too low to meet the country's needs. As a result, Nigeria relies on its neighboring countries of Chad, Cameroon, and Niger for imports of live cattle, most of which are brought into the country by nomadic herders. The National Bureau of Statistics (NBS) findings on foreign trade showed a significant 68.4% increase in national spending on live cattle imports between 2015 and 2021 (Ibemere, 2021). The perennial plague of Nigeria's livestock sector is gross underproduction, despite an annual growth rate of 22.7% and a net value of 30 trillion naira. This is largely owing to the fact that 70% of beef and 25% of poultry consumed in Nigeria is imported (Iyayi, 2020).

In addition to cattle, an important source of meat in Nigeria is sheep and goats. The indigenous breeds of sheep and goats are raised in households in semi-intensive or free-range systems. They are mostly kept as a quick and available source of income, and their sales may not be sufficient to meet the demand for large-scale processing, so their demand is also supplemented by imports. On the other hand, sheep rearing in Nigeria is usually optimized for the Eid-al Adha festival period, when sales of live sheep and consumption of mutton are highest. Sheep are fattened about 3 to 4 months before the Islamic festival and sold at a substantial profit.

Recently, modern farmers have begun to intensively expand the production of these small ruminants and market their products to meet the growing demand.

Poultry farming is the most widespread livestock production in Nigeria. Traditionally, animals are raised in free-range systems, accounting for 45% of total national production, while semi-intensive and intensive poultry farming account for 33% and 22%, respectively (Herrero, 2020). Relatively, poultry is consumed less by Nigerians than beef and other red meat products because they are considered more expensive and unaffordable to the average citizen (Abe-gunrin et al., 2021). Poultry meat is consumed most during the Christmas season and some poor Nigerians even save up to afford a live chicken. This limited consumption of poultry meat in the pre-Christmas period by the average Nigerian probably contributes to the fact that domestic production in the country is just enough and the importation rate of poultry meat and products is not that high.

The poultry sector of the Nigerian livestock industry is consistently the most active and thriving sector within the livestock industry. It is the sector where individuals and companies are most actively engaged in livestock production and has more intensive production activities. This growth can be attributed to

the availability of information on poultry production to novice and veteran farmers. It has also contributed to the affordability and ease of acquiring livestock for production. Farmers have quick access to inputs such as medicines, vaccines, and feeds. The year-round availability of the market for poultry products for restaurants and processing plants has also had a very positive impact on the growth of the sector.

On the other hand, the red meat animal sector is largely dominated by small-scale farmers and there are very few commercial farms for intensive production of these animals. Some of the challenges responsible for the low participation and performance of this sector are the high cost of production, inaccessibility of credit, and illiteracy, to name a few. The Nigerian livestock sector is not without hope. The ways recommended to harness the imminent potential are *i*) genetic improvement of indigenous livestock breeds, *ii*) adoption of intensive but sustainable production systems to maximize productivity, *iii*) availability of cheap inputs to reduce production costs, and *iv*) value chain development (Iyayi, 2020).

Meat processing industry

Meat processing is the set of activities performed on raw meat to change the form of the meat to meet the needs of the final consumer. In Nigeria, meat processing includes, at its simplest, the slaughter of live animals, evisceration, cutting of the carcass, and preparation of large portions for retail sale (open stalls). However, these activities are limited to public slaughterhouses or local slaughterhouses where butchers or open-stall meat vendors purchase and slaughter their animals individually (Adeyemo et al., 2009). Private slaughterhouses go beyond these basic activities of slaughtering and cutting large portions by thoroughly washing animals, cutting them into small portions, packaging, sealing, branding, labelling, and storing fresh meat. Cattle, sheep, goats, and pigs are predominantly slaughtered in government slaughterhouses, while chickens and other less popular meat animals are processed in private establishments.

Currently, the processing of meat for retail is done in extremely dirty conditions with no regard for health and safety. Animals are transported to slaughterhouses in inhumane conditions, kept in filthy pens, slaughtered, and processed on the bare floor in open spaces

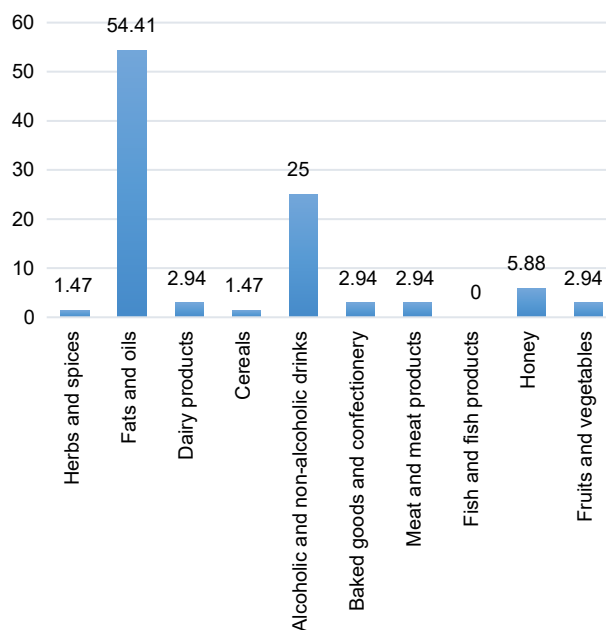


Fig. 2. Adulteration frequency of common food items consumed in Nigeria based on a survey response from 68 respondents

Source: Opia, 2020.

(Adeyemo et al., 2009; Tekki et al., 2012). The dirty conditions of retail meat production have led many health-conscious Nigerians to become active in the meat processing business, which was otherwise in the hands of uneducated and less safety- and health-conscious people. These businesses are small-scale operations that slaughter an average of 8–10 cattle, 15–20 goats, rams, pigs, and thousands of poultry per day. They operate in a hygienic environment and ensure that meat processing standards are met (Fig. 2).

The largest percentage of red meat eaten in Nigerian homes and *bukatarias* (local restaurants) comes from public abattoirs while few and more informed elites patronize the new emerging businesses of the small-scaled processors (Osazuwa-Peters, 2021). However, this does not imply the absence of industrial-sized meat processors – they just happen to be more established in poultry meat production e.g., Zartech Farms and Obasanjo farms. Similarly, there are more small- and medium-size poultry meat processors than there are for red meat, and they account for 80% of poultry meat products in the Nigerian market.

Meat market

Meat markets describe designated places where meat is offered for sale. Meat outlets are usually butcher stores, supermarkets, hypermarkets, grocery stores, online stores, and wet markets. In Nigeria, prominent meat markets are located in close proximity to the city's central slaughterhouse, such as the Oko-Oba meat market in Lagos and the Bodija meat market in Ibadan. The strategic location facilitates the transportation of meat from the slaughterhouse to the point of sale. The system of selling meat in Nigeria is very unorganized, lacks price regulation, and is unhygienic. Adesokan et al. (2021) described this system of meat marketing as an informal system of food marketing common in developing countries because of their poor economic conditions and because of its unsanitary and untaxed disorganization, supplying meat products at affordable prices to equally poor consumers.

Fresh red meat is sold on the basis of standardized cuts and regulated prices per kilo in an extremely hygienic and refrigerated environment in developed nations around the world. What comprises the sale of fresh red meat in Nigeria is the direct opposite of everything enumerated. The meat is offered on wooden boards in open-air stalls where dust, dirt, and flies abound (Fig. 3). Consumers buying the meat are also in the habit of touching the meat to physically assess it or get a feel for the weight so they can set an appropriate price to haggle with the vendor/butcher.



Fig. 3. Hygienically processed and packed fresh meat cut from a meat processing company in Nigeria (phot. Bukola Adenuga)

While some meat vendors are stationary in the market, some vendors hawk their meat in small wooden crates, sometimes with only a flimsy piece of netting to protect the meat from the elements (dust, flies, sunlight, etc.). Refrigeration of meat for sale is virtually non-existent in the Nigerian meat market, as electricity is also non-existent. Many butchers suffer losses when their meat is not sold in full every day because the price of a second day's meat drops drastically, especially if it has been out in the open all day and its fresh red colour from the day before is now a dull brown colour. In the worst case, this meat goes bad and must be disposed of.

In the poultry meat market, on the other hand, there is a higher level of hygiene in the sale and handling of meat. Throughout Nigeria, a high percentage of fresh poultry meat is sold frozen. It is sold in small or large stores, supermarkets, or even in open-air stalls. The system of selling poultry suggests that Nigerians can adopt a sanitary system of processing and distributing red meat. However, decades of unregulated acts in public slaughterhouses and the sale of meat in the open, which has resulted in cheap and affordable meat, have conditioned people to see this as the norm. It is therefore not surprising that more and more informed Nigerians are no longer buying this type of unhealthy meat as a new generation of red meat markets grows. This new generation of markets offers hygienically treated meat for sale, with a regulated pricing system that assures consumers that they are getting quality meat at a good price. Many of these new meat sellers are available online and offer online catalogues where consumers can shop and have a purchase delivered to their homes.

The bushmeat market is another thriving segment in the Nigerian meat industry. This market contributes significantly to total meat consumption in Nigeria annually. In WildAid's study, which surveyed 2000 people, it was found that over 70% of Nigerians living in urban areas have eaten game meat at least once in their lives (Unah and Knights, 2021). There are markets dedicated to the sale of bushmeat, such as the Oluwo market in Lagos, where all kinds of wildlife are offered for sale. Bushmeat can also be found hanging on poles along highways to be sold fresh or processed through hard smoking to motorists. Similar to the red meat market, less attention is paid to hygiene in these

markets. Carcasses of dead game with bullet holes or cuts from the snare are offered for sale unprocessed on wooden tables. Some vendors, however, process meats such as antelope and snakes by smoking them. To date, there is no standard commercial processing for bush meat despite its significant consumption in Nigeria.

MEAT FRAUD INCIDENCES IN NIGERIA

Food fraud is any intentional or deliberate attempt by a company or individual to deceive the consumer and make an illicit profit from the sale of a product (Commission Regulation EU, 2019). Food fraud comes in several forms: substituting ingredients, adding undeclared substances, diluting, reducing, mislabeling, etc. Fraudulent activities in buying and selling are a common phenomenon in many modern markets and Nigeria is no exception (Otu and Okon, 2019). The concept of fraud in the Nigerian food industry is not foreign or unfamiliar to many Nigerians who shop in the local markets. Nigerians, more than any other nation, face food fraud that has become their reality in hindsight. Nearly 200,000 Nigerians die annually from food poisoning and more than 90,000 are hospitalized for foodborne illnesses (Oyefeso, 2019). Locally produced unbranded and unpackaged products are highly vulnerable and susceptible to fraud.

Meat is very susceptible to fraud, especially when it is processed. It can be difficult to determine the type of origin, cut, or even the quality of the meat. Although meat is relatively widely available in Nigeria, it is still not easily affordable, especially in a volatile economy like Nigeria, where the slightest change in production factors immediately leads to an increase in food prices. Recently, meat production, processing, and sales have been severely affected by additional factors such as increasing insecurity and insurgency in the northern region of Nigeria, where most red meat animals are produced or found in large numbers. Also, the continuous increase in fuel prices and ethnoreligious clashes between herdsmen and farmers, livestock traders, and butchers are some of the factors affecting the availability of meat in Nigerian markets (Ajulo, 2022). These factors have led to widespread fraudulent activities such as mislabeling of meat, sale of expired meat, sale of meat of questionable origin, and product smuggling in the Nigerian meat industry.

Cases of meat fraud have been reported in several countries around the world, including the European horsemeat scandal (Smith and McElwee, 2021), the Chinese rat meat scandal (Dai and Jiang, 2013), and the Brazilian rotten meat scandal (Oxford Analytica, 2017). The incidents have shaken the media wave and consumers' interest in the authenticity of meat products has been reawakened, making them no longer just passively interested in verifying the meat products they consume (Kendall et al., 2019). In Nigeria, however, cases of fraud in the meat sector were far too underreported (Fig. 4) and the media do not give them the attention they deserved in order to alert consumers to the dangers of meat fraud. One can only speculate about the reasons for this lack of attention. Oyekunle (2018) attributes this to poverty and consumer illiteracy while Onyeaka et al. (2021) observed that despite the increasing food safety problems that have led to the deaths of hundreds of thousands of Nigerians, many are unaware of these dangers in the food industry.



Fig. 4. Fresh meat cuts offered for sale on wooden boards with no sanitary measures in place (phot. Bukola Adenuga)

The sale of contaminated meat is a major challenge in the Nigerian meat sector. Meat products have been reported to contain unhealthy levels of bacterial loads (Adesokan et al., 2021), aspergillosis and mycotoxins (Atanda et al., 2013), high levels of metal deposits (Ekanem et al., 2020), and even antibiotic residues (Omeiza et al., 2012). On September 19, 2022, the Nigeria Security and Civil Defence Corps (NSCDC), Kwara State Command, arrested a butcher

for allegedly selling contaminated meat after receiving a tip-off from a concerned person. Further analysis of the meat by the State Department of Health confirmed that the meat was not fit for consumption (Rabiu, 2021). In Lagos, Nigeria's most populous metropolitan state, a news reporter uncovered the sale of toxic brown ponmo (cow skin, a product derived from cowhide) to unsuspecting consumers. The leather-like material of ponmo is repeatedly scorched by these processors with toxic materials such as old shoes, tires, old motor oil and plastics to remove hair on the hide. This results in harmful fumes and blackened products contaminated with toxic chemicals and carcinogenic heavy metals (Lambo, 2021).

Another common practice is the use of paracetamol tablets and soda drinks when cooking meat to make it softer or more tender. Paracetamol is a drug used to treat mild headaches or fevers and was never designed for cooking and is therefore not approved for food safety. This practice was devised to reduce production costs but comes at the expense of consumer health (liver and kidney failure). The National Agency for Food and Drugs Administration and Control (NAFDAC) has issued a warning to all food processors and sellers to refrain from using the compound as a tenderizer (NAN, 2020). Similarly, the agency warned meat and fish sellers against using formaldehyde as a preservative to keep meat fresh (Odunsi, 2021).

In 2018, there were reports of a rather unusual incident in Bayelsa, a state located in the South-South region of Nigeria, where chicken meat was allegedly substituted with vulture meat and donkey carcasses. The suspected perpetrators sell this meat at very low prices to establishments such as restaurants, bars, and canteens that process them, making it more difficult for the end consumer to identify the species they are eating (Falade, 2018). Four other individuals were arrested in Edo State for illegally selling donkey meat (Peterson, 2021). Donkey meat is banned for human consumption in Nigeria after the Nigerian government recently banned the slaughter of donkeys, the consumption of donkey meat, and the illegal export of donkey hides to protect the animals from endangerment of extinction (Nwaiwu-Nnewi, 2020). Suya, a popular Nigerian beef kebab, has also been exposed as a product of fraudulent acts by butchers who sell dead animals to suya producers. Reportedly, 7 members of the same family died as a result of eating such a product in Abia state (Ukanwa, 2022). These instances of fraud in the meat sector show that meat processors are unaware of the impact of their cost-cutting measures on the health of consumers of their products. The apparent disregard for public health and consumer choice is exacerbated by the indifferent attitude of government officials who are supposed to ensure compliance with food safety regulations (Ukanwa, 2022). Table 3 shows a summary

Table 3. Reported cases of meat fraud in Nigeria

State/Region	Incident	Fraud category	Product involved	Reference
Kwara	Sale of contaminated meat	Adulteration	Raw meat	Rabiu (2021)
Lagos	Use of toxic materials for meat product processing	Adulteration	Ponmo (cow hide)	Lambo (2021)
Osun	Use of unauthorized substance as tenderizer	Adulteration	Cooked meat	NAN (2020)
Bauchi	Use of toxic chemical as preservative	Adulteration	Fresh meat	Odunsi (2021)
Bayelsa	Substitution of chicken with vulture	Misrepresentation	Processed and cooked chicken	Falade (2018)
Edo	Sales of illegal meat	Black or Grey Market	Donkey meat	Peterson (2021)
Lagos	Exhume and sale of unfit-for-consumption poultry products buried by law enforcement agents	Misrepresentation Adulteration	Fresh poultry meat	Ogbeche (2015)

of incidence of meat fraud gathered from online news sources. In many of the cases mentioned, suspects are typically fined and rarely brought to justice, while the public is urged to watch what they eat. This pedestrian approach to dealing with food fraud is why these problems still exist.

SUSCEPTIBILITY OF MEAT PRODUCTS TO FRAUD IN NIGERIA

Five major categories of food fraud as recognized by the EU are: adulteration/product tampering, counterfeiting, document forgery, grey market, and mislabelling (European Commission, 2021). Many of the instances highlighted in Figure 5 can be found in the Nigerian meat market. The vulnerability of meat products to fraud in Nigeria is fueled by several factors, including limited governance structures, limited traceability systems, and lack of public awareness. These conditions create an environment where fraudulent practices can thrive and compromise the quality, safety, and authenticity of meat products.

Consumers' ignorance or disregard of meat fraud is another of the causes of the increase in fraudulent

activities by meat producers. Often, many meat buyers want quality or large quantities of meat for less than the going rate, and in an effort to satisfy their customers, these sellers resort to trickery and malicious means to achieve this. Secondly, due to the rising cost of living, resulting in high food prices, many Nigerians cannot afford the high prices of meat and meat products, so they look for cheaper alternatives. A good example of this is the high reliance of Nigerians on brown ponmo (cow skin) as a meat alternative. Despite numerous studies (Tijani and Ajayi, 2016; Tijani, 2017) showing that brown ponmo offers no nutritional benefits, and despite the federal government's warning against eating ponmo because of its mitigating effects on the leather industry, many continue to eat it anyway.

Meat producers also continue to face problems of limited or poor infrastructure, poor storage facilities, inadequate access to clean water, irregular power supply, lack of maintenance of meat processing machinery, and poor transportation systems. Products from these producers are usually contaminated and of low quality. Also, inadequate veterinary staff in slaughterhouses, abattoirs, and meat-producing plants to check the suitability of animals for processing has encouraged



Fig. 5. Categories of food fraud
Source: European Commission, 2021.

fraudulent practices such as processing sick or dead animals. Dead animals or animals unfit for consumption must be properly disposed of. However, some unscrupulous producers and processors buy these dead animals in large quantities and sell them as processed meat (Ukanwa, 2022). They are usually sold cooked and seasoned so that consumers do not know what they are eating.

Food production is generally not well-regulated in Nigeria. There is too much focus on ready-to-eat and packaged foods, medicines, and other fast-moving consumer goods (FMCG) by food safety agencies. Furthermore, there is a failure to pay close and adequate attention to unbranded and unpackaged foods, which make up the bulk of consumption among the middle class and less privileged Nigerians (Tijani, 2017). Street vendors (hawkers, stall holders), local canteens (bukateria), and roadside snack vendors are responsible for the majority of food consumed outside the home in Nigerian cities (National Bureau of Statistics, 2020). It is important that all food vendors maintain high standards to ensure that quality and authentic products are available to all consumers.

LEGAL FRAMEWORK FOR THE PREVENTION OF MEAT PRODUCT ADULTERATION IN NIGERIA

Across the world legal measures and laws exist to monitor and prevent meat product fraud. In the European Union, fraudulent activities involving food product are covered by Regulation (EC) No 178/2002 of the European Parliament and of the Council (Brooks et al., 2021). In Nigeria, the legal framework for combating meat product adulteration involves various laws and institutions which are not consolidated in a single enactment but are spread across different statutes (Uzoamaka, 2019). Many of these laws are longstanding from as far back as 1917 and they do not get reviewed often due to the lengthy and slow legislative process involved in the Nigerian national assembly (Okoruwa et al., 2022). Furthermore, the majority of these laws are proactive in nature; that is, they often focus on the final products and primarily target removing unsafe food from retail markets and punishing responsible parties after a lengthy investigatory process. This reactive approach may limit the effectiveness of prevention and early detection of meat product

adulteration (Okoruwa et al., 2022). Some major acts targeting meat products are listed below.

Criminal Code Act

Section 244 of the Criminal Code Act in Nigeria specifically addresses the offense of dealing with diseased meat. It states that anyone who knowingly brings the carcass of an animal that died of disease into a slaughterhouse or sells the carcass of a diseased animal is guilty of a misdemeanor and is liable to imprisonment for two years. This provision serves as a deterrent and provides a legal basis for prosecuting those involved in the adulteration of meat products.

Animal Diseases Control Act

Section 9(1) of the Animal Diseases (Control) Act CAP 17 LFN 2014 includes provisions related to the disposal of diseased animals. If an animal dies of a disease or is slaughtered in a manner contrary to the provisions of the Act and its carcass is deemed infected with a disease by a veterinary officer, the carcass must be disposed of through burning or as directed by the veterinary officer. These provisions help prevent the consumption of meat from diseased animals.

Meat Edict, 1968

The Meat Edict was amended in 1966 to empower the veterinary department to oversee the cleanliness, safety, and authenticity of meat and meat slaughterhouses across Nigerian states. Key provisions of the law include the maintenance of satisfactory sanitary environments, registration of slaughterhouses, the requirement for animals slaughtered for public consumption to be slaughtered in registered slaughterhouses, meat inspection by veterinary officers, condemnation of unwholesome meat by veterinary officers, stamping of fit-for-consumption animals by veterinary personnel, and prosecution of defaulters (Olukole, 2008). The Meat Edict acts as an important monitoring tool to ensure the quality and safety of meat products.

Hides and skin act: 6th Oct 1942 Ordinance

This law makes provision for the process, handling, preservation, and exporting of hides and skin. It provides guidelines for flaying, drying, preservation, and marketing of hides and skin, particularly those intended for export. However, it does not address the

processing and handling of these animal by-products as food products, as they were never intended for such use. Consequently, the diversion of hides and skins into the meat market as edible meat products violates established laws.

Federal Competition and Consumer Protection Act, 2018

The Federal Competition and Consumer Protection Act was created to develop and promote fair, efficient, and competitive markets in the Nigerian economy, thereby enabling citizens to access safe and quality products while protecting their rights. Part XV of the Act ensures consumers' right to information about a product in clear, understandable language, price, informative labelling, fair dealings, protection of consumers from false and misleading product representations, and preservation of consumers' right to quality and safe products. At the same time, part XVI of the law requires manufacturers to properly label their products and recall dangerous goods from the market.

The enforcement and implementation of these laws to prevent meat fraud are of the utmost importance. It ensures that instances of non-compliance are promptly addressed, and appropriate sanctions or punishments are administered by the relevant authorities (Uzoamaka, 2019).

Nigerian Agencies Tasked with Ensuring Authenticity in the Meat Industry

The National Agency for Food and Drug Administration and Control (NAFDAC), with its subunit Food's Safety and Applied Nutrition (FSAN) Directorate, oversees production in small- to medium-sized food establishments, provides inspection guidelines, and establishes the minimum good hygienic practices (GHP) required in the production, processing, and packaging of food of animal origin. The primary objective is to ensure both consumer protection and the safety of foodstuff. These agencies conduct inspections of production facilities and issue the required quality assurance certificates for products in cooperation with the Standards Organization Nigeria (SON).

The Standards Organization Nigeria (SON) is responsible for setting and implementing standards for all domestic and imported products. It establishes and ensures the application of standard measurement

procedures to products that require them. It also ensures that products meet all established standards and conducts inspections to ensure that all required safety information is included on product packaging. The Federal Competition and Consumer Protection Council (FCCPC) is the federal agency that handles complaints and protects consumers from unfair and deceptive trade practices. They monitor and investigate unhealthy competitive practices between businesses and among traders in the markets. They provide a platform for aggrieved or defrauded customers to fight back against companies or institutions that have violated consumer protection laws. To protect and promote exemplary behavior among Nigerian citizens, the National Orientation Agency (NOA) is charged with creating awareness, promoting positive attitudes, and fostering peace and harmony among citizens. The NOA works with food safety agencies, federal ministries, etc., to sensitize Nigerians on how the actions of these organizations benefit them. They also organize workshops and seminars to educate the public on the potential benefits and risks of certain activities. In addition, they provide information on food safety, health, and sanitation.

Other organizations involved in ensuring healthy practices in the Nigerian meat industry include the Federal Ministry of Health (FMOH), the Federal Ministry of Agriculture and Rural Development (FMARD), the Federal Ministry of Environment (FMEnv), the Federal Ministry of Industry, Trade and Investment (FMITI), the Federal Ministry of Education (FME), and the private sector.

IMPACT OF MEAT FRAUD ON NIGERIAN CONSUMERS

Food safety practices are a significant concern for Nigerian consumers, as evidenced by a self-reported study conducted in Ilorin among 869 meat consumers where 40 percent of the respondents showed "knowledge and practice levels of food safety" (Odetokun et al., 2022). Unhealthy and unsafe meat consumption can lead to various health risks, including exposure to allergens, toxic chemicals, and microbial contamination, resulting in direct, indirect, short-term, or serious health consequences (Onyeaka et al., 2022). A recent incident in Kogi State exemplifies these risks, where

the government banned meat consumption following the mysterious death of 20 cattle, some of which were discovered at the point of sale by health officials (Sahara Reporters, 2022). Unfit-for-consumption meat is often sold at lower prices to processors, who subsequently distribute it to unsuspecting consumers, thereby exposing them to the risk of zoonotic diseases. The outbreak of gastroenteritis in the Yagba West area of Kogi State in 2017, which resulted in 62 deaths, serves as an illustrative case where zoonotic diseases were transmitted through the consumption of contaminated meat (The Nation, 2017).

These incidents have significantly raised the awareness of Nigerian meat consumers, prompting them to be more cautious about their meat purchases. However, caution should be exercised regarding the reliability of shared health tips and advice on preventing meat adulteration, as many of these can be misleading, misinformative, or even pose greater health risks. For instance, some consumers have adopted the unhygienic practice of biting into the raw hide of fresh meat to assess its tenderness. Additionally, a social media platform user has suggested boycotting fresh meat in open markets that do not attract flies, alleging the use of insect repellents (Ezirigwe, 2018). Such practices of verifying meat authenticity are dangerous and expose consumers to additional foodborne hazards.

In Nigeria, where nearly 50% of the population is estimated to be Muslim, there is a lack of widespread understanding and proper implementation of halal meat concepts among butchers, processors, and consumers. Annabi and Lolade (2015) critical study on the perspectives of abattoir workers reveals that halal certification involves comprehensive adherence to specific practices throughout the entire production chain. However, several factors in Nigeria contribute to the meat being unworthy of halal certification, such as inadequate knowledge of the health and physiological condition of animals before slaughter, inhumane treatment, unclean slaughter environments, and a high risk of contamination. Consequently, many meat products in Nigeria may be falsely certified as halal based solely on the recitation of the shahada, without meeting the necessary halal criteria.

While some fraudulent acts in meat production, such as misrepresenting the sex of the animal, its origin (wild-caught or farm-raised), manipulating scales,

or adding bones to deceive buyers, may not directly impact consumer health, they significantly undermine consumer confidence and the right to know what they have purchased or are consuming (Kendall et al., 2019). To avoid being deceived, many consumers prefer trusted or verified brands, often resulting in increased expenditure.

IMPACT OF MEAT FRAUD ON THE MEAT INDUSTRY AND ECONOMY

Illegal beef production, particularly in unlicensed operations, accounts for 42.9 percent of reported food fraud in the global beef supply chain (Elliott et al., 2019). The coexistence of illegal and legal producers leads to unfair competition and advantages that often result in meat products being offered at lower prices. Licensed meat production facilities adhere to regulated standards to ensure product quality and authenticity. However, when fraudulent producers appear on the scene, genuine products may be imitated, safety practices may be ignored, or products unfit for human consumption, such as Bovine Spongiform Encephalopathy (BSE) meat, may be produced. These practices negatively impact the meat industry and cause losses for real companies that must compete in an unfair market. The closure of over 700 fast food outlets in Nigeria, including popular brands such as Mr. Bigg's, Tantalizers, Tastee Fried Chicken, and Sweet Sensation, has been attributed to low clientele and unfair competition from bukaterias and mama-put food outlets, resulting in a higher unemployment rate and loss of revenue for the economy (Abiola, 2019).

Illegal importation of meat poses a threat to the Nigerian meat industry. The poultry meat sector is particularly affected by this economic threat. However, Nigerian poultry production giants such as CHI Farms, Zartech, and Ajanla have played an important role in ensuring a level playing field by implementing stringent biosecurity measures, investing in modern technology and equipment, and collaborating with supervisory authorities to enforce strict adherence to safety and quality standards in their supply chains to produce quality products which consumers trust. Additionally, in light of government plans to crack down on illegal meat imports, CHI Farms planned to double production by expanding operations and improving

storage facilities while maintaining product quality in order to meet the increasing demand for quality poultry products and contribute to the overall growth of the Nigerian poultry industry (Umeh, 2019).

Other far-reaching effects of meat fraud in the Nigerian economy are evident in the lack of export of Nigerian meat products to international markets. This is due to the World Trade Organization's dissatisfaction with production standards (Onyeka, 2016). To address the problems of hygiene, poor quality, and fraud in the industry, the introduction of national meat laws has been proposed. In addition, religiously certified foods require the establishment of a halal certification body in Nigeria. This certification process would cover all stages of meat production, including rearing, feeding, slaughter methods, handling, processing, packaging, storage, and transportation. Such measures would ensure the inspection and certification of meat products and reassure consumers that the meat they consume meets global requirements and standards.

ANALYTICAL METHODS FOR MEAT FRAUD IDENTIFICATION AND PREVENTION IN NIGERIA

Detecting meat fraud is critical in Nigeria's burgeoning food industry. Recently, food manufacturers have realized the financial benefits of selling processed products. Nigerian indigenous chicken breeds are now sold in packaged form. Kilishi (beef jerky), a popular beef snack usually sold without a brand name, is now available in packaged form in stores. Meatballs can also be found in both physical and online markets. In addition, food sales have evolved greatly to include bulk cooked foods delivered directly to consumers. Given this boom in the industry, it is critical that consumer welfare and confidence is maintained through proper authenticity testing of meat products. Considering the high percentage of Muslim population in Nigeria, verification of meat for non-halal ingredients is of utmost importance.

With the critical lack of research and studies on meat product authenticity testing, there is an urgent need for Nigeria to take a cue from other African countries such as South Africa, Egypt, and Tunisia. These countries have devoted scientific resources to analytical studies for the prevention and detection of fraudulent meat products. Some of these studies

include next-generation sequencing (NGS) analysis to detect contamination and mislabeling in processed and commercially available meat products in South Africa (Chaora et al., 2022), near-infrared hyperspectral imaging (NIR-HSI), a nondestructive imaging method used to distinguish between the four categories of patty (Edwards et al., 2023), and various mass spectrometry techniques to determine the regional origin of lamb (Erasmus et al., 2016, 2017, 2018). The analytical methods used by these researchers are summarized in Table 4. When these methods are grouped, the following categories can be identified.

DNA-based methods

The DNA-based method for species identification is one of the most widely accepted methods for identifying meat species. Since all living organisms have a unique genome, DNA can be extracted and a specific gene fragment can be detected with relatively high accuracy using sophisticated techniques (Adenuga and Montowska, 2023). DNA-based methods are known for their high sensitivity, multiple species identification, and quantification. In addition, DNA barcoding techniques facilitate the accurate identification of species in meat products without any prior knowledge of using a database known as the Barcode of Life Database (BOLD) (Ratnasingham and Hebert, 2007), which contains known reference sequences by comparing them to the DNA sequence of the unknown species. This technique as applied by (D'Amato et al., 2013) to verify the authenticity of the commercial labels of regular and game meat products in South African local markets, showed that 76.5% of the game meat samples were substituted. A similar approach can be replicated in Nigerian meat markets to verify game meat products' authenticity.

PCR-based methods

There are numerous DNA-based methods for authenticating meat species, but polymerase chain reaction (PCR) plays a central role in most assays (El Sheikha et al., 2017). PCR in conjunction with post-PCR analyses such as restriction fragment length polymorphism (PCR-RFLP) and sequencing (PCR sequencing) underpin the reliability of PCR experiments in species identification. In addition, species-specific PCR and multiplex PCR allow accurate and rapid species

Table 4. Analytical techniques employed for meat products authentication in some African countries

Country	Analytical method	Method category	Meat product	Aim	Activity	Outcome	References
1	2	3	4	5	6	7	8
South Africa	Near-Infrared Hyperspectral Imaging	Spectroscopy	Raw beef patties	To discover a cheap, novel, and non-destructive technique for meat authentication	Eight hundred beef patties were analysed to assess the potential of NIR-HSI to distinguish between the four patty categories	It was possible to distinguish between the four patty categories with accuracies $\geq 97\%$,	Edwards et al. (2023)
South Africa	Next-Generation Sequencing	DNA	Minced meat, biltong, burger patties, and sausages	To identify processed meat product mislabeling	Sequencing, Quality control, and filtering	Sausage samples showed the highest level of contamination with 46% of the samples having mixtures of beef, pork, or mutton in one sample.	Chaora et al. (2022)
South Africa	Real-time PCR	PCR	Ready-to-eat meat products	To detect animal species in commercial products	DNA extracted from 44 ready-to-eat meat products was screened for the presence of commonly reported undeclared animal species using Real-time PCR	27% of the meat products tested contained undeclared species.	Sreenivasan Tantuan and Viljoen (2021)
Tunisia	Visible reflectance spectroscopy technique	Spectroscopy	Meat from pasture-fed and stall-fed lambs	To discriminate meat produced in conventional systems pasture or stall-fed	The colour and the reflectance spectrum of different fat tissues were measured	The study confirmed the efficiency of the visible reflectance spectroscopy technique applied to perirenal fat in feeding systems authentication	Hajji et al. (2019)
South Africa	Isotope ratio mass spectrometry (IRMS)	Mass spectrometry	Homogenized and defatted meat of the <i>longissimus lumbo-rum</i> muscle	Origin verification and authenticity	Powdered meat samples were broken down into gases which were then fed into an isotopic mass spectrometer to obtain isotope ratios	95% and 90% correct classification of the samples for the estimation model and validation models	Erasmus et al. (2018)
South Africa	Proton-transfer mass spectrometry (PTR-MS)	Mass spectrometry	Lamb meat and fat from the <i>longissimus lumbo-rum</i> (LL) muscle	To use volatile fingerprints obtained through PTR-MS analysis as an authentication tool	Measuring the volatile fingerprints of lamb meat and fat samples, chemometric analysis (PLS-DA), classification of the samples based on their region of origin and overall origin	PLS-DA models successfully classified the samples based on their region of origin and overall origin with high accuracy	(Erasmus et al. 2017)

Table 4 – cont.

1	2	3	4	5	6	7	8
South Africa	isotope ratio mass spectrometry (IRMS)	Mass spectrometry	Homogenized and defatted meat of the <i>longissimus lumborum</i> (LL) muscle	To differentiate between lambs from farms with different vegetation types	measuring the stable isotope ratios ($^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$)	The differences in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values showed promising potential for reliable authentication, particularly in determining the region of origin	Erasmus et al. (2016)
South Africa	ELISA, LCD array, Species-specific PCR, DNA sequencing	Immunoassay, DNA, PCR	139 processed meat products collected from retail outlets and butcheries	To assess the extent of mislabeling and fraudulent practices in meat products	A commercial DNA-based LCD array was used to initially screen, confirmed using species-specific polymerase chain reaction (PCR) and, in some cases, DNA sequencing	68% (95 out of 139) of the samples contained species that were not disclosed on the product labels, undeclared plant protein in meat	Cawthorn et al. (2013)
South Africa	DNA barcoding.	DNA	14 beef and 132 game label	To determine the authenticity of commercial labels of meat products, especially game meat	BLAST searches in GenBank for sequence comparison, maximum likelihood phylogenetic analysis	76.5% of the game meat samples were substituted	D'Amato et al. (2013)
Kenya	PCR-HRM analysis	PCR	107 meat samples from seven common live-stock animals	To detect species substitution	PCR-HRM analysis was performed on these samples using primers targeting the three mitochondrial genes	10.3% species substitution was observed	Njaramba et al. (2021)
Egypt	Species-specific PCR-based method	PCR	Commercial meat products, uncooked beef burgers, sausages, kofta, and luncheon	To determine potential adulteration and/or contamination with donkey, chicken, or human tissues	Species-specific PCR-based analysis was performed	87.5% of the analyzed beef samples were adulterated or mislabeled with one or more species	Omran et al. (2019)
Egypt	Species-specific PCR-based method	PCR	96 beef meat and meat product samples	To detect adulteration of crude and processed beef meat with horse and donkey tissue	PCR was used to detect the presence of horse and donkey key tissue. Specific primers were used in PCR reactions.	6 out of 96 beef meat samples were positive for the presence of donkey tissue	Abd El-Razik et al. (2019)
Egypt	Polymerase Chain Reaction (PCR) and the Raw Meat Flow-ThroughTM Test.	PCR	30 beef meat product samples (hawawshi, sausage, and burger)	To detect species adulteration in meat products	DNA from the samples was extracted and subjected to PCR analysis, and adulterated samples detected by PCR were reanalyzed using the Raw Meat Flow-Through Test	80% of the examined samples contained undeclared meat species	Ahmed et al. (2020)

Table 4 – cont.

1	2	3	4	5	6	7	8
Egypt	SNP-based PCR-RFLP and forensically informative nucleotide sequence (FINS) methodologies	PCR	25 fresh red meat samples	To discriminate six red meat species (cattle, buffalo, goat, sheep, camel, and donkey) to ensure meat authenticity and prevent species substitution	PCR amplification of the mitochondrial 12S rRNA gene for all target species. FINS methodology was used for unambiguous identification. SNP-based PCR-RFLP to generate unique restriction fragment length polymorphism patterns for each species	One buffalo sample was substituted with cow meat, and one goat sample was substituted with sheep meat	Galal-Khalaf et al. (2021)
Tunisia	PCR-RFLP and species-specific Multiplex PCR	PCR	six commercial turkey sausage brands and fresh meat	To detect meat species in commercial meat products to ensure traceability and consumer confidence	gene using universal primers in PCR amplification of 359 bp cyt b. Enzymatic digestions of the amplified fragments	7 animal species were efficiently detected in pure and mixed meat samples. Triplex species identification	Gargouri et al. (2021)
Uganda	PCR-sequencing	PCR	229 bushmeat sample	To investigate bushmeat misidentification in northern Uganda.	PCR targeting specific genes and sequencing were performed to identify the species in the samples using NCBI BLAST	27.9% of the samples had disparate results between the species reported and the analysis using NCBI BLAST	Dell et al. (2021)
Tanzania	PCR-sequencing	PCR	151 bushmeat samples	To identify the species of origin of bushmeat in the Tanzanian market and confirm seller-reported species of origin	PCR and sequence analysis of the CytB gene were performed to determine the species of origin for each sample	30% of the bushmeat samples were misidentified by sellers	Schilling et al. (2020)
Egypt	Multiplex PCR coupled with 12S rDNA sequencing.	PCR	Frozen beef liver samples and cold cut samples.	To detect meat adulteration in frozen beef liver and cold cut samples	Multiplex PCR to identify different types of meat residues. PCR-sequencing of 12S rRNA gene was performed to identify the species of frozen beef liver samples	62.5% of the processed beef samples were found to be contaminated with poultry residues	Galal-Khalaf (2021)
Egypt	PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism)	PCR	Meat mixtures	To differentiate between mixtures of different animal species	PCR amplification using universal primers. Product digestion with two different restriction endonucleases	Unique banding pattern for each species or mixture, allowing for accurate identification	Farag et al. (2022)

identification. Abd El-Razik et al. (2019) achieved a sensitivity of 0.01% (w/w) for donkey or horse tissue in beef using their species-specific PCR assay to detect unwanted meat species in processed beef. Since many laboratories in Nigeria have PCR machines, PCR-based research for meat control should be encouraged.

Mass spectrometry-based methods

MS is an analytical method for detecting and measuring the masses of various molecules. This method is used to analyze nonvolatile compounds in a meat sample. Modern mass spectrometers can be programmed to detect specific peptides without the need for a bioinformatics database (Watson et al., 2017). High-performance liquid chromatography (HPLC) is a chromatographic technique used to separate, identify, and quantify the components of a sample. HPLC-MS identifies species in meat by identifying specific peptide markers. This allows for more specific identification and reduces overall laboratory time (Watson et al., 2017). Currently, HPLC is often combined with high-resolution mass spectrometry (MS). Other methods combined with MS include isotope ratio mass spectrometry (IRMS) (Erasmus et al., 2018) and proton transfer mass spectrometry (PTR-MS) (Erasmus et al., 2017).

Spectroscopy-based methods

Spectroscopy-based analyses are rapid, accurate, and nondestructive methods for determining the chemical composition of a variety of materials, including meat products. Spectroscopy techniques commonly used for meat authentication studies include near-infrared (NIR), Fourier transform infrared (FTIR), Raman, nuclear magnetic resonance (NMR) spectroscopy, and ultraviolet-visible (UV-Vis) and fluorescence spectroscopy. The operating principle of spectroscopic methods is based on the ability of biological, chemical, and physical components in meat products to emit measurable spectra when excited at specific wavelengths (Li et al., 2020; Saleem et al., 2022). These emitted spectra contain valuable information about the composition and properties of the samples. Subsequently, the spectra obtained are usually subjected to various chemometric analyses, such as multivariate statistical methods, to facilitate their interpretation and provide meaningful insights (Rohman, 2019). In Tunisia, the visible reflectance spectroscopy technique was used

to identify and discriminate lamb from conventional pasture and confinement. The result showed a range from 76.4% to 100% correctly identified meat origin (Hajji et al., 2019).

Immunoassays

Immunoassay techniques are a collective term for tests that use antibodies to detect the presence of specific proteins for species identification. Some important immunoassay techniques include enzyme-linked immunosorbent assay (ELISA), lateral flow immunoassay (LFI), and colorimetric immunoassay. The technique is based on the basic principle of immunochemistry, namely, the formation of an immune complex from an antigen and its specific antibody. It is highly adaptable and can also be used to detect genetic material. ELISA and lateral flow assays use specific monoclonal or polyclonal antibodies to identify targets labelled with appropriate antigens (Banerjee et al., 2023). Immunoassays are known to have a short test duration, contain inexpensive materials, are easy to understand, and do not require specially trained personnel (Asensio et al., 2008; Mandli et al., 2018). In their study, Cawthorn et al. (2013) used ELISA and LCD array methods and species-specific PCR and DNA sequencing to determine the degree of mislabeling in 139 processed meat products such as ground beef, burger patties, deli meats, sausages, and jerky from South African meat stores.

MULTI-STAKEHOLDERS' APPROACH IN ADDRESSING FRAUD IN THE NIGERIAN MEAT SECTOR

A comprehensive national food control system involving key stakeholders such as the government, food industry, consumers, and academic and scientific institutions is crucial for effectively managing and preventing food hazards, ensuring compliance with all food safety measures, and providing consumers with healthy and unadulterated products (Opia, 2020). The role of scientific institutes in combating meat fraud cannot be underestimated, as they conduct research and offer evidence-based solutions to address the issue. Researchers play a vital role in studying the prevalence and patterns of meat fraud, developing innovative detection methods, and establishing quality standards

for meat products. Their expertise can inform policy decisions and support industry practices aimed at ensuring the authenticity and safety of meat products. According to a report by Maestroni (2014), access to food control laboratories and related services is a fundamental requirement for generating surveillance data to manage food risks in a nation. However, the establishment and operation of laboratories come with significant costs, including expenses for equipment, tools, chemicals, and reagents. This can pose a challenge for individuals or institutions without sufficient financial resources to embark on such endeavors (Opia, 2020). Therefore, a more sustainable approach is to establish government-accredited laboratories that are readily available and accessible to producers, researchers, and consumers throughout the country (Maestroni, 2014). This would promote efficiency and collaboration and ensure the availability of reliable testing and analysis facilities for the detection of meat fraud.

Numerous reports have highlighted that food processors and retailers prioritize quantity, profit margin, and volume over the quality, freshness, and authenticity of raw materials or products they produce (Ezirigwe, 2018; Omemu and Aderoju, 2008). Therefore, the responsibility of regulating the meat sector lies with all industry stakeholders, including meat producers, processors, and distributors. It is crucial for them to reassess their objectives and establish consultative forums where members adhere to regulated standards, ensuring that every stage and level of production is properly vetted and scrutinized to meet accepted standards. Industry stakeholders need to work closely with scientific institutes and embrace the outcomes of research to incorporate them into their production strategies. For instance, in a study conducted on street-vended meat pies sold in Lafia, Nasarawa State, Nigeria, it was found that although the level of microbial contamination in the meat pies was within the acceptable threshold for human consumption, improper storage beyond two days could render them harmful to health (Okoro et al., 2021). Therefore, it is crucial to properly label such products, clearly indicating the type of meat used, production date, and sell-by or best-before date to prevent intentional and unintentional fraudulent activities, such as the sale of expired meat products.

There is a significant amount of work to be done by the Nigerian government to effectively secure the

meat industry against the risks associated with meat fraud. A crucial first step is the complete overhaul of the entire production chain within the meat industry. It is imperative that animals raised for consumption undergo comprehensive monitoring and vetting by national veterinary agencies, starting from day zero up to the point of slaughter. Abattoirs and slaughterhouses should be renovated to meet standardized operating practices, and strict enforcement and appropriate punishment should be applied to any violations.

The government must ensure that all meat production businesses, their products, and trademarks are duly registered with the appropriate agencies such as NAFDAC, SON, and CAC (Corporate Affairs Commission). This registration will facilitate the implementation of a functional traceability system for meat products. Additionally, the issue of unbranded, unlabeled, or unregistered meat products must be addressed as it is a major source of product adulteration in the country. Regular and standardized routine checks should be conducted on production grounds, products, and market surveys to ensure compliance with established standards. Establishing a regulatory framework that promotes integrity and accountability is vital for the meat sector. Government agencies should facilitate information sharing and coordination among stakeholders, support research and development efforts, and provide financial incentives to encourage compliance with industry standards.

CONCLUSIONS

In Nigeria, the meat industry grapples with substantial challenges related to safety, hygiene, and product authenticity. This is well-reflected in the body of literature by Nigerian scholars, who have extensively examined various aspects of the meat sector, including production enhancement, spoilage prevention, industry challenges, and market constraints. Notably, a considerable focus has been placed on ensuring the safety of meat products. Consumers have been duly cautioned regarding ready-to-eat meat items, as demonstrated in the work of Erhirhie et al. (2020). Furthermore, recommendations for public awareness and improved coordination within the food supply value chain have been put forth as means to cultivate a comprehensive national food safety system (Omojokun,

2013). Some scholars argue that enhancing the oversight of the meat slaughter process by local authorities (Nwanta et al., 2008) and empowering butcher associations to enforce standards within meat markets (Grace et al., 2012) can significantly elevate product quality within the sector.

While these approaches outlined by the scholarly community are pivotal for promoting product and consumer safety, a concurrent understanding of product fraud, its limitations, and prevention has been relatively underrepresented. This article underscores the vulnerability of Nigerian meat products to fraudulent practices, stemming in part from consumer preferences for cost-effective items and the limited coordination within the meat sector due to the absence of robust standards enforcement in areas such as product metrology, sales, and packaging.

Through this review, it has become evident that a considerable research gap exists within the domain of meat product authenticity. Existing research, albeit limited, is often subsumed under the broader subject of food safety. It is imperative to communicate to the Nigerian populace that food fraud extends beyond product imitations, specifically within the realm of meat. In publishing this article, we aim to lay the foundation for food safety researchers and regulatory agencies in Nigeria to initiate investigations into meat product verification. By showcasing methods, objectives, and findings from analogous research conducted in other African nations, we seek to encourage future studies. We recommend that future research endeavors to incorporate consumer opinions on meat fraud as a starting point. Understanding what consumers perceive regarding possible adulteration in their consumption is a foundational step toward progress.

Furthermore, we anticipate that forthcoming research will delve into verifying product species claims, geographical origins, and halal certifications, among other aspects. As research continues to evolve, we aspire to reach a stage where consumers can employ home-test kits to confirm product claims independently. Ultimately, we encourage Nigerian food safety authorities to play an active role in the realm of meat safety, ensuring that all food items, whether packaged, unpackaged, or locally produced, meet established standards.

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Publication 2 (P2)

Adenuga, B. M., Montowska, M. (2023). A systematic review of DNA-based methods in authentication of game and less common meat species. *Comprehensive Reviews in Food Science and Food Safety*, 22, 2112-2160. <https://doi.org/10.1111/1541-4337.13142>

COMPREHENSIVE REVIEW

A systematic review of DNA-based methods in authentication of game and less common meat species

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Funding information

National Science Centre, Grant/Award
Number: 2020/37/B/NZ9/00082; Polish
Ministry of Science and Higher
Education's program, Grant/Award
Number: 005/RID/2018/19

Abstract

Despite the numerous studies on food safety and authenticity, especially for meat and meat products, not enough studies have been conducted focusing exclusively on game species and other unusual meat animals. As a result of the European horse scandal, the horse is currently the target of many meat authenticity studies. With this review, we aim to present various DNA-based methods that have been used by researchers to identify, detect, and quantify game, uncommon meat animals, and wildlife species. Polymerase chain reaction (PCR) is considered the standard method for DNA analysis in meat authenticity testing. However, in this paper, we present several other methods that may or may not involve the PCR technique. For this purpose, we systematically reviewed 131 articles selected according to various criteria such as target animal species, method of analysis, year of publication, and so forth. The result of our study shows the most studied game and uncommon meat species, PCR- and non-PCR-based methods for game meat analysis, and DNA-based methods in wildlife conservation. With this study, researchers can find detailed information about frequent game species used as adulterants for regular meat products and the DNA-based techniques to identify them.

KEYWORDS

adulteration, authenticity, conservation, DNA, game, meat products, PCR, wildlife species

1 | INTRODUCTION

Meat is an important part of the human diet and one of the main sources of dietary protein. Game meats are considered a good source of high-quality protein with low fat and cholesterol contents; in addition, the sensory quality of this type of meat is its most endearing attribute for consumption and the reason why it is highly sought after. The term game meat is used to describe the meat of animals that

are not domesticated in the traditional way. Game meat is obtained from hunted animals living in the wild. However, some types of wild species are raised in captivity, while others are hunted in the wild either legally or illegally (Hoffman & Cawthorn, 2014). In Poland, game is admitted to trading by the Veterinary Inspection. People consume this type of meat for several reasons. Some of them are the appeal of eating unconventional meals and a healthy diet based on lifestyle choices. Deer meat, for example,

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has higher protein and minerals content, lower fat content, and no antibiotics and growth promoters. Game meat has also captured the heart of many meat lovers because it is overly flavorful and considered tastier than conventional meats.

Key to the repeated consumption of game meat by regular consumers is confidence in the authenticity of game products and the integrity of the producer/seller (Niewiadomska et al., 2020). This is noteworthy that one of the biggest barriers to consuming game meats is adulteration, such as substitution or addition of parts of the meat or all of the meat by less valuable counterparts, misrepresentation of the origin of the animals in the case of wild-caught or farmed animals, and misrepresentation of the geographic origin of the animals. The most common reason for meat adulteration is economically motivated. For this reason, there are several government agencies (i.e., European Food Safety Authority [EFSA], United State Department of Agriculture—Food and Drug Administration [USDA-FDA], National Agency for Food and Drug Administration and control [NAFDAC] in Nigeria) that provide transparency in the meat business. However, wildlife is not only susceptible to adulteration, it is also a prime target for exploitation, animal mistreatment, welfare abuse, poaching, and illegal trade. Several wildlife species are on the International Union for Conservation of Nature (IUCN) Watch List as “Critically Endangered,” and many others are classified as “Endangered” or “Vulnerable,” for instance, as in the case of six crocodile species (Zhang et al., 2021).

Illegal wildlife procurement is an ongoing constraint on wildlife trade and conservation. Despite existing agreements under the Convention on International Trade in Endangered Species of Fauna and Flora (CITES) to regulate wildlife trade, several endangered species still fall through the cracks, while some are overexploited (Cardeñosa et al., 2017).

Therefore, to protect both wildlife and consumer choice, research in meat authentication, that is, species identification to detect replacement of meat by cheaper meat species as well as to detect undeclared animal ingredients or their proportion, is essential for the following reasons: (i) to detect adulteration of wild meat with conventional meat and vice versa; (ii) to prevent animal-to-person transmission of zoonotic infection with animal diseases, for example, from consumption of bats, raccoon, or fox meat; (iii) to prevent some microbial infections, for example, from decomposing animals; (iv) to prevent contact with allergens; (v) to prevent consumption of religiously prohibited foods; (vi) to stop illegal capture and trade of wildlife and their resources such as hides, ivory, tusks, and so forth; and (vi) to enable proper identification of wildlife species.

Over the past two decades, methods for authenticating meat based on various molecular products have been described in the literature, each with its own advantages and disadvantages (Li et al., 2020). Technologically processed meat is at the forefront of meat product adulteration due to its complex and heterogeneous composition (Naveena et al., 2017), making it difficult to use simple, morphological, or organoleptic approaches to identify its composition. Meat species in the food chain have been previously identified based on protein markers (Montowska et al., 2014), lipid metabolites (Trivedi et al., 2016), and DNA nucleotides (Guntarti et al., 2017; Hossain, Ali, Hamid, Hossain, et al., 2017). DNA-based technologies have the advantage of stability, uniformity, and polymorphism (Asing et al., 2016a; Druml et al., 2016) over other methods when detecting meat products. In addition to that, real-time polymerase chain reaction (RT-PCR) offers the additional advantage of quantification of meat species (Druml et al., 2016).

In the last 6 years, wildlife species have been authenticated (identified and/or quantified) using DNA-based assays. Methods such as polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) were used to differentiate wild boar and pig (Koseniuk et al., 2021); DNA barcoding in the research by Kane and Hellberg (2016) identified kangaroo, emu, and bison meat among the 48 ground meat products tested; multiplex PCR (mPCR) has also been used to differentiate beef and buffalo meat (Bhat et al., 2016). Forensic wildlife investigations on the other hand have used molecular protocols based on the forensically informative nucleotide sequencing (FINS) to identify monitor lizard and its traded products (Rajpoot et al., 2017), short tandem repeat and single-nucleotide polymorphism (SNP) genotyping to forensically differentiate wild boar, pigs, and their hybrids (Lorenzini et al., 2020), and DNA mini-barcode assay to identify sharks, animal skins, or furs in highly processed finished products (Cardeñosa et al., 2017; Janjua et al., 2017). It is important to note that most of the research on meat authentication has focused on domestic (farm-reared) meat and a smaller percentage has focused on wild meat identification. In this article, we aim to provide a systematic review of the literature addressing wild meat as a target species. The aim of this review is to (i) provide an overview of the main DNA-based methods published in the literature between 2016 and 2022 for the identification of game meat; (ii) provide a listing of the most commonly adulterated game species during this period; (iii) provide a listing of game meats that have been used as adulterants for regular meat products; (iv) compare the effectiveness of each DNA-based method; and (v) highlight some DNA-based methods used in wildlife conservation and forensic analysis.

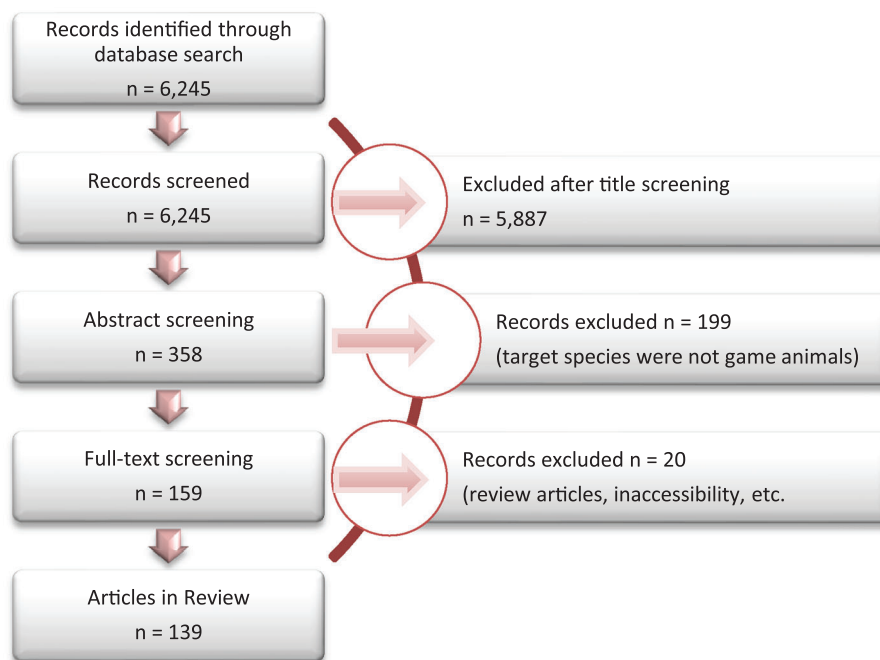


FIGURE 1 Flow chart of the study selection process.

2 | METHODOLOGY

In this review, the systematic approach to data collection is presented. In the initial phase of writing, a preliminary search was conducted using Google and Citation Gecko. Then, following the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) protocol for systematic reviews, a literature search was conducted at SCOPUS on April 28, 2022, using the advanced search option. The search terms molecular probes, polymerase chain reaction (PCR), DNA-based technique, meat, exotic meat, bush meat, game species, wild animals, meat product, deer, wild boar, wild rabbit, limit of detection (LOD), food analysis, and species identification were used to limit the search to papers dealing with game species authentication.

The search result was filtered to exclude older publications from 2015 and above, as this review focuses on more recent entries. Papers from English-language journals and in their final stages were considered. The search result yielded 6245 documents that were segmented by year of publication and exported to a Microsoft Excel spreadsheet for further review (Figure 1).

3 | OVERVIEW OF THE RESEARCH ON GAME MEAT AUTHENTICATION AND WILDLIFE CONSERVATION

The majority of the papers were related to the identification of microbes. After eliminating these and all other irrelevant articles, we were left with 340 articles on meat

authentication, from which we finally extracted 141 articles on game meat authentication and wildlife conservation (Figure 2). Of these studies, 29 dealt with the identification of wildlife products for forensic purposes. From the studies reviewed, it appears that the majority of DNA-based research on wildlife meat identification is based on PCR-based methods. PCR-based methods were referred to as the “gold standard” in some of the papers. This assertion is reinforced by the comparison of the effectiveness of PCR methods with other DNA-based methods in some of the literature. A few studies also combined multiple methods, mostly for the purpose of improving or comparing existing methods. Nanopore sequencing, which is considered a fourth-generation sequencing method, was used in only one study.

There are a large number of studies using mitochondrial DNA for analysis, suggesting that fewer studies have been performed on nuclear genes. As a result, many of the studies were qualitative rather than quantitative. These papers examined over 30 different wild meat species, with species such as buffalo and horse being studied more frequently than others (Figure 3). A geographic overview of the studies in this review shows that more work on wild meat authentication came from the Asian region than from other regions (Figure 4).

3.1 | Sample collection and validation

Sample collection and preparation is one of the crucial moments in meat authenticity testing

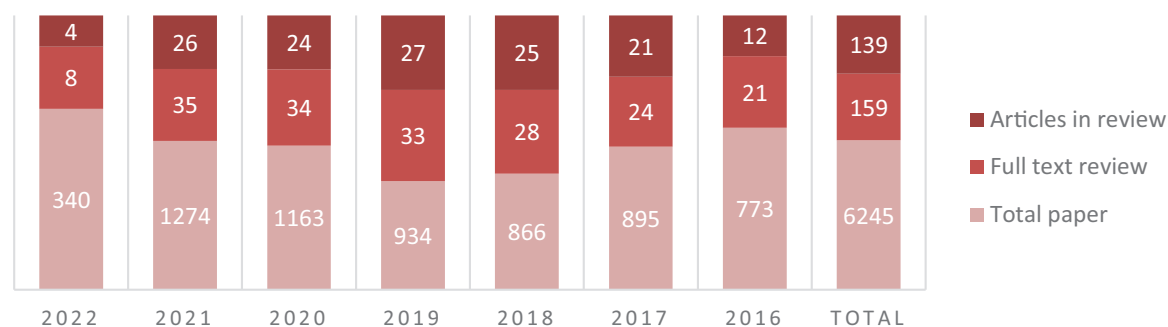


FIGURE 2 Number of articles reviewed by year.

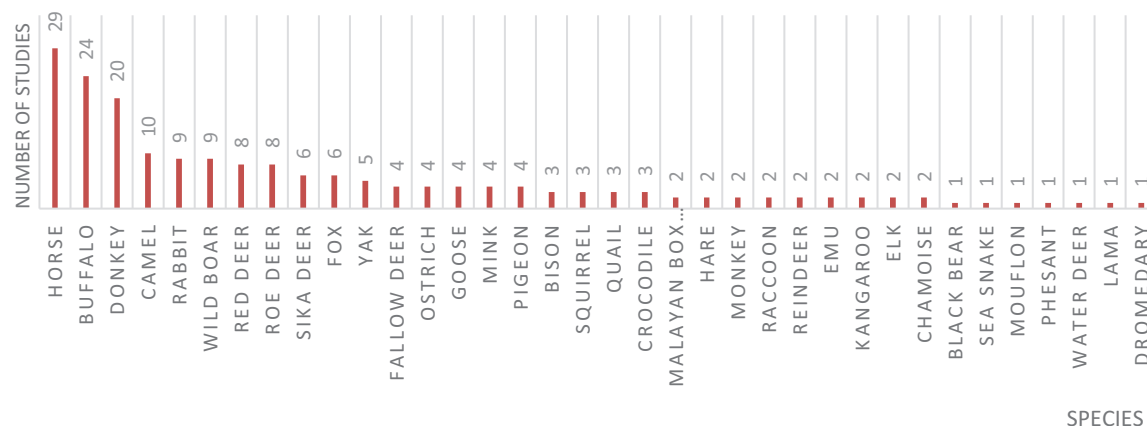


FIGURE 3 Frequency of studies on some game and less common meat species.

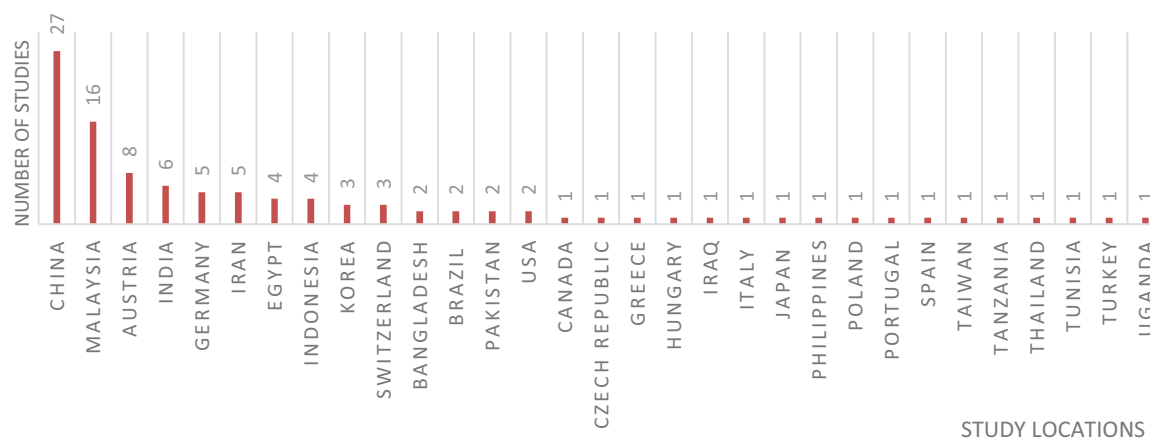


FIGURE 4 Studies frequency by location.

(Stachniuk et al., 2019). Therefore, adequate care is usually taken during this step to ensure that the correct samples are collected and properly handled. The samples collected in the studies reviewed were muscle tissue, raw meat, blood, sausages, and other commercially available meat products. Samples generally came from markets (local markets, butcher shops, supermarkets, wet markets, street vendors, and online outlets), animal farms, slaugh-

terhouses, and various government organizations or research institutes. Other sources that are not as popular include restaurants, food companies, and hunting.

Validation or authentication of meat samples for research is equally important, as an inauthentic sample defeats the purpose of the research. The means used in the works studied were morphological verification, DNA sequencing, and acquisition of authentic

samples from government/educational institutions. However, some studies validated their acquired samples with the research (Dell et al., 2021; Kane & Hellberg, 2016; Scales et al., 2021; Schilling et al., 2020).

3.2 | Target species

The horsemeat scandal in Europe in 2013 was the main reference point for many studies on meat authenticity and was an excellent justification for many of these studies. As a result, there have been more papers (29) identifying horse species than any other species. In addition to horses, the following game species were also frequently studied: buffalo, donkey, camel, deer, wild boar, rabbit, fox, mink, and yak (Figure 3).

3.2.1 | Buffalo

Carabeef or buffen (buffalo meat) is one of the most popular meats consumed worldwide (Hossain et al., 2016). There are different breeds of buffalo on all continents, such as the African buffalo and the Asian water buffalo. Like cattle, buffaloes belong to the Bovidae family and therefore share some physical similarities, which further makes it difficult to distinguish their meat morphologically. As a result, carabeef and beef have been adulterated with each other over the years. The acceptability and value of buffalo meat vary culturally and geographically. In Europe and China, where it is widely accepted and eaten, it has a higher value than beef and is considered a delicacy (Hossain, Ali, Hamid, Hossain, et al., 2017; Wang, Hang, et al., 2019). In India, however, buffalo meat is less valued and is considered an adulterant of more valuable meat such as mutton (Naveena et al., 2017). In Brazil, the lack of adequate legislation to regulate the production of carabeef is also cited as a reason for the meat's undervaluation in that country (Dantas et al., 2019). Egypt is mentioned as a country that accepts buffalo meat very well (Sakaridis et al., 2013), but on the contrary, Egyptians prefer mutton and beef to carabeef (Galal-Khallaf et al., 2021).

3.2.2 | Camel

Camels or dromedaries are popular in Middle Eastern and northeastern African countries. The harsh climatic conditions of these regions make it difficult for traditional livestock such as goats, sheep, and cattle to thrive (Hoffman & Cawthorn, 2014). Instead, horses, donkeys, and camels, which are better suited, are the predominant animals in such areas. Unlike horses and donkeys, camels can be eaten for religious reasons in these Muslim-majority countries, and their meat is readily available. For their

project, which involved authenticating camel and donkey meat, Galal-Khallaf et al. (2021) were able to easily obtain camel meat from a local butcher shop in Egypt, while donkey hair was used instead of donkey meat because it was not available. Camel meat is also available in other non-Muslim countries such as Australia and China (Hoffman & Cawthorn, 2014). Because of its limited availability, camel meat is rarely adulterated (Hai et al., 2020). Even Galal-Khallaf et al. (2021) did not detect adulteration in their camel samples.

3.2.3 | Deer

Deer are antlered ungulates consisting of several species found in different places of the world. Roe deer, red deer, and fallow deer are native primarily to Europe, while elk, fallow deer, sika deer, axis deer, and white-tailed deer are farmed in the United States and Canada (Hoffman & Cawthorn, 2014). They are abundant in the wild, but are also widely farmed in New Zealand. Meat from deer is highly prized by consumers for its exotic flavor, low-calorie content, and essential fatty acid content (Kaltenbrunner et al., 2018c). It is accepted in most cultures and religions and is consumed in most geographic areas. In China, it is becoming increasingly popular and is sold as a recreational food (Guan et al., 2018). Venison is the term often used for commercial deer products without specifying exactly which animal they come from (Kaltenbrunner et al., 2018b). Such generalization could lead to inaccurate identification of the deer species. Deer meat is more expensive than conventional meat because it is not as readily available as other species. The availability of fresh deer meat is usually seasonal, that is, during hunting season and only the frozen ones available in the off season. Given the current shortage of processors of hunted deer meat in the United States (Anderson Dennis, 2020; Bestul Scott, 2022; Doyle Ceile, 2021), deer meat is likely to be more expensive and more susceptible to adulteration with meats such as beef and pork. Studies examining the authenticity of deer products are mainly from Europe (Druml et al., 2016; Kaltenbrunner et al., 2018b, 2018c, 2018d; Nesvadbová et al., 2019), with a few from Asia (Guan et al., 2018; Kim et al., 2020; Xue et al., 2017).

3.2.4 | Donkey

Like the horse, the donkey is an animal not generally accepted as a source of meat. But like other unconventional sources of meat, it is considered a delicacy in some other cultures and geographic regions. In China, donkey meat is highly sought after for its high protein content and other byproducts used in traditional Chinese medicine (Zhao, Hua, et al., 2019a). The adulteration of donkey meat with

other meat in regions where it is not very acceptable may be due to its availability as a cheap meat substitute. In Islamic communities, eating donkey meat is forbidden, and mislabeling donkey meat as halal (permissible) is considered a heinous offense (Al-Ali & A, 2019). Despite this, there are still cases of adulterated donkey in beef products in countries such as Pakistan (Asghar et al., 2021) and Tunisia (Gargouri et al., 2021). Zhao et al. (2019b) tested the applicability of their developed authentication method on 46 commercial samples labeled as donkey burgers and found that four products contained horse meat and no donkey meat. This means that not only is donkey meat substituted as beef, but horse meat is also used to replace donkey meat in places where its demand is high.

3.2.5 | Fox

Fox meat is very unconventional that, while edible, is not accepted by most consumers of game meat. Foxes are animals that can be farmed for their fur, but not for their meat. In a food blog, blog writer Charlie Holland describes that fox meat is stinky, fibrous, and tough unless marinated overnight with vinegar and other seasonings. In addition, as a predator, fox is likely to be a host to many diseases and parasites, making it highly unsuitable for consumption (Holland, 2021). Studies have been conducted on fox meat detection to ensure that this valueless meat is not processed into acceptable meat products (Wang et al., 2021; Zheng et al., 2020).

3.2.6 | Mink

The meat of farm-raised mink is also usually discarded after their pelts are harvested because the animals were fed drugs and hormones during their rearing that are supposed to make their pelts beautiful but can be harmful to human health when consumed (Niu et al., 2022). Mink caught in the wild are also inedible because their meat contains high levels of toxins and heavy metals derived from their predatory activities. In a country like China, where mink farming is widespread, there is growing suspicion that fraudulent individuals are buying mink carcasses and selling them to illegal food processors, where they are substituted as beef or mutton buns (Niu et al., 2022).

3.2.7 | Horse

Horse meat is probably considered one of the most unconventional meats to consume. Popoola et al. (2021) in their work, in which participants completed an online survey

that included a free word association task with meat from cattle, bison, and horses, words such as “aversion” and “unethical” were associated with eating horse meat. For most people, horses are considered pets and the thought of eating horse meat is repugnant to them, but this does not mean that horse meat cannot or should not be consumed. Rather, it is that it is usually undeclared in processed meat products (Ramos et al., 2016), depriving consumers of the freedom to choose whether or not to consume it. According to the Canadian Department of Justice website, horse-meat may be sold for human consumption as long as it is properly labeled as such (Naaum et al., 2018). The major consumer concern with the intentional or accidental consumption of horse meat is the presence of phenylbutazone, a nonsteroidal anti-inflammatory drug given to horses that poses a serious health risk to humans when consumed in food (Dodman et al., 2010).

3.2.8 | Rabbit

Rabbits are classified as proper game or ground game (Mazmanyany, 2020). Although rabbits are now domesticated for food and fur, they are still abundant and hunted in the wild for sport or food. Wild rabbits are distinguished by the fact that their meat is leaner than that of their farm-raised counterparts (Hoffman & Cawthorn, 2014). Rabbit meat is available worldwide and fits into many modern diets such as keto, paleo, low carb, and so forth. High consumption of rabbit meat is seen in China, which is the largest producer (Mazmanyany, 2020). Still, several cases of adulteration have been reported in China, some of which involved the adulteration of rabbit meat. In the study of Ahamad et al. (2017) on the identification of rabbits, squirrels, and rats in the food chain, it was pointed out that the familial similarities between these species and the great availability of the latter in the wild could lead dishonest producers to substitute them as rabbits. Despite the increasing interest in the inclusion of alternative meat sources in the regular diet, not enough research has been conducted to correctly identify rabbits and their byproduct (fur). In our review, only about 8% (9/110) of the papers reviewed addressed the identification of rabbit meat.

3.2.9 | Wild boar

Wild boars are abundant in nature. They represent one of the most important game species in Europe (Nauman & Paulsen, 2017). Pigs are known for their high fertility, and wild boars are no exception: they have up to six litters per

parity. This high number of wild boars is an advantage in European countries, where they are hunted and consumed as a delicacy. However, in a Muslim-majority country such as Indonesia, the large number of wild boars poses a threat to the authenticity of the meat sold in the market there (Adiningsih et al., 2018). While pork is not consumed in the country, over 40% (4/9) of papers investigating the authentication of wild boar meat were produced there. These researches were done to verify the authenticity of a popular beef product (bakso) that was not adulterated with wild boar meat.

Wild boars and domestic pigs are genetically very similar but can be distinguished by their body length and skull shape. However, when domestic swine meat is adulterated with wild boar meat or vice versa, it is usually difficult to distinguish the two even at the genetic level. Several papers in the literature addressed the distinction between wild boar and domestic swine. Adiningsih et al. (2018) used *HaeIII* to digest a specific region of the Cyt b gene of wild and domestic swine in the PCR-RFLP assay. Kaltenbrunner et al. (2019) examined two SNP loci using RT-PCR assays to differentiate the two species. Kaltenbrunner et al. (2020) also used RT-PCR assays to detect and quantify wild boar and domestic swine meat in processed foods. Koseniuk et al. (2021) distinguished the two species by examining genetic polymorphisms in the NR6A1 and MC1R genes.

3.2.10 | Yak

Yaks are hardy, densely built, long-furred Bovidae that share many similarities with domestic cattle. They occur both in the wild and as domestic animals. Yak meat is still sold at a higher price despite its similarity to beef in texture and appearance, perhaps because consumers believe their meat is free of drugs and additives (Zhao, Xu, et al., 2019a). Yak and other game meats are eaten as delicacies in China despite their high cost (Guan et al., 2018; Xue et al., 2017). In the United States, yak meat is sold at twice the price per kilogram as beef. For this reason, Kane and Hellberg (2016) suspected a case of economically motivated adulteration in their study when a tested yak burger sample was identified as beef instead. Most studies on adulteration of bovine meat have always excluded studies on yak, which is probably the reason why adulteration of yak meat goes unnoticed (Wang, Liu, et al., 2019). To distinguish yak from cattle, Zhao, Xu, et al. (2019a) genotyped 10 SNP marker loci that accurately distinguished the two species. Other authors used RT-PCR (Wang, Liu, et al., 2019), PCR-RFLP (Guan et al., 2018), a universal primer PCR assay, and DNA barcoding technique (Kane & Hellberg, 2016).

4 | DNA-BASED METHODS FOR GAME SPECIES IDENTIFICATION

DNA is the blueprint of life and no two individuals are 100% genetically alike. The uniqueness of this biochemical macromolecule is what DNA-based authentication research has harnessed. The cells of an individual contain the genome, which contains the totality of all genes in the cell. A specific gene fragment can be detected using sophisticated methods to identify an unknown species with relatively high accuracy. The stability of DNA despite very harsh conditions such as thermal, chemical, mechanical, or technological processing of meat products makes it a reliable method for detecting biological origin of species. The 110 papers reviewed for the purposes of the present review used a variety of DNA-based methods to identify and/or quantify game meat. Approximately 95% of these studies used PCR alone or in conjunction with other methods, but there are others that do not rely on PCR techniques.

4.1 | Conventional PCR-based methods

PCR is an *in vitro* biochemical procedure used, in the simplest case, to amplify a specific segment of DNA. The thermocycler, which is essentially a thermoblock with holes into which the PCR reaction mixture in test tubes is placed, is the machine that makes this possible (Garibyan & Avashia, 2013). The PCR test is widely used in the authentication of meat and meat products because it requires little material, is easy to perform, and is highly reproducible (Li et al., 2020). The sensitivity, specificity, stability, and reliability of the PCR technique and its products have made it the preferred method for the analysis of game meat for numerous authors (Table 1). PCR is the standard for DNA-based species identification. The limitations of conventional PCR methods have led to the development of other methods that are dependent, partially dependent, or independent of it. The most important PCR method is simplex PCR or basic PCR. mPCR is a combination of two or more simplex assays in one reaction tube. Further advances in PCR assay technology have led to the development of more sensitive and less labor-intensive assays such as RT-PCR and digital droplet PCR (ddPCR).

4.1.1 | Simplex PCR

Simplex, basic, end-point, or conventional PCR is traditional PCR with one target species in a single PCR reaction mixture, that is, a single reaction test tube contains the target DNA template, a single primer pair, nucleotides,

TABLE 1 Summary of the conventional PCR methods in the review

Target species	Main method	Sample source	Target gene	Applicability/ validity test	Outcomes	Detection limits	Reference
Beef, chicken, pork, turkey, and horse	ddPCR, real-time PCR, DNA barcoding	Grocery stores and specialty stores	COI, Cytb, 12S	100 raw meat sausages	95% contained the predominant species matching the label	Not stated	
Pork, beef, horse, sheep, turkey, and chicken	ddPCR	Produced in lab and acquired from previous experiment	Beta actin, prolactin receptor, growth hormone receptor, growth factor beta 3	All participants measure the concentration of copies per microliter for all expected meat species in the calibration samples	Conversion factors show variations between the laboratories especially for the poultry chicken RSD (24.8%) and turkey RSD (24.5%)	LOQ for chicken: 0.5%, turkey: 0.67%, beef: 0.5%, pork, sheep, horse: 1%	
Beef, pork, horse, sheep, chicken, and turkey	ddPCR	Retailers	EURL-AP protocol, 16s, cytb	20 commercial meat product and binary mixtures made in the lab	12 samples contained traces of DNA from other animal species than those declared	0.1 pg/ μ L for pork and chicken, 0.01 pg/ μ L for beef, horse, sheep, and turkey	
Fox, mink, and raccoon dog	ddPCR	Breeding farms, local market	F gene and actb gene	16 different retail samples	Three samples were adulterated by fur-bearing animals	0.001%; LOQ 1% (w/w) for three species	
Pork, beef, horse meat, duck, ostrich meat, and chicken	Direct PCR	Farms	Cyt b, COI, 16s rDNA	Ninety-three raw meats, food products tested assay validation and fraud survey	About 14% unlisted meat species detected in all samples; the highest fraud ratio was in the street food	Less than 1.0 mm ³ of meat sample, 0.32 ng of DNA template	
Duck, buffalo, goat, sheep, and dog	Direct PCR	Local super-markets	COI	117 samples of food and food products from several local markets in Thailand	26 contain unlisted meat species; the highest fraud detected in the street food	12,500 mtDNA copies for dog, duck, and buffalo; 25,000 mtDNA for goat; 50,000 mtDNA copies for sheep; between 0.1 and 0.32 ng for mixed DNA	
Beef and wild boar meat	Real-time PCR Eva green	Local markets	Cyt b, AG3A	Five commercial meatballs sold in specific Indonesian communities	There are no amplification peaks of DNA extracted from meatball markets	48 pg, which is 3% of wild boar meat in meatball formulation	

(Continues)

TABLE 1 (Continued)

Target species	Main method	Sample source	Target gene	Applicability/ validity test	Outcomes	Detection limits	Reference
Horse	Real-time PCR Eva green	Local retail markets	Cyt b gene, 18S rRNA	67 different commercial samples acquired at local markets were evaluated	Two samples, one canned sausage and one cooked hamburger, were positive for horse detection	0.1 pg	
Horse, donkey, and bovine	Real-time PCR Eva green	Local breeder, government institute, farm, local markets	Cyt b, 18S rRNA	33 samples selected from different product groups	Neither horse- nor donkey species-specific amplification was observed in 21 of the market samples	0.5% for donkeys, 0.2% for horse species; LOQ 1.4% for donkeys, 0.6% for horses	
Wild boar meat	Real-time PCR Eva green	Slaughter houses, local markets	Cyt b	The validated q-PCR method was employed for DNA analysis of meat species in commercial meatballs samples	There were no amplifications observed during analysis of DNAs extracted from commercial meatball samples	5 ng/ μ L	
Pigeon and chicken	Real-time PCR Eva green	Government institute, local markets	Cyt b	Validated using eight of practical food samples, including steak, patty, nugget, cutlet, meatball, fried meat, and braised meat	All of processed samples containing chicken or pigeon were successfully amplified and identified	0.1 pg for both chicken and pigeon meat	
Beef, sheep, pork, horse, rabbit, chicken, turkey, and quail	Real-time PCR Eva green		Cyt b	PCR products mixture, a blind test was performed	Identification of the two species. Identification of both single source species samples and samples with a DNA mixture of two species	0.1 ng of target DNA	
Beef and buffalo	Multiplex PCR	Open market	Cyt b	Tested on lab mixture of cooked, mixed, and pure meat	Presence of three bands in the mixed meat of cooked Rista and one band for pure meat Rista of mutton, beef, and buffalo meat	1%	

(Continues)

TABLE 1 (Continued)

Target species	Main method	Sample source	Target gene	Applicability/ validity test	Outcomes	Detection limits	Reference
Beef and buffalo	Multiplex PCR	Open market	Cyt b	Tested on lab mixture of cooked, mixed, and pure meat	Presence of three bands in the mixed meat of cooked kebab and one band for pure meat kebab of mutton, beef, and buffalo meat	1%	
Rabbit, squirrel, and rat	Multiplex PCR	Commercial wet and super markets	ATP6, cyt b eukaryotic internal control	Spiked chicken meatballs made in the laboratory; 72 meatballs of eight different halal brands were screened	Rat, rabbit, and squirrel targets were amplified from all adulterated samples. Absence of rat, rabbit, and squirrel meat in Malaysian food products	0.01 ng DNA for pure meat and 0.1% for meatballs	
Beef and buffalo	Multiplex PCR	Wet markets and super-markets, government institution	Cyt b, ND5, 16s RNA	Screening seven meat curries and 45 burgers samples collected from Malaysian commercial outlets	Two of the seven beef curries were found to contain beef; five of the seven curries contained buffalo	0.01 ng of source DNA and 0.1% adulteration in commercial products	
Pig, buffalo, and beef	Multiplex PCR	Wet market, government institution	Cytb and ND5	Commercial meatballs of five different brands for beef and pork were tested	80% of the analyzed beef meatball was found to contain both beef and buffalo and 20% contain only buffalo species	0.02 ng of total DNA extracted from muscle tissues	
Crocodile	Multiplex PCR	Market, government institutes	ATP6, cyt b, eukaryotes internal control	Screening five commercially available crocodile products, namely spicy crocodile meat, crocodile soup, crocodile herbal soup, crocodile jerky, and over-the-counter crocodile balm	This qualitative detection was not affected by the very complex natures of the matrices	0.001 and 0.01 ng DNA, in pure meat and 1% under mixed matrices	

(Continues)

TABLE 1 (Continued)

Target species	Main method	Sample source	Target gene	Applicability/ validity test	Outcomes	Detection limits	Reference
Chicken, camel, pig, sheep, donkey, goat, and beef	Multiplex PCR	Markets	COX1, 18s rDNA	Twenty unknown samples of raw meats bought randomly from markets	No false detection was observed for the specific primers of each seven species	6.25 ng/ μ L	
Horse, donkey, pig, and other ruminants	Multiplex PCR	Research institute	Cytb, 12s RNA, 16s RNA	15 raw and treated samples tested in the study	Meat species were accurately determined in all combinations by multiplex PCR	0.001 ng	
Dog, chicken, cattle, pig, horse, donkey, fox, and rabbit	Multiplex PCR	Farmers markets, retail markets, or online shops	16s rRNA, ATPase 6, 12S rRNA, cyt b	Universal primer-multiplex PCR assay was used for the detection of target animal mtDNA in 103 commercial meat products	Meat balls and sausages were detected to be mixed with chicken meat	Chicken, horse, and fox: 0.1 ng/ μ L mtDNA; dog, cattle, pig, donkey, and rabbit: 0.05 ng/ μ L mtDNA	Wang, Liu et al., 2019
Beef, buffalo, chicken, cat, dog, pork, and fish	Multiplex PCR	Wet markets, supermarkets, government institution	Cyt b, ND5, and 16s rRNA	Laboratory-prepared model and commercial burgers and meatballs of beef, chicken, and pork of various brands were tested	The analysis of a total of 15 Malaysian meatball and burger products revealed that 86.7% of beef products (13 out of 15 tested) were adulterated.	Pig, cat, dog, cow, chicken, and fish: 0.001 ng; buffalo: 0.01 ng	
Buffalo, beef, pig, and duck	Multiplex PCR	Local abattoirs, retailers, and markets	COI	The developed assay was used to test 182 commercial meat products	Meat ball had the highest number of adulteration (56.7%); vacuum-packed meat was the lowest number 15.4%	1 pg for DNA, 0.1% (w/w)	
Sheep, goat, beef, pig, donkey, horse, and ostrich	Multiplex PCR		Cyt b, t-Glu-cytb, 12S rRNA, COI	Two multiplex PCR techniques were used and compared with each other	First multiplex system showed cross-reactivity; the second multiplex showed an inability to amplify all five DNA species simultaneously	Not stated	

(Continues)

TABLE 1 (Continued)

Target species	Main method	Sample source	Target gene	Applicability/ validity test	Outcomes	Detection limits	Reference
Yak meat and beef	Multiplex PCR	Four main yak distribution areas	SNP loci	20 samples of yak dried beef samples were tested	One sample produced from cattle beef was being sold as “yak dried beef”	About 0.04 ng/ μ L of DNA	
Beef and water buffalo meat	Multiplex PCR	Slaughterhouse	12S	161 commercial samples of meat cuts labeled as cattle from different suppliers	Approximately 21.7% (35/161) of the samples labeled and presented as bovine meat cuts were actually water buffalo meat cuts	Cattle: 2.23 ng/ μ L; buffalo: 2.31 ng/ μ L	
Cattle and donkey	Multiplex PCR	Blood samples collected from donkey and cow	16S rDNA and NADH dehydrogenase four	10 blood samples from cow and donkey each, 18 cow–donkey blood mix	16s rDNA primer was specific for cow, NADH primer was specific for donkey	10% of mixed blood for cattle and donkey	Al-Ali & Al-Dahmoshi, 2019
Red deer, roe deer, and water deer	Multiplex PCR	Conservation Genome Resource Bank, or purchased from online and local markets	Cyt b, 12S rRNA, D-loop	Two sets of meat DNA mixtures were prepared	The detection limit of 0.1% for meat DNA mixture	Red deer, roe deer, and water deer: 0.1, 1, and 0.1 pg, respectively; 0.1% for meat DNA mixture	
Buffalo and cattle	Multiplex PCR	Abattoirs, post-mortem hall	Cyt b gene		Two discrete bands of expected amplicon size as per species were reflected	Not stated	
Wild boar	Multiplex PCR	Farms and abattoirs, research consortium, hunting events, processing plants, slaughtered animals, living animals	Whole genome	The three indel markers were tested on different pig and wild boar samples individually and then combined in a multiplex protocol for efficient daily use application	All three indels amplified efficiently and reliably with detectable products	Not stated	

(Continues)

TABLE 1 (Continued)

Target species	Main method	Sample source	Target gene	Applicability/ validity test	Outcomes	Detection limits	Reference
Buffalo, cattle, goat, and sheep	Multiplex PCR	Research institute, local open markets, and super-markets	16S ribosomal RNA, cyt b-specific	Several types of commercial meat and milk food products	Fraudulent food was not detected in the food samples tested in the present work	0.1 ng/ μ L	
Donkey, sheep, cattle, pig, and horse	Multiplex PCR	Local market	12S rRNA, 18S rRNA	The total DNA extracted from different ratios of six mixtures (mixed donkey meat into horse meat) of 90%, 50%, 25%, 10%, 5%, and 1%	Ct values and the estimate content of donkey meat as well as the recovery rate satisfy acceptable criterion	0.1% (5 pg/ μ L) for donkey, sheep, cattle, and pig, 0.01% (0.05 pg/ μ L) for horse	
Cow, buffalo, goat, sheep, chicken, duck, and pig	Multiplex PCR	Butchers, wet and super-markets	Cyt b and ND5	A total of 46 meat products were tested in this study	100% of the tested beef meatballs were mislabeled; four out of 10 beef meatballs were found positive for both beef and buffalo	0.005 ng for pig, buffalo, duck, goat, and sheep; cow and chicken: 0.01 ng	
Horse, pigeon, camel, rabbit, ostrich, beef, turkey, dog, chicken, duck, cat, and goose	Multiplex PCR	Local retailers and markets	ND5 and 6, 12S and 16S rRNAs, COI and CO II, D-loop, and cyt b	A total of 55 samples including raw and heat-processed products purchased from markets as well as online supermarket platforms	Six of 15 (40%) beef samples, three of 10 (30%) horse samples, two of 10 (20%) camel samples, three of 10 (30%) ostrich samples, and three of 10 (30%) turkey samples contained meat ingredients that were unlisted	Up to 0.05–0.1 ng	
Monkeys, dogs, rats, pigs, and cats	Multiplex PCR	Research institute or local meat markets	ND5, ATP6, cyt b	Spiked beef meatballs with each nonhalal meat; validate 115 meat samples from a local market	Fourteen samples with pork adulteration in fresh meat and ground meat, two samples in processed meat products	Monkey, rat, and pig had the lowest concentrations (0.01 ppm). Lowest DNA concentrations for cats and dogs were 0.1 and 1 ppm, respectively	

(Continues)

TABLE 1 (Continued)

Target species	Main method	Sample source	Target gene	Applicability/ validity test	Outcomes	Detection limits	Reference
Horse and rat, pork, beef, and poultry meat	Simplex PCR	Markets and imported	Cyt b	30 commercial meat samples	All samples show no bands after electrophoresis for both species targeted	Not stated	
Cattle meat, water buffalo meat, and sheep meat	Simplex PCR	Slaughterhouse and beef shop	D loop	Cooked samples and buffalo meat in beef meat mix raw samples	Differentiation between cattle, water buffalo, and sheep meat	0.5% for fresh meat and 1% in cooked meat mix	
Goat, sheep, buffalo, cattle, yak, pig, deer, and camel	Simplex PCR	Slaughterhouse and research center	Mitochondrial genome, 16S rRNA	170 collected commercial meat products were analyzed	The results showed that mutton, beef, camel, and donkey meat products were the mostly substituted	For goat, sheep, deer, buffalo, cattle, yak, and camel DNA templates, between 5 and 20 pg	
Donkey, chicken, human	Simplex PCR	Restaurants, butchers, hypermarkets, and local shops	12S rRNA and two STR loci TP0X and D18S51	40 different commercial beef meat products	Beef origin products showed a percentage mixing with another undeclared species; Kofta sample showed adulteration with chicken, donkey, and human	Not stated	
Chinese mink	Simplex PCR	Government institute, local super-markets, and farmers' markets	Cyt b	The 10 reference meat mixtures prepared for the study were analyzed	All of the commercial meat products, mixed with 10% by mass of mink meat, were presented as positive	0.1 ng/ μ L	
Beef donkey horse	Simplex PCR	Street vendors and retail markets		Testing 96 meat and meat product samples	Six of the samples were positive for donkey tissue	0.01% for minced beef meat blended by either donkey or horse meat	
Murines lab rats, lab mice, wild rat and wild mice, cattle, sheep, duck, and donkey	Simplex PCR	Farmers' markets, government institute	Cyt b	A series of four murine meat mixtures processed like ham sausage manufacture with elevated temperature	PCR products showed that murine meat products were detected in all mixtures of meat pellets	0.1 ng for murine species	

(Continues)

TABLE 1 (Continued)

Target species	Main method	Sample source	Target gene	Applicability/ validity test	Outcomes	Detection limits	Reference
Beef, chicken, and equine species	Simplex PCR, RMFT	Restaurants and super-markets		30 samples of beef meat products	Samples were found to be adulterated with chicken and equine meat	RMFT: 1%	
Deersika deer, wapiti, reindeer, Chinese water deer, samba, muntjac, Pere David's deer, and roe deer	Simplex PCR	12 published deer genomes consisting of all subfamilies and three closer nondeer species	CEP295NL	Amplification of DNA samples of Chinese water deer	Successful PCR amplification despite species-specific mutations in the gene	Not stated	
Mink	Simplex PCR	Farm and agricultural products market	Cyt b	Mink meat and mixed market pure meat sample	The developed method-identified mink meat was mixed with common edible meats at a ratio of 10%	0.5 ng/ μ L, higher than 1% in meat mixed with mink meat	
Malayan box turtle	Real-time PCR SYBR green	Wet markets, supermarkets, government institution	Cyt b, 18S rRNA	100 commercial samples of traditional Chinese herbal jelly powder, lab-made dummy products, commercial meat products	About 22% of traditional Chinese medicine (TCM) samples were found to be MBT positive; none of the commercial meat products yielded a positive amplification signal for MBT	0.001% under various matrices; LOQ 0.00001, 0.00170 $\pm$ 0.000228 $\pm$ 0.000198 $\pm$ 0.000191 $\pm$ 0.000191 $\pm$ 0.000191 DNA for the pure meat, binary mixtures, meatball, burger, and frankfurter	Asing et al. 2016a
Beef, bison, black bear, duck, emu, goat, turkey, veal, lamb, chicken, pork yak, elk, and kangaroo	Real-time PCR SYBR green, DNA barcoding	Online specialty meat distributors and retail outlets	COI	48 samples collected in this study	Nine samples contained multiple species, 39 samples containing single species	Not stated	

(Continues)

TABLE 1 (Continued)

Target species	Main method	Sample source	Target gene	Applicability/ validity test	Outcomes	Detection limits	Reference
Control meat beef, chicken, horse, turkey, and camel	Real-time PCR SYBR green	Stores	Mitochondrial DNA	Acquired sample products	DNA molecules of chicken, horse, camel, beef, and turkey meat were detected successfully in 34 commercial samples	0.2 ng; LOQ 0.1 ng of turkey DNA in a reaction	
Fox-, dog-, mink-, and rabbit- derived ingredients	Real-time PCR SYBR green	Slaughterhouse veterinary hospital	Nuclear gene	Tested on mixed and diluted DNA sample	The number and size of the electrophoretic bands were in accordance with the number and species of the added DNA mixture in the multiplex PCR analysis	Not stated	
Wild boar meat	Real-time PCR SYBR green	Local retailer, slaughter- houses, research institute, local markets	D-loop	Fifteen samples from different districts in Yogyakarta, Indonesia were evaluated using steps as in validated method	The tested commercial meatball samples did not contain WBM in their formulation	4.68 and 2.34 ng using DNA extracted from WBM and wild boar in meatball product	
Pork, cattle, buffalo, chicken, and turkey	Real-time PCR SYBR green	Not stated	18S RNA, D-loop	The developed assays were used to test raw meat admixtures at 5.0%, 1.0%, 0.5%, and 0.1% spike level	The results indicate a good agreement of the amount of DNA detected and proportion of the raw meat admixture	0.05% corre- sponding to a target species DNA template concentra- tion of 0.025 ng	
Poultry: chicken, duck, goose, wild goose and quail, horse, cattle, sheep, goat, donkey, and pig	Real-time PCR SYBR green	Markets, butcher- ies, farm, food processing enterprise	ND4, CO II, 12S rRNA, 16S rRNA, COI	Fifty-seven commercial products were collected and verified using this method	Results showed that only hams were not adulterated among all six kinds of meat products; raw meat products had highest adulteration ratio	0.01–0.1 ng of absolute LOD, and 0.1%–1% of relative LOD	

(Continues)

TABLE 1 (Continued)

Target species	Main method	Sample source	Target gene	Applicability/ validity test	Outcomes	Detection limits	Reference
Beef, pork, sheep, goat, chicken, turkey, buffalo, horse, cat, and dog	Real-time SYTO	Local markets, research and government institute	16S, cyt b	The performance of the method was evaluated on 14 meat products purchased from local markets in Belo Horizonte, Brazil	The method detected the presence of genetic material from all reported species on product labels and those species were not declared on label	2.05–10.24 pg of target DNA	
Wild boar and domestic pig meat	Real-time PCR TaqMan probe	Austria and Germany, local suppliers	Vertnin, NR6A1, intergenic chromosome 9	34 commercial meat products declared to contain wild boar and one declared to contain domestic pig	Outcome revealed that domestic pig DNA was frequently present, even in 14 out of 15 products declared to consist of 100% wild boar	Not stated	
Beef, pork, chicken, rabbit, horse, and mutton	Real-time PCR TaqMan probe	Local grocery stores, food manufacturer	Cytochrome c oxidase subunit I (cox I) gene	We tested our rapid real-time PCR protocols on a hamburger patty, chicken nugget, sausage, and hamburger steak	The meat species of patty was identified as beef. Chicken in chicken nuggets was confirmed, as well as chicken-derived egg in a hamburger steak	Approximately 0.1% of the target DNA	
Deer red, sika, fallow), roe deer, and poultry	Real-time PCR TaqMan probe	Research Institute, local super-markets, and meat markets	Lactoferrin, epidermal growth factor pseudogene, myostatin	Assay was tested by analyzing DNA extracts from targets and nontargets	Real-time and Ref assay: increase of the fluorescence signal observed. MY70 assay: amplification of the target myostatin gene in a number of mammal and poultry species	Ref assay My70: 0.1%; LOQ Ref assay My70: 0.25%	
Beef, buffalo, and pig	Real-time PCR TaqMan probe	Wet market, government institution	ND5, cyt b	12 frankfurters, 12 meatballs, and 12 burgers were procured from various Malaysian outlets and were analyzed using the tetraplex qPCR assay	71% of beef frankfurter, 100% of beef meatballs, and 85% of beef burgers were adulterated with buffalo meat	0.1% adulteration in the ternary admixes	

(Continues)

TABLE 1 (Continued)

Target species	Main method	Sample source	Target gene	Applicability/ validity test	Outcomes	Detection limits	Reference
Horse, sheep, beef, and pork	Real-time PCR TaqMan probe	Previous work	Cyclic-GMP-phosphodiesterase-beta-actin, growth hormone receptor, prolactin receptor, myostatin	16 meat samples containing one or more of the meat species for analysis were examined	Nine out of the 16 products analyzed contained undeclared horse fractions in proportions varying from as little as <1% to very high quantities like 90% for a lasagna bolognese	0.01 ng; LOQ 1% for the respective animal species	
Roe, red, fallow, and sika	Real-time PCR TaqMan probe	Research institute, supermarkets, and meat markets	Reported in previous study	Whether the same LOD and LOQ were achieved when the DNA of one of the other three species was present in surplus	For each of the target species, an asymmetric LOD of 0.1% (w/w), Ct value ≤ 37 , and an asymmetric LOQ of 0.5% (w/w) (RSD < 25%) were obtained	Concentration of 0.1% (w/w); LOQ 0.5%	
Sika deer	Real-time PCR TaqMan probe	Research institute, supermarkets, and meat markets	Kappa-casein precursor gene	Analyzing commercial meat products declared to contain sika deer meat	The sika deer-specific real-time PCR assay was found to be applicable to processed food	0.3%; LOQ 0.5%	
Red deer	Real-time PCR TaqMan probe	Research institute, local spice and meat suppliers	Protein kinase C iota gene	Analyzing model game sausages with known composition and different red deer content	DNA isolates from meat extract mixtures and DNA isolates from meat mixtures could be used as calibrators	1% (w/w) in red deer DNA, pig DNA was 0.05%; LOQ 0.4%	
Fallow deer	Real-time PCR TaqMan probe	Research institute, supermarkets, and meat markets	MC1-R gene	DNA mixtures, meat extract mixtures, meat mixtures, and model game sausages containing known contents of fallow deer were analyzed	Highest accuracy was obtained when the composition and the fallow deer concentration in the calibration mixture were similar to the composition and the fallow deer content in the sample	0.001%; LOQ 0.4%	

(Continues)

TABLE 1 (Continued)

Target species	Main method	Sample source	Target gene	Applicability/ validity test	Outcomes	Detection limits	Reference
Wild boar and pig	Real-time PCR TaqMan probe	Samples collected across Europe, research institute, local spice and meat suppliers	SNP g.299084751C>T in the NR6A1 gene, SNP rs81416363	Analysis of samples from 64 domestic pig individuals, including 14 breeds and six cross-breeds plus a total of 30 wild boar samples from five different countries	HRM was employed to verify ambiguous result	Assay chr9d 1.0% (w/w), other assays 0.2% (w/w)	
Bovines (cattle, zebu, yak, buffalo), and equines (horse, donkey, and mule)	Real-time PCR TaqMan probe	Minced meats	Lcor-universal	Four minced meat samples of beef and horsemeat with different weight ratios were assessed	The relative contents of cattle- and horse-derived ingredients were accurately detected in all samples, with good accuracy and precision	0.05 ng genomic DNA, which is approximately 18 genome copy equivalents; LOQ 5%	
Squirrel, rat, rabbit, and cat	Real-time PCR TaqMan probe	Wet market, supermarket, government institute	Cytb, ATP6 gene, 18srRNA	Total of 72 burgers and 72 frankfurters were tested	The experimental results revealed that no target species were present in the burger and frankfurter products	0.003 ng of target DNA in a 20- μ L and of 0.1% in mixed matrix	
Crocodile	Real-time PCR TaqMan probe	Wet market, supermarket, government institute	18S rRNA, cyt b	Seven different commercial crocodile food and TM products were cross-tested	All model chicken meatballs adulterated with <i>Crocodylus porosus</i> , commercial crocodile products, and TM samples revealed a range of Ct values	0.004 ng of target DNA in a 20- μ L of reaction mixture	
Chicken, turkey, guinea fowl, quail, and pheasant	Real-time PCR TaqMan probe	Grocery store, breeder farm, local stores	Cyt b, 12S rRNA	Control of poultry products	All three quantification methods tested had reasonable recovery rates	0.5% (w/w)	

(Continues)

TABLE 1 (Continued)

Target species	Main method	Sample source	Target gene	Applicability/ validity test	Outcomes	Detection limits	Reference
Donkey	Real-time PCR TaqMan probe	Research institutions and local markets	Cyt b, 18S rRNA	The meat mixtures treated under six conditions were used to confirm the applicability	Ct values of the target species in the boiled, fried, and autoclaved meat mixtures were relatively higher than in other meat mixtures	0.001 ng	
Horse, donkey, and mule/hinny	Real-time PCR TaqMan probe	Market, farm	Creatine kinase muscle MCK	158 market samples tested	In 78 heat-processed donkey meat samples and four samples were hinnies. In 60 heat-processed horse meat samples and nine samples were identified as mules	0.01 ng/ μ L for donkey, horse, and mule/hinny	
Camel and cow DNA adulterant	Real-time PCR TaqMan probe	Agricultural markets	12S ribosomal DNA	Three independent sets of dairy and meat mixtures were tested	Efficient technique for the identification of camel and cow DNA in foodstuffs	Camel milk = 0.005–0.0025 ng, camel yogurt = 0.05–0.001 ng, camel milk beverage = 0.001–0.0005 ng, and camel meat were 0.00025–0.0001 ng	
Chamois, roe, deer, pork, and beef	Real-time PCR TaqMan probe	Local suppliers	From previous work	Twenty-eight market samples of stews were analyzed	One product of declared deer meat also consisted meat of roe	0.1 ng of target DNA	
Roe deer	Real-time PCR TaqMan probe	Slaughterhouse	Myostatin, lactoferrin	DNA isolates from 12 meat samples were analyzed	Result was correct for all meat samples	Five copies of the roe deer specific target sequence per 5 μ L	

(Continues)

TABLE 1 (Continued)

Target species	Main method	Sample source	Target gene	Applicability/ validity test	Outcomes	Detection limits	Reference
Sheep/goat, fox and murine mouse, rat, and hamster	Real-time PCR TaqMan probe	Research center, local markets, farms	Lcor-universal	Mixed samples	Three different animal-derived ingredients and the reference could be detected and distinguished simultaneously	0.05 ng of target species DNA for sheep/goat-, fox-, and murine-specific sequences, and 5 pg for the internal reference	

and the elongation enzyme, Taq polymerase (Garibyan & Avashia, 2013). The target gene locus flanked by these primers synthesizes millions of copies of the specific region at a series of regulated temperatures, which are visualized at the end of the experiment using agarose gel electrophoresis, electropherograms, or other visualization methods. Simplex PCR was applied to detect rat and horse meat in 30 locally produced and imported canned and processed meat products in the Philippines after the infamous Europe horse scandal to verify whether imported meat products contained the declared species (Ramos et al., 2016). The result of the experiment showed no visible band on the gel, indicating that the meat products tested did not contain horse and rat meat.

In a proteomics-based OFFGEL electrophoresis and basic PCR comparison experiment of Naveena et al. (2017), the sensitivity of basic PCR in detecting game meat was demonstrated when percentages as low as 0.5% and 1.0% (w/w) of buffalo meat mixed with beef were detected in raw and cooked meat mixtures. The authors noted that the assay was rapid (24–36 h), and the PCR thermal cycler and associated consumables were relatively cheap and available. Xue et al. (2017) developed a novel simplex PCR assay for the simultaneous detection of eight animal species, including four wild species (deer, buffalo, yak, and camel). A universal primer was designed to target the variable regions of mitochondrial DNA of all animal species. The assay was specific (no cross-reactivity), sensitive (detection limit between 6 and 20 pg in a 20- μ PCR mixture), and commercially applicable. The simplex PCR assay was used to identify contaminated food in the research of Omran et al. (2019). Forty commercial beef products were tested and a percentage of adulteration of 72.5%, 12.5%, and 2.5% from chicken, donkey, and human, respectively, was detected.

Despite the many advantages of the simplex PCR, Naveena et al. (2017) and Ramos et al. (2016) recom-

mend the use of more sensitive techniques such as DNA sequencing for PCR product verification to avoid ambiguous products and ensure specificity. Ramos et al. (2016) also suggest RT-PCR for faster visualization of results. Xue et al. (2017) on the other hand noted that despite the effectiveness of the technique they developed, it should be used in combination with another method for optimal performance.

4.1.2 | Multiplex PCR

The mPCR technique allows the detection of more than one species in a single PCR test. This method can detect multiple species in a meat mixture by combining multiple primer sets in a single PCR test tube along with the different target DNA templates (Wang, Hang, et al., 2019). Kumbhar et al. (2020) described this assay as a promising tool for species identification in meat mixtures. Unlike simplex PCR, mPCR requires optimization of all test conditions: annealing temperature, template concentrations, and primer concentrations before the test can be performed. Particular attention is paid to the specificity and melting point of the primers to ensure specific binding and avoid cross-amplification, since the whole principle of the mPCR reaction relies on the specific binding of primers to their target template (Al-taghlubee et al., 2019). After successful optimization, the assay is then developed stepwise, starting with a simplex, duplex, triplex, and so forth (Hossain, Ali, Hamid, Asing, et al., 2017).

Taking advantage of the specificity of mPCR, Hossain, Ali, Hamid, Asing, et al. (2017) developed a duplicate gene detection assay targeting duplicate genes from cattle, buffalo, and pigs in the food chain. According to them, detecting multiple targets in a single assay is cheaper and ensures less time in the lab. However, to improve the reliability and acceptability of PCR results, product

sequencing is recommended (Ahmad Nizar et al., 2018; Hossain et al., 2019). To demonstrate the importance of primer specificity in an mPCR, Al-taghlubee et al. (2019) compared two previously published mPCR assays. The first used a universal forward primer and a specific reverse primer targeting the cytochrome b (cyt b) gene of sheep, goat, cattle, pig, and donkey, whereas the second used a species-specific forward and reverse primer targeting the following genes: cyt b, t-Glu-cytb, 12S rRNA, and COI in pig, lamb, ostrich, horse, and cattle. Cross-reactivity was observed in the first experiment, while specific amplicons for each species were found in the second experiment. The major shortcoming of the second experiment was the lack of complete amplifications, as not all amplicons were visible despite the specificity of the primers. This amplification failure of mPCR was also noted in another study by Thanakiatkrai et al. (2019). It can be safely stated that mPCR technologies, despite their limitations, have been successfully used by many authors to identify various game animals, for example, crocodile (Ahmad Nizar et al., 2018), buffalo (Mansoor Bhat et al., 2016), lamb, rabbit, and squirrel (Ahmad et al., 2017), horse and donkey (Alikord et al., 2017), yak (Zhao, Xu, et al., 2019a), and deer (Kim et al., 2020).

4.1.3 | Real-time PCR

Species identification is based on qualitative (present or absent) or quantitative (amount present) detection. RT-PCR, also known as qPCR when quantitative, is an advanced PCR technique that allows for the immediate detection of DNA product amplification while it is occurring in the reaction tubes in the thermal cycler. This real-time detection chemistry is enabled by fluorescent (light-emitting) chemical compounds that, when added to the PCR reaction mixture, can monitor amplification of target DNA. RT-PCR techniques can be classified based on the fluorescent phenomena used to detect the progress of amplification: (i) the double-stranded DNA intercalation molecules or binding dyes such as SYBR Green I, SYTO-9, and EvaGreen and (ii) the fluorophore-labeled oligonucleotides such as the hydrolysis TaqMan probes (Dalsecco et al., 2018; Meira et al., 2017). SYBR Green I, SYTO-9, and EvaGreen are simple, inexpensive, nonspecific binding, reliable fluorescent detection methods (Asing et al., 2016a; Dalsecco et al., 2018; Meira et al., 2017). TaqMan probes, on the other hand, are specific short-length amplicons carrying a reporter and a quencher dye that binds to the target DNA during amplification. The specific binding of the TaqMan probes increases the specificity of the assay (Hossain, Ali, Sultana, et al., 2017).

Quantitative detection is important for identifying game meat products when specific claims are made on the label. For example, in Austria, when a product is labeled “game,” at least 38% of the meat content must come from game species (Kaltenbrunner et al., 2020). There are two main real-time quantification methods: absolute and relative quantification (Druml et al., 2016). Absolute quantification of DNA in an unknown sample is estimated by plotting the CT (cycle threshold) value of a serially diluted or calibrated DNA obtained from a known fresh, raw, and uncontaminated meat tissue against the logarithm of the DNA concentration (Druml et al., 2016; Türkanoglu Özçelik et al., 2019). The CT value refers to the PCR cycle in which fluorescence is detected above background fluorescence (Chung, 2018). Relative quantification allows quantification of DNA from both raw and processed meat by simultaneous analysis of the unknown sample with a reference or housekeeping gene (Dolch et al., 2020). Using the relative quantification technique, Druml et al. (2016) recorded recoveries of 97%, 132%, and 112% for cooked deer sausage, deer ragout, and roasted deer, respectively. Hossain, Ali, Sultana, et al. (2017), on the other hand, achieved target recoveries of 84%–115% when analyzing sausages and meatballs made from beef, carabeef, and pork. Generally, for qPCR, the acceptance range of assay efficiency is between 90% and 105%, regression slope between 3.1 and 3.6, and R^2 value $\geq .98$. Given the great importance of RT-PCR techniques, some authors have cited some disadvantages of this method. According to Türkanoglu Özçelik et al. (2019), the detection of accumulated specific and nonspecific products limits the use of dye-based RT-PCR. Kissenkötter et al. (2020b) have noted that the qPCR method is time consuming, requires sophisticated equipment and laboratories, and can only be performed by trained personnel. Despite the sensitivity of the method, the quality, integrity, and relative quantity of DNA recovered from samples can affect the reliability of data obtained by the RT-PCR assay (Hai et al., 2020).

4.1.4 | Digital droplet PCR

A more recent improvement in RT-PCR technology is ddPCR. Invented 11 years ago, ddPCR, which is based on the water/emulsion droplet technique, has enabled researchers to achieve accurate quantification of target nucleic acid in meat samples (Yu et al., 2021). To determine copy number of target species in mixtures using this technique, a ddPCR mixture containing primers, probes, and target template DNA is added to a ddPCR system equipped with microfluidic cartridges that generate emulsions. The system divides the reaction mixture into approximately 20,000 droplets into which the target DNA sequences are

randomly interspersed. These droplets are pipetted into PCR tubes and amplified. The products of the PCR run are then discretely analyzed using dedicated quantitative software. The concentration of target DNA templates in the sample is determined as copies per microliter (Basanisi et al., 2020; Köppel et al., 2019; Naaum et al., 2018; Yu et al., 2021).

Basanisi et al. (2020) examined meat products to detect and quantify the presence of five common (beef, pork, mutton, chicken, and turkey) and one uncommon (horse) meat species. The sensitivity of the ddPCR assay for DNA concentrations of each species was 0.1 pg/ μ L for pork and chicken and 0.01 pg/ μ L for the other species. Köppel et al. (2019) used the ddPCR assay to determine the amount of beef, pork, chicken, turkey, sheep, and horse meat that could be quantified in cooked sausages. To ensure the accuracy and repeatability of the results, six other laboratories jointly performed a laboratory comparative analysis of 20 sausage samples. The average mean values obtained by all laboratories were within acceptable ranges of the reported values. Yu et al. (2021) identified and quantified meat from mink, raccoon, and fox in regular meat products. The detection and quantitation limits of their test were set at 0.1% (w/w) and 0.01% (w/w), respectively.

These authors, who have applied the ddPCR method to verify the presence of unusual meat species in meat products, praised the method for its quantitative accuracy (Basanisi et al., 2020), high repeatability (Köppel et al., 2019), inhibitor tolerance (Yu et al., 2021), and strong qualitative discriminatory ability (Naaum et al., 2018). The high cost of this instrument severely limits its use and commercial application. Furthermore, the precision required for a perfect result can only be achieved by trained professionals.

4.1.5 | Direct PCR

Direct PCR is a method that, although it has not been used in many works, is equally effective in species identification. The working principle of this technique is to save time and cost of the experiment by skipping the steps of DNA extraction and purification common to all other PCR assays and starting directly with PCR amplification. It is as simple as using a tissue sample as the target DNA template. Direct PCR can be performed as a simplex, multiple, or real-time experiment. Apart from the obvious advantages, direct PCR turned out to be as sensitive as conventional PCR and offered little to no risk of contamination or sample loss (Thanakiatkrai et al., 2019). Thanakiatkrai et al. applied this method twice (Thanakiatkrai & Kitpipit, 2017; Thanakiatkrai et al., 2019) to identify 10 species, includ-

ing three game species of interest: buffalo, horse, and ostrich. In their studies, a pre-PCR solution consisting of a small piece of the target meat sample and phosphate-buffered saline (PBS) was used as the DNA template for PCR. The assay was able to detect undeclared meat species in the commercial samples tested. In addition, the assays were 100% reproducible. Sensitivity was determined at less than 1.0 mm³ meat sample and 0.32 ng DNA template.

4.2 | Post-PCR methods

The common practice that follows conventional PCR product amplification is visualization. The most frequently used method is agarose gel electrophoresis, in which the PCR product is passed through an electrically charged gel matrix. The products are visualized as bands and their size is determined with a DNA ladder. Some studies (Ahmad Nizar et al., 2018; Kim et al., 2020; Zhao, Xu, et al., 2019a) have used a newer technique known as capillary electrophoresis. Similar to gel electrophoresis, this method works by applying electrical currents to a capillary cartridge filled with gel and individually connected to an electrode to allow accurate separation of oligos. Results are presented as both a gel image and an electropherogram. Capillary electrophoresis has been described as a sensitive, rapid, inexpensive, and efficient post-PCR imaging method (Hossain et al., 2016; Zhao, Xu, et al., 2019a). However, to combat the limitations of PCR assays, such as product ambiguity or contamination, other specific post-PCR methods have been used to efficiently verify products, as discussed below (Table 2).

4.2.1 | Polymerase chain reaction–restriction fragment length polymorphism

PCR–RFLP is a widely used technique in meat authentication studies. Products from PCR amplifications are subjected to enzymatic restriction (cutting) by specific endonucleases, resulting in a specific fingerprint in the product that is then used to identify the target species. Publicly available web domains provide online tools for researchers to perform *in silico* analyses to determine species-specific endonuclease and endonuclease sites for their targets (Ali et al., 2017). To detect Malaysian box turtle in food and traditional Chinese medicines, Asing et al. (2016b) used the PCR–RFLP technique to restrict the 120 base pair (bp) long product of the *cyt b* gene with the *Bfa*I endonuclease. According to them, the result of their RFLP assay is highly reliable and useful for legal and forensic applications. This method has also been used to genotype

TABLE 2 Summary of the post-PCR methods in the review

Target species	Main methods	Sample sources	Target genes	Applicability/ validity test	Outcome	Detection limits	Reference
Cattle buffalo and pork	PCR–RFLP double gene targets	Wet markets and supermarkets, government institution	Cyt b and ND5	Model beef and buffalo frankfurters, 0.1% autoclaved frankfurters; halal branded beef, chicken and pork frankfurters	Substitution of beef with buffalo in commercial frankfurters; chicken and pork frankfurters contained beef and buffalo	0.1% adulteration	
Wild boar and pigs	PCR–RFLP	Blood samples collected from wild boar hunters and Polish pig breeders	MCIR gene, NR6A1	Authors recommend that a larger sample of genomes is studied to verify this method	Both genes enable the discrimination of wild boar and pigs	Not stated	
Malayan box turtle	PCR–RFLP	Wet markets and supermarkets, government institution	Cyt b gene	Mixed-meat products, laboratory-prepared dummy meat products, commercial meat products, and traditional Chinese medicine (TCM) products	No MBT-specific PCR product in meat products collected from commercial sources, 40% of the tested TCM products were MBT positive	PCR products = 0.01%, LOQ: pure meat DNA = 0.0001 ng	Asing et al., 2019b
Macaques meat monkey	PCR–RFLP	Malaysian markets	D loop, 18S rRNA	Boiling, microwaving, and autoclaving of sample; in silico analysis of 11 <i>Macaca</i> species	By digesting the PCR products with AluI and CViKI-1, distinctive restriction fingerprints for macaque species were demonstrated	Detect much lower than 0.1% level of adulteration, 0.001 ng DNA	
Quail, pigeon, and chicken	PCR–RFLP	Commercial markets, research farm	12S rRNA	14 meat samples, including labeled quail and pigeon meat samples from different commercial markets, were tested	Three samples were identified as chicken in labeled quail meat and one sample was identified as chicken meat in labeled pigeon meat	It was possible to identify mixed quail chicken and pigeon–chicken meat products at all ratios tested 1:9, 2:8, 3:7, 4:6, and 5:5	

(Continues)

TABLE 2 (Continued)

Target species	Main methods	Sample sources	Target genes	Applicability/ validity test	Outcome	Detection limits	Reference
Sea snakes	PCR-RFLP	Samples and shed skins were obtained from a snake Farm	Cyt b, 12S and 16S ribosomal RNA	Blood, shed skin, raw meat, cooked meat, sea snake–fish binary admixture, and sea snake–pork binary admixture	Different cooking methods did not affect the PCR–RFLP pattern	Not stated	
Rabbit, squirrel, and rat	PCR-RFLP	Fresh muscle tissues or specimens obtained from target and nontarget species	Cyt b, ATP6, 18S RNA	Chicken and beef frankfurters were adulterated by spiking squirrel, rat, and rabbit	All commercial frankfurters did not have any rabbit, rat, and squirrel adulteration	0.1% contamination	
Wild boar meat, beef, and pork	PCR-RFLP	Wet market	Cyt b	Testing the digestion capabilities of six enzymes in differentiating between beef pork and wild boar meat	Only <i>HaeIII</i> was the only specific enzyme to differentiate between pork, Sumatran wild boar, and beef meat	Not stated	
Crocodile and chicken	PCR-RFLP	Wet market, stores	Cyt b, ATP6, 18S RNA	Model chicken meatballs deliberately spiked with crocodile meat, halal branded chicken, and 21 traditional medicine products	No commercial meatballs were positive for crocodile DNA; traditional medicine products contained crocodile	0.01% of crocodile meatball matrices	
Goat, sheep, buffalo, cattle, yak, deer, pig, and camel	PCR-RFLP	A slaughterhouse and experimental animal center	Mitochondrial DNA	15 commercial samples were screened	Two samples contained pork in addition to declared species, while others had only one species.	2.0% visibility on agarose gel. DNA detection limit of pigs was 0.001 ng. For goat, sheep, deer, buffalo cattle, yak, and camel, it was between 0.01 and 0.05 ng.	

(Continues)

TABLE 2 (Continued)

Target species	Main methods	Sample sources	Target genes	Applicability/ validity test	Outcome	Detection limits	Reference
Red deer, sika deer, roe deer, fallow deer, mouflon, wild boar, hare, and cattle	PCR-RFLP	Slaughterhouses, hunting wild animals	Cyt b	Meat and meat juice processed at high temperatures	The cooking process did not affect the RFLP pattern.	Not stated	
Camel	PCR-RFLP	Slaughterhouse, retail meat shops, local market, research center, and law enforcement agencies	Cyt b, 12S rRNA	16 unknown meat samples submitted by the law enforcement agency	Three samples out of 16 were from camel	Absolute limit of detection is 10 pg, relative LOD is 45 pg	
Dromedary, rabbit, goat, turkey, rat, donkey, and pork	PCR-RFLP	Local suppliers	Cyt b, d-loop	Identification of animal species in turkey sausage products	Turkey sausage contained both turkey and undeclared chicken meat; no dog and cat meat in the turkey sausage samples	Not stated	
Cow, buffalo, goat, sheep, chicken, duck, and porcine	PCR-RFLP	Butchers, wet markets and general stores, government institution	Cyt b and ND5 genes	50 burgers and frankfurters (beef, chicken and pork) available in the local food market	Out of 19 samples labeled as beef, 16 were found to contain buffalo DNA; chicken DNA was detected in two samples	0.5% (w/w) for adulterated boiled and autoclaved beef	
Cow, buffalo, goat, donkey, and a dog	PCR-RFLP	Slaughterhouse, government veterinary hospital	Cyt b	Mixed samples of beef buffen and chevon-mutton	Unambiguous identification of the species origin of meat	Not stated	
Beef, buffen, chevon, mutton, and pork	PCR-RFLP	Butcher shops, pig meat shop	12S rRNA gene	Tested individual meat samples as well as mixture of different meat	This digestion resulting in a pattern that could identify and differentiate each of the species employed in the study	Not stated	

(Continues)

TABLE 2 (Continued)

Target species	Main methods	Sample sources	Target genes	Applicability/ validity test	Outcome	Detection limits	Reference
Wildebeest, buffalo, impala, zebra, giraffe, topi, eland, warthog	PCR sequencing	Local sellers	Cyt B gene	A total of 151 samples were selected for molecular characterization of the wildlife species of origin	46 represented a mismatch between seller-reported and molecular species identification	Not stated	
Bushmeat species in northern Uganda	PCR sequencing	Hunters and local sellers	Cytochrome b gene, 12 s rRNA	229 bushmeat samples from 23 Ugandan communities	The overall rate of species misrepresentation among samples was 27.9%; molecular analysis showed that 25 of bushmeat samples were tissue of domesticated animals	Not stated	
Bison	PCR-SFLP; DNA barcoding	Retail outlets online	COI	45 bison product purchased from a variety of retailers	Three of the 45 samples were mislabeled.	Not stated	
Cattle, buffalo, goat, sheep, camel, and donkey	SNP-based PCR-RFLP, FINS	Local butchers and markets	12S rRNA gene	Identification of 25 commercial meat samples at the species level	Two out of 25 meat samples were mislabeled; all camel samples were correctly labeled.	Not stated	

wild boar and domestic swine (Koseniuk et al., 2021), based on SNPs in the NR6A1 and MCR1 genes of each species. The authors were able to show a clear distinction between the two species and also to identify admixture based on the PCR-RFLP test. In the macaque study (Ali et al., 2017), *AluI* and *CViKI*-1 enzymes were used to validate a 120-bp monkey-specific PCR product. The authors pointed out the ease with which the assay could be performed without expensive instrumentation. He et al. (2018) confirmed the cheapness and simplicity of the restriction fragment length assay in their work authenticating pigeon and quail meat products.

PCR-SFLP (satellite fragment length polymorphism), another variant of PCR-RFLP, was used in a study that confirmed the presence of other species in bison meat products (Scales et al., 2021). This method was used to further increase the specificity of the DNA barcoding result that identified three samples of 45 tested products as domestic cattle. PCR-SFLP identified two samples as cattle, while the last sample was identified as bison with ancestral cattle DNA. Galal-Khallaf et al. (2021) on the other hand developed an SNP-based PCR-RFLP for the successful identification of cattle, buffalo, goat, sheep, camel, and donkey meat. In this work, a universal primer adopted from a previous study was used to amplify the 12sRNA gene region of all species. The amplified products were sequenced, aligned, and analyzed for regions containing SNPs. These regions were subjected to restriction endonuclease *TasI* after thorough *in silico* assessments. Using this method to examine 25 commercial meat samples, four of five buffalo meat products were correctly identified, and all camel products (5) were found to be authentic. The authors used the FINS method, in which a phylogenetic tree is constructed from the bioinformatic analysis of publicly available reference sequences and the obtained target sequences to further confirm species identification.

Overall, the techniques from PCR-RFLP show that it is a very good test for intraspecies identification. However, it is important to recognize the specific endonuclease and cleavage sites in the products before using the restriction length techniques, which can be time consuming and labor intensive.

4.2.2 | PCR sequencing and DNA barcoding

PCR sequencing and DNA barcoding are a molecular system of meat authentication that is particularly well suited for wild meat products whose biological origin is unknown. The authors (Dell et al., 2021; Kane & Hellberg, 2016; Naaum et al., 2018; Scales et al., 2021; Schilling et al., 2020) who applied these methods to verify game meat

obtained their samples from hunters, local bushmeat vendors, grocery stores, and online stores. The product sequencing and barcoding techniques are similar, but the main difference is the target gene amplified. Kane and Hellberg (2016), Naaum et al. (2018), and Scales et al. (2021) applied DNA barcoding techniques to screen various game meat products (burger patties, ground beef, hot dogs, sausages) purchased from stores, retail outlets, and online vendors. The general working principle used by this technique involves amplification of the ~650-bp fragment of the mitochondrial gene encoding cytochrome c oxidase subunit I (COI). The PCR product is purified and bidirectionally sequenced to generate the raw product sequences, which are trimmed and edited using bioinformatics software such as MEGA and Geneious. Consensus nucleotide sequences, which are aligned with existing databases such as the Barcode of life database (BOLD) and the National Centre for Biotechnology Information (NCBI), are generated for each sample studied. The databases contain existing entries for almost all mammalian species and are therefore suitable for unknown species identification.

PCR sequencing, using the same techniques as DNA barcoding, does not rely on amplification of a single target gene for species identification. Schilling et al. (2020) amplified 480- to 490-bp fragments of the *cyt b* gene to identify 151 fresh and locally processed (smoked, dried, highly salted, etc.) bushmeat samples purchased from known middlemen in Tanzania, while Dell et al. (2021) amplified 1420 bp of *cyt b* and 212 bp of 12s rRNA in 229 bushmeat samples to understand the basis for species misidentification in local markets in Uganda. Similar to how the DNA barcoding principle works, the PCR sequencing technique purifies the amplified PCR products, sequences them, and matches the resulting nucleotides to nucleotide databases such as NCBI to enable definitive species identification. As a result of their experiment, Schilling et al. (2020) found that 46% of the samples were misidentified by the vendors. The most commonly misreported species were giraffe (47%), buffalo (36%), wildebeest (20%), and impala (20%). Dell et al. (2021), on the other hand, reported waterbuck as the most frequently identified species (31.5%) in the study. Sixty-one of the total samples belonged to species other than those reported by the vendors.

In addition to the identification of unknown species, the technique has been used by several authors (Hossain et al., 2019; Xie et al., 2021; Zhu et al., 2021) to improve the validity, consistency, and authenticity of PCR assays they have developed, while others (Dalsecco et al., 2018; Kaltenbrunner et al., 2018d; Thanakiatkrai et al., 2019) have used it to verify the authenticity of purchased meat samples for research purposes. Both DNA barcoding and sequencing are severely limited by their inability to identify more than

one species in a product containing meat mixtures (Naaum et al., 2018).

4.2.3 | Next-generation sequencing DNA metabarcoding

DNA metabarcoding using next-generation sequencing (NGS) is a significant improvement on both PCR sequencing and DNA barcoding techniques. While these two methods allow the sequencing and identification of a single species in a single assay, NGS DNA metabarcoding surpasses them by allowing the sequencing and identification of multiple species present in a single sample (Cottenet et al., 2020). Many authors consider this method to be a game changer in meat species identification, especially in the identification of species in foods whose origin is unknown (Chaora et al., 2022; Cottenet et al., 2020; Zhang et al., 2020), significantly limiting the use of species-specific identification methods to verify the presence or absence of target species. The methodology or assay pipeline of the NGS metabarcoding assay is quite comprehensive, but in simplified terms it includes three main steps: DNA extraction, NGS library preparation and sequencing, and bioinformatics data analysis.

After DNA is extracted from a meat sample, an NGS library is created by either fragmenting long strands of DNA into short strands of similar size or using PCR amplicons. A specific nucleotide sequence of 20–40 bp, called an adapter tail, is added to the fragments or amplicons at both ends. Gel electrophoresis or magnetic beads are then used to select the amplicons or fragmented DNA sizes of interest, which are then sequenced. The prepared library is then quantified using the Bioanalyzer system™ or by qPCR analysis to provide information on library concentration and fragment sizes.

The most common sequencing system is manufactured by Illumina and uses a sequence-by-synthesis (SBS) method. The sequencer contains a flow cell with oligos attached to its surface. The oligos are designed to be exactly complementary to the adapter sequences of the library. When the library is denatured and added to the flow cell, the forward strands bind to the complementary matched oligos and the reverse strands of the library sequence are completed, binding the library to the flow cell. The forward strands are discarded and the bound library forms a bridge with the next oligonucleotides. Polymerization occurs and the complex is then denatured to form single-stranded molecules. This process is repeated until all clusters are formed. Sequencing of the strand with fluorescent oligonucleotides, which are then read by a camera, is performed until all sequences and indexes are read. The read sequences are demultiplexed, that is, split into indi-

vidual samples and mapped to a known reference. The data generated (BAM or SAM) are compared to a database to identify the different species or organisms present in the sample.

Bioinformatics pipelines are developed to analyze the raw NGS data using sophisticated software and ultimately produce meaningful results. The metabarcoding technique is suitable for the analysis of processed food samples because NGS-assisted sequencing of short-length fragmented DNA can be used. Chaora et al. (2022) used this technique to sequence 186 bp of 16s mitochondrial RNA from pork, beef, sheep, chicken, turkey, goat, ostrich, duck, and kangaroo to identify mislabeled processed meat products in South African retail stores. Similarly, Cottenet et al. (2020) tested 45 ground meat products from European and Asian markets and achieved sensitivities of up to 1% (w/w). A large and comprehensive database is essential for successful application of the NGS metabarcoding technique. In addition, researchers should pay attention to good laboratory practices (Cottenet et al., 2020) to avoid sequencing extraneous DNA, as Chaora et al. (2022) observed in their study, in which an ostrich meat sample was contaminated with beef DNA due to human error in the lab.

4.3 | Unconventional PCR-based methods

In contrast to conventional PCR methods, several other efficient methods were used in conjunction with PCR to extend its applicability. Several authors who have applied these unconventional PCR-based methods to determine meat authenticity complain about the complexity, cost, time, ambiguity, and tediousness inherent in PCR testing and have therefore developed new methods that complement PCR but overcome its shortcomings (Table 3).

4.3.1 | Amplification refractory mutation system

Amplification refractory mutation system (ARMS) is a simple PCR-based method that targets and amplifies only a specific point mutation in the target organism. The strength of this method lies in its specific primer design for amplification of the mutation region. Waqas et al. (2019) used this method to detect donkey, horse, cattle, buffalo, goat, and dog by designing species-specific forward primers that were thoroughly screened to ensure that they contained species-specific variations and a single universal reverse primer that contained conserved sequences in the beta-actin (ACTB) gene for all species. Amplification of these specific mutation primers increased product

TABLE 3 Summary of unconventional PCR-based methods in the review

Target species	Main method	Sample source	Target gene	Applicability/ validity test	Outcome	Detection limit	Reference
Donkey, horse, cow, buffalo, goat, and dog	ARMS PCR	Whole blood samples collected from research institutions	ACTB gene	Analyzing five samples of each species to determine sample variation	Assay could differentiate between buffalo and cow clearly	5 ng template DNA concentration	
Horse and beef	FRET-based PCR	Commercial sources	16S rRNA gene	Detection of horsemeat in adulterated processed beef samples	Eight out of 22 samples indicate positive adulteration of horsemeat in beef samples	0.01% adulteration	
Cattle, pork, equine, sheep, goat, bison, water, buffalo, hare, rabbit, fallow deer, red deer, reindeer, roe deer, chicken, turkey, guinea fowl, Muscovy duck, mallard duck, goose, pigeon, emu, ostrich, camel, lama, giraffe, springbok, kudu, kangaroo, red fox, cat, dog, and brown rat	LCD array	Local markets and industrial suppliers. Certified reference DNAs were purchased from biotechnology companies.	16S rRNA gene	Beef and turkey powders were spiked with 1% pork and 1% chicken powders. Twenty different beef cuts were spiked with 1% horse, pork, reindeer, bison, and water buffalo. Twenty replicates of duck spiked at 1% with chicken and guinea fowl	All the adulterant species, including pork and reindeer, were successfully detected in all replicates with different signal values, varying from 50,000 to 2500.	≤1% (w/w)	
Horse, pork, beef, and sheep	LFI-PCR	Local markets or farmers.	Beta-actin gen, growth hormone receptor, prolactin receptor	Individual target test with PCR products, binary DNA mixtures	Beef-specific PCR product gave a low positive signal	0.01% of horse DNA and 0.02% of pork DNA in beef and sheep. As low as 1.6 fmol of the PCR product specific to horse. 6.2 fmol of pork and 0.4 fmol specific to beef	

(Continues)

TABLE 3 (Continued)

Target species	Main method	Sample source	Target gene	Applicability/ validity test	Outcome	Detection limit	Reference
Donkey meat	LFI-PCR	Local farm, shops, verified suppliers, research institute in Tianjin	D-loop	Binary mixtures of donkey meat and beef, raw or processed in three different ways, were tested using the developed method	No donkey meat was identified as adulterant in any tested product except for donkey burger; however, four out of 46 donkey burger samples were found free of donkey meat.	As low as 0.001% (w/w) in raw meat mixture; 0.01%, 0.01%, and 1% (w/w), deep frying, boiling, and autoclaving, respectively, in donkey–beef mixtures	
Goose meat	LFI-PCR	Government institute, local farm markets, restaurants, local grocery stores, and online retailers	12s RNA gene	Twenty-five commercial products, including raw goose meat, braised/marinated meat products, chicken roll cooked, fish meal, and animal feeds	The presence or absence of goose ingredient for all tested commercial products was in compliance with their labels.	0.01% (w/w) of goose in uncooked binary mixtures and 0.1% (w/w) in cooked binary mixtures	
Camel meat	LFI-PCR	Local markets and research institutes	COI gene	20 processed camel meat products	Two products purchased online were identified as containing no camel meat	0.1% (w/w) of camel meat in beef for both the raw and cooked samples	
Sika deer	LFI-PCR	Government institution	D-loop region	15 commercial venison products	One dried venison product did not contain any venison ingredient	0.01% (w/w) of raw and cooked venison with PCR-LFI, 0.1% with electrophoresis	
Horse	LFI-PCR	Local markets and supermarkets in Changchun, China	Cyt b gene	47 real food samples were used to test the assay	Food samples containing horse were detected; donkey, beef, and chicken were negative on the sensor	As little as 0.01% horse in the meat mixture and 1 pg of target DNA	

specificity and within-species identification. According to Waqas et al. (2019), ARMS is simple and straightforward, and the amplification result does not require post-PCR confirmation testing.

4.3.2 | Fluorescence resonance energy transfer

Fluorescence resonance energy transfer (FRET) techniques involve the emission and absorption of energy between light-sensitive molecules. TaqMan Probe PCR and molecular beacon assays are also based on FRET technology. In an FRET-based method developed by Qin et al. (2019), a self-signal-driven fluorescent colorimetric protocol was developed to distinguish horse meat from beef. Here, the authors designed a simple reverse primer and forward primer containing a loop in addition to the conventional primer sequence and a double-stranded hybridization after the loop that also has fluorescent and quencher-labeled bases. PCR amplification of this specially designed primer results in increased fluorescence detection of the equine DNA, which was also quantified. This procedure is simple and does not require a post-PCR visualization technique. Using the fluorescent colorimetric PCR protocol based on FRET, Qin et al. were able to detect horsemeat with a PCR cycle of only 15 cycles and a detection limit of 50% mixed horsemeat and beef. However, with an increased amplification of 30 cycles, a detection of 0.05% was achieved. The assay has higher performance and detection efficiency compared to conventional PCR.

4.3.3 | Meat low-cost and -density array

Low-cost and -density (LCD) array technology for meat is another PCR-based testing technique that is versatile and can be easily used to identify numerous species from a single sample. It is a simple and efficient method that is used commercially in Germany and in government laboratories in the United States for testing meat products (FSIS-USDA, 2021). In this method, as described by Cottenet et al. (2016) and FSIS-USDA (2021), a specific region between 115 and 165 bp of the vertebrate mitochondrial 16S rRNA gene is amplified with a universal primer in a conventional PCR. Cottenet et al. (2016) used the LCD array to identify an extensive list of animal species that included common meats (beef, pork, chicken), some closely related species (poultry, Bovidae), game (deer species), unusual meats (horse, fox, kangaroo), and other less conventional meats (lama, camel, kudu).

Amplicons from the assay are hybridized to a microarray chip already equipped with a species-specific capture probe for all target species. Subsequent visualization of the experimental result is facilitated by the addition of streptavidin-labeled horseradish peroxidase, which produces a visible color change (a dark precipitate) that is analyzed by imaging software. Cottenet et al. (2016) tested 20 beef cuts spiked with 1% horse, pork, reindeer, bison, and water buffalo to determine the sensitivity of the assay. All targets were successfully detected at signal levels between 50,000 and 2500, and a detection limit of <1% (w/w) was achieved. The success of the LCD meat array technique depends heavily on the hybridization step as a deviation in temperature and buffer composition can affect its sensitivity and specificity.

4.3.4 | Lateral flow immunoassays and DNA strips

PCR-based lateral flow immunoassays (LFI) and DNA strips are methods developed out of the need for cheaper, less labor-intensive, time-saving, and easy-to-use post-PCR analyses. These methods eliminate the need for laborious techniques such as gel electrophoresis and offer the advantage of rapid (2–3 min) presentation of PCR results. In the simple LFI method, primers targeting the target species region are labeled or modified with different antigens. Zhao et al. labeled their donkey-specific (Zhao, Hua, et al., 2019) and goose-specific (Zhao, Li, et al., 2020) primers with fluorescein isothiocyanate (FITC) at the 5' end for the forward sequence and with biotin for the reverse sequence. Chen et al. (2021), on the other hand, denatured their horse-specific PCR products into single strands before hybridizing them with nucleic acid probes labeled with FAM and biotin. This labeling system allows accurate binding of the amplicon on the antibodies that have been immobilized on the strip (Zhao, Hua, et al., 2019).

The strip, which is usually developed in the laboratory, consists of a plastic support to which a dip pad, a glass fiber conjugate pad, a nitrocellulose membrane, and an absorbent pad that are all overlapped are attached to ensure the flow of the sample through the strip (Magiati et al., 2019). Three or two lines of antibody are applied to the strip to serve as test line 1, 2, and control. Red lines in test lines 1 and 2 indicate the presence of target DNA, while a single red line in the control line indicates a negative result. The result is invalid if no lines are seen on the strip. In the experiments by Zhao et al. (Zhao et al., 2021; Zhao, Hu, et al., 2020; Zhao, Hua, et al., 2019; Zhao, Li, et al., 2020), testing products from different meat species (donkey, goose, camel, and sika deer) subjected to various

sensitivity and stability tests such as boiling, autoclaving, oven cooking, and frying, they found that thermal treatment weakens the color signal of the LFI strip, while frying activities have the opposite effect.

The DNA strips function similarly to the LFI strips. Denyinghot et al. (2022) developed this approach as a rapid on-site technique for detecting five nonhalal meat species (monkeys, dogs, rats, pigs, and cats) in halal-certified foods. In this method, the products of biotin-labeled primers are hybridized to a probe. The labeled amplicons bind to the antibodies fixed on the DNA strips, which allow red staining indicating the presence or absence of a target. Products were visible within 15 min, and 100% detection accuracy was found for beef meatball samples containing 10% and 1% of nonhalal targets (Denyinghot et al., 2022). With the LFI method, multiplexing or viewing results simultaneously on a single strip can be challenging; however, Denyinghot et al. (2022) were able to depict the outcome of an mPCR on a single DNA strip by varying the tagged primers on their strip.

4.4 | Non-PCR-based methods

The non-PCR-based methods for meat species identification, unlike the other methods discussed, do not require or rely on the PCR technique at any point in the assay. As mentioned earlier, PCR methods and PCR-based methods have several limitations, and researchers are seeking alternative and more effective methods for their analyses. These non-PCR-based methods allow for convenient species identification (nanopore sequencing), the use of time-saving and less expensive approaches (isothermal amplifications), and precise and highly specific techniques (locked nucleic acid [LNA] biosensors) (Table 4).

4.4.1 | Loop-mediated isothermal amplification

Loop-mediated isothermal amplification (LAMP) is a cost-effective, sensitive, and rapid method for the detection of game meat in food mixtures (Liu et al., 2017). The LAMP assay, based on isothermal nucleotide amplification technology, was developed in the 1990s as an alternative to PCR technology to simplify and accelerate the nucleic acid amplification process (Zhao et al., 2015). LAMP techniques have been little used in meat authentication research, especially for the identification of game meat species. Liu et al. (2017) used LAMP to identify raccoon meat in food mixtures. Several advantages of LAMP over conventional PCR have been noted. For instance, LAMP does not require an elaborate machine to regulate temperature because the test can be performed at a uniform temperature, so a heating

block or incubator is sufficient (Liu et al., 2017). The specificity of the LAMP assay is enhanced by the design of its four or six special loop primers that target up to six to eight regions on the DNA target templates. The primers designed by Liu et al. (2017) are four sets consisting of two inner primers (forward inner primer [FIP] and backward inner primer [BIP]) and two outer primers (F3 and B3) that recognize six different regions of the target sequence. After a short amplification time, usually about 1 h, the amplification products are distinguished by closed-tube methods such as turbidity or fluorescence or by open-tube methods such as gel electrophoresis or lateral flow (LF) dipstick. Compared to RT-PCR, the LAMP assay has equally high specificity and works excellently with direct meat samples, whereas the TaqMan probe assay is affected by inhibitors in the direct sample. LAMP is suitable for on-site screening of meat products, and its simplicity and affordability make it a good solution to meat authentication challenges in developing countries (Liu et al., 2017).

4.4.2 | Recombinase polymerase amplification

Recombinase polymerase amplification (RPA) is another isothermal nucleic acid-based amplification technology that allows rapid on-site molecular examination of meat products (Kissenkötter, Böhlken-Fascher, Forrest, et al., 2020b). Three major enzymes play a role in the RPA assay—recombinase, single-stranded DNA-binding proteins, and polymerase. The recombinase binds to primers 30–35 bp in length, targeting a specific region in the target template. The recombinase–primer complex penetrates the DNA double strand at the complementary sites of the primer sequence, resulting in the formation of an open loop at both ends. The uncomplemented strands at both ends of the target templates are held in place by the single-stranded DNA-binding proteins, which help prevent primer displacement, while the DNA polymerase works to extend the newly formed complementary strands (Kissenkötter, Böhlken-Fascher, Forrest, et al., 2020b; Zhao et al., 2015). The amplification process of RPA occurs at a temperature between 25° and 42°C (Hsu et al., 2021), and it takes only 20–40 min to generate millions of copies of a single target DNA copy (Zhao et al., 2015).

Real-time measurement of RPA amplification is possible by using an exonuclease probe that is cleaved by an exonuclease after binding to its complementary sequence, resulting in the emission of fluorescence. This technique was used by Kissenkötter et al. (2020b) in their study, which aimed to develop two RPA assays for real-time detection of mitochondrial genes from pork and horse meat to identify possible meat adulteration, and was

TABLE 4 Summary of the non-PCR-based methods in the review

Target species	Main methods	Sample sources	Target genes	Applicability/validity test	Outcome	Detection limits	Reference
Raccoon meat	LAMP, real-time PCR	Animal breeding farm and local market	Cyt b, d-loop region	Tested on lab meat mixture	As low as 0.5% of raccoon dog component could be directly detected from meat samples	LAMP assay: low as 0.1 pg/ μ L, TaqMan real-time PCR: as low as 0.01 pg/ μ L	
Pig, cattle, sheep, horse, chicken, turkey, duck, and rabbit	Nanopore sequencing	Local supermarket and standard genomic DNA provided by a biotechnology company	Whole genome	High-accuracy and the fast base calling run were analyzed to identify the time after which all species are correctly detected	The first hour read of fast base calling could identify all species in the meat mixture except chicken	Not stated	
Pork and horse DNA	RPA	Local supermarket chain	ND2, ATP 6–8 genes	Fifty milligrams of minced beef meat or beef salami was mixed with 10%, 5%, 1%, 0.5%, or 0.1% of pork or horsemeat to validate the RPA assays	Both pork and horse RPA assays can detect a contamination with meat of their target species down to 0.1% in both minced meat and salami products	LOD 95% of pork was 22 DNA molecules/reaction = 59 pg, 2 DNA molecule/reaction = 5.4 pg for horse and 0.1% of target DNA in meat mixtures	
Cow, sheep, arctic fox, and pig meat	CPA	Animal farm, hypermarket	12S ribosomal RNA, 16S ribosomal RNA genes	The double-test line test strip was tested with two or three meat-derived DNA products	Nontarget meat DNA could not affect the binding of species-specific labeled primers to the target meat CPA products	Minimum of 0.3 ng of arctic fox and pig target DNA in 20 μ L reaction mixture, 1% for minced sheep meat blended by arctic fox meat	(Continues)

TABLE 4 (Continued)

Target species	Main methods	Sample sources	Target genes	Applicability/validity test	Outcome	Detection limits	Reference
Donkey meat	LNA probe-based electrochemical biosensor	Veterinary hospital	Cyt b gene, ND2 gene, 18S RNA	Analysis of donkey adulteration in cooked sausages	The designed biosensor could detect target DNA in food products processed by vigorous pressure and heat	1% donkey meat containing 10 ng/ μ L of related DNA in consumable sausage prepared from donkey–beef dual blends. Lower LOQ: 148 pM	
Cattle, buffalo, sheep, goat, pig, and chicken	RPA–LFI	Postmortem house, experimental abattoir	Cyt b and D-loop	Ten precoded genomic DNA samples from different meat, different meat admixtures	Species-specific RPA–LF assays of cattle, buffalo, and pig could identify the species in each coded samples; the species in binary meat admixture were identified	Up to 10 pg of genomic DNA. Up to level of 1% presence of tissue of cattle, buffalo, and pig origin	
Formosan Reeves' muntjac meat	RPA–LFI	Government and research institutes, local markets	Cyt b gene	Seven common Taiwanese cooking methods including boiling, pan-frying, roasting, stewing, and stir-frying	DNA extracted from Formosan Reeves' muntjac meat gave positive results regardless of the type of meat preparations, from raw to all different cooking methods	Not stated	

TABLE 5 Summary of the DNA-based methods for wildlife conservation

Study country	Target species	Method	Purpose	Target	Reference
Ireland	Fallow deer, red deer, and sika deer	Real-time PCR	Species identification through noninvasive sampling method	ZFX and SRY gene	
Italy	Wild boar and domestic pig	Real-time PCR assays of SNP	Discrimination of wild boar, domestic pig, and admixed genotype in gene pools	MCIR and NR6A1 gene	
India	Sambar deer	PCR sequencing	Provision of information on the mtDNA genetic variability of Sambar populations across India	Mitochondria DNA	
Thailand	Elephant	Multiplex PCR	Identification of elephant species and ivory	NADH dehydrogenase 5 and cytochrome b gene	
Thailand	Elephant	Real-time PCR, melt curve analysis	Identification of elephant species and ivory	Cyt b and ND5	
Germany	Wolf, cat, and bear	SNP genotyping using microfluidic arrays	Species identification through noninvasive sampling method	c-Myc proto-oncogene, MCIR	
Serbia	Fallow, red, and roe deer	End point PCR, real-time PCR	Species identification for wildlife forensic research	12S rRNA gene	
India	Monitor lizard	Forensically informative nucleotide sequencing (FINS)	Species identification and illegal trade monitoring	Cyt b, 12S rRNA, and 16S rRNA	
USA	Steller's sea cow bone	PCR–electrophoresis	Species identification and illegal trade monitoring	Cytochrome b gene and D-loop	
China	Shark and processed shark products	Multiplex PCR, mini-barcode	Identifying processed shark products for species conservation	Whole COI and partial COI region	

(Continues)

TABLE 5 (Continued)

Study country	Target species	Method	Purpose	Target	Reference
Pakistan	Red fox, black bear, and <i>Lutra</i> spp.	DNA mini-barcoding	Identification of the source species of the highly processed and chemically treated samples	COI gene	
Thailand	Elephant	Mini-snapshot multiplex assay	Identification of elephant ivory and its processed products	COI fragments	
China	Sika deer and red deer	Fast PCR–RFLP	Authentication of deer antler velvet product	Cytochrome b region	
Hongkong	Shark	Multiplex real-time PCR, melt curve analysis	Detection of nine of 12 CITES-listed sharks in illegal trades	Internal transcribed spacer 2 (ITS2)	
Brazil	Shark	DNA barcoding	Identification of illegal shark trade and species mislabeling	COI gene	
Thailand	Asian or Malayan tapir	PCR-sequencing	Species identification	Cyt b	
Australia	Rhinoceros	Bayesian phylogenetic analyses	Forensic identification of rhinoceros horn in the illegal wildlife trade	Cytochrome-b region	
Brazil	Sharks	Multiplex PCR, DNA barcoding	Identification of shark species in illegal shark fin seizures	Nuclear ribosomal ITS2 locus, COI	
USA	Sharks	Multiplex mini-barcoding PCR	Identification of shark DNA in beauty care and pet food products	COI	
Australia	Short beaked echidna	PCR-sequencing	Determination of country of origin of echidna and use of noninvasive sampling techniques	D-loop region	
USA	Coyote	SNP genotyping by multiplexed amplicon sequencing	Affordable species identification through noninvasive sampling method	Mitochondrial 12s gene	

(Continues)

TABLE 5 (Continued)

Study country	Target species	Method	Purpose	Target	Reference
China	Cervus Antlers	Species-specific PCR	Authentication of <i>Cervus</i> antlers products	Mitochondrial complete genome	
Indonesia	Sea turtles	PCR-sequencing	Identifications of sea turtles in illegal trading	Fragment of mtDNA	
South Africa	Cheetah	Single nucleotide polymorphism (SNP) array from double-digest restriction site-associated DNA sequencing data	To monitor the legal and illegal cheetah trade in South Africa	218 SNP loci	
Mexico	Sea turtles	Mini-barcoding and real-time PCR	Identification of sea turtle in processed foods	COI	
Australia	Hog deer	STR marker genotyping	Hog deer population study	29 STR markers	
Japan	Dog, cat, rabbit, pig, goat, horse, deer, raccoon dog, monkey, cattle, chicken, sheep, and bear	Singleplex and multiplex PCR and melting curve analysis	Identification of species in crime investigations involving animals	Cytochrome b, NADH dehydrogenase 5, cytochrome c oxidase 3, ATP synthase 8	
China	Chicken, duck, sheep, pig, cattle, horse, pigeon, rat, mouse, dog, and human	PCR-STR	Species identification	22 short tandem repeat (STR) loci	
Kenya	Bovidae, Equidae, Felidae, Hominidae, Elephantidae, Rhinocerotidae, and Suidae	Real-time PCR, HRM, DNA barcoding	Vertebrate species identification for wildlife conservation	COI, 16S rRNA, cyt b	

performed on-site in a solar-powered suitcase. When validating the method, as little as 0.1% (w/w) contamination of pork and horse meat could be detected in a 50-mg minced meat and salami products with the horse and pig RPAs. Using a fluorescence reader, they were able to obtain amplification data in real time. In addition to real-time data acquisition, the results of the RPA assay can also be visualized by gel electrophoresis or an LF strip. The RPA-LF assay according to Kumar et al. (2021) involves hybridization of amplified RPA products with antibody (biotin and fluorescein isothiocyanate) labeled probes. The LF dipstick is added to the product solution, and color formation on the test and control lines is observed (Hsu et al., 2021).

The RPA technique offers numerous benefits, which some authors have noted. Kissenkötter et al. (2020b) found that meat contaminants such as myoglobin and hemoglobin had no effect on test performance when the purification step was skipped. Second, the RPA test was rapid—it could detect DNA from pigs and horses within 15 min—and portable. Hsu et al. (2021) noted that the RPA test is superior to PCR because it can amplify target DNA in a less stringent temperature range. There is also the possibility of multiplexing with an RPA assay.

4.4.3 | Cross-priming amplification

Cross-priming amplification (CPA) is an isothermal nucleic acid amplification technology in which more than one primer pair is used in an amplification reaction, one of which is a cross-primer (Xu et al., 2012). The cross-primer is a primer that does not fully complement the target at the 5' end, allowing displacement of one strand on the target template and creating another priming site at the 5' end of the generated product (Xu et al., 2012). Target isothermal amplification, facilitated by a Bst DNA polymerase with strand displacement function, occurs at a stable temperature of 63°C for 1 h (Zheng et al., 2020). The products from CPA amplifications contain an eclectic mixture of single-, double-, or partially double-stranded amplicons. Zheng et al. (2020) used this method to enable concurrent identifications of two of the studied species (cow, sheep, Arctic fox, and pork) in one test strip. For this purpose, they prepared arctic fox and pig DNA dilutions in varying proportions, and mixtures of arctic fox and sheep meat. A total of 0.3 ng of arctic fox and pig DNA was the detectable limit in a 20- μ L reaction and 1% (w/w) of minced sheep meat blended to fox meat was the detection limit for the meat mixtures. They designed five universal CPA primers and four additional species-specific labeled primers. Using a CPA test strip, they succeeded in simultaneously identifying both species in the DNA dilutions

by double labeling their detected product with biotin and FAM antibodies for one target, and biotin and digoxin for the other target. The CPA test in conjunction with a CPA test strip for product visualization is a rapid, simple, and sensitive method for the detection of game meat (Zheng et al., 2020).

4.4.4 | Nanopore sequencing

The nanopore sequencing technology invented by Oxford Nanopore Technologies plc enables real-time sequencing of long strands of DNA. The technique as described by the manufactures works by passing a double-stranded DNA loop, held in place by a tether, through a charged, electrically resistant membrane in which protein nanopores are embedded (Oxford Nanopore Technologies, 2020). Passage of the single strand through the nanopores causes a disruption in the ion current, which is recorded by the nanopore device and translated into sequences in real time. About 400 bp of sequences are read per second. The DNA double strand unwinds completely and is read/sequenced as a single strand. MinION and Flongle are portable handheld devices that allow researchers to use these devices in the field.

This technique has been used extensively for microbial sequencing but less so for meat species identification. For this reason, Kissenkötter, Böhlken-Fascher, and El Wahed (2020a) combined this technology with an offline BLAST search tool to identify horse, chicken, turkey, beef, sheep, duck, rabbit, goat, and donkey in meat products. In their research, the accuracy, sensitivity, and speed of the nanopore sequencing technology were established within the first hour of base calling. The reads generated were sufficient to identify all 10 target species in a meat mixture. However, they pointed out some limitations of the technique that could affect its widespread adoption. First, sequencing assays are generally not quantifiable. Second, the nanopore sequencing technique reads very long DNA sequences, and its applicability to processed or heat-treated samples may be limited due to DNA fragmentation. Third, it is expensive and requires the expertise of a trained staff.

4.4.5 | LNA probe-based electrochemical biosensor

Nucleic acid-based biosensors are analytical devices with oligonucleotides of known sequences interwoven in or with signal transducers used to detect specific DNA regions in a biological species (Palchetti & Mascini, 2008). When biosensors hybridize to their specific targets, they

are referred to as genosensors (Palchetti & Mascini, 2008). Because of the instability, low sensitivity, and specificity of DNA probes used as genosensors, LNAs have been developed to overcome these limitations (Mansouri et al., 2020). LNAs are synthetically produced nucleic acids that have been modified to exhibit greater stability by locking the sugar ring in its structure with a methylene bridge that prevents it from rotating back to an s conformation but instead maintains a stable and perfect n conformation (Arkitek Scientific, 2020). DNA probes containing LNAs exhibit high stability and complementary binding to their target template.

Therefore, an LNA probe-based electrochemical biosensor, when used for the detection of game meat adulteration, such as Mansouri et al. (2020) for the detection of donkey meat in beef sausages, exhibits excellent specificity, sensitivity, reliability, and accuracy (Arkitek Scientific, 2020). The LNA probe biosensor developed by Mansouri et al. (2020) was made of simple, inexpensive, and nontoxic materials. Compared with qPCR analysis for detecting donkey meat in beef, the LNA probe-based biosensor showed higher sensitivity than qPCR. Biosensor technology for species detection promises to be a cost-effective, rapid, and reliable technique. Although currently limited by the large and immobile devices, it is hoped that it can be miniaturized into a portable and table-sized device in the future (Mansouri et al., 2020).

4.4.6 | Raw Meat FlowThrough™ Test

As the name implies, the Raw Meat FlowThrough™ Test (RMFT) is a simple, rapid, and portable on-site test for the detection of raw meat adulteration (Solus Scientific, 2016). This technique is a sensitive test with a 1% detection limit and is considered highly reliable with no known “false positives” (Ahmed et al., 2020). An RMFT procedure is usually completed in less than 12 min. The principle of operation explained by Solus Scientific (2016) involves the extraction of species-specific proteins from a finely ground sample that is diluted with a specific reagent and placed in the well of the RMFT test device, which are labeled with the letters T (test) on the left side and C (control) on the right side. A pink staining reagent, which can be used to visualize the binding of the species-specific protein to the T-stain on the test, is added to the well area. Within minutes of addition, two pink spots appear next to the letters T and C on the well area of the instrument, indicating a positive result. A single pink color on the C side of the well indicates a negative color.

Ahmed et al. (2020) used the RMFT procedure to analyze eight samples of beef products suspected of containing

equine and poultry species content. The poultry RMFT successfully detected poultry meat in the sample, while the equine RMFT did not detect any equine species in the sample. The equine RMFT used by Ahmed et al. (2020) can only detect horse species, not other equids such as mules or donkeys. In conclusion, they recommended the RMFT for rapid and authentic verification of raw meat components for researchers and quality control laboratories, and also emphasized its specificity in distinguishing between horses and other equine species.

5 | PRIMER DESIGN AND SPECIFICITY CHECK

Primer design is an important step in most DNA-based methods for authenticating game meat. Poorly designed primers can cause an experiment to fail before it even begins (Waqas et al., 2019). DNA primers are a pair of 18- to 30-bp-long nucleotide sequences used in an amplification reaction along with other reagents for specific replication of a target DNA fragment or gene. Primers bind to the target template at the 3' ends and extend using DNA polymerase and deoxynucleotide triphosphates. In meat authentication studies, primers are designed for either mitochondrial genes or nuclear genes. Mitochondrial genes are maternally inherited, are highly conserved within species, and are located in the mitochondria of cells. The large number of copies of mitochondrial genes in the cell is a major reason for their wide use in studies of meat animal identification, in addition to point mutations that allow discrimination of closely related species (Nesvadbová et al., 2019). Mitochondrial genes targeted in the literature for primer designs to detect wildlife species are as follows: NADH dehydrogenase subunits 4, 5, and 6; 12S, 16S, and 18S rRNAs; COI and COII; and D-loop region, ATP synthase 6, and cyt b. The COI gene is so highly conserved among species that it has been called the barcode gene of life.

In contrast, nuclear genes are the genes located in the nucleus of a eukaryotic cell. It is known that nuclear genes have a fixed copy number in the cell, which makes them very suitable for gene quantification analysis. It is difficult for researchers to quantify the amount of mitochondrial genes in a meat sample because copy number and content vary in different tissues. Core genes that have been used in studies to quantify wild meat include cyclic GMP phosphodiesterase, beta-actin, growth hormone receptor, prolactin receptor, and myostatin (Iwobi et al., 2017); kappa-casein precursor gene (Kaltenbrunner et al., 2018c); protein kinase C iota gene (Kaltenbrunner et al., 2018b); and MC1-R gene (Kaltenbrunner et al., 2018a).

For the design of efficient primers and/or probes (universal or species specific) targeting regions of interest in mitochondrial or nuclear genes, the following conditions must be considered: (1) a gene region with little sequence variation within the target species but with dissimilarity between other nonrelated species should be selected when designing species-specific primers, whereas a region with homologous sequences for all species of interest should be selected for universal primers; (2) the melting temperature, G–C content, and sequence length should be within acceptable ranges; and (3) primer dimer and hairpin structures should be avoided, which is possible using online software tools (Klymus et al., 2020; Wang et al., 2021; Waqas et al., 2019).

Specificity testing closely follows primer design. Specificity testing ensures the efficiency of an amplification assay by ensuring that the designed primers bind correctly to the intended target. In primer design, one way to ensure product specificity is the presence of base mismatches at the 3' end of primers (Zhao, Xu, et al., 2019a). The following are some of the methods the authors used to ensure specificity in their experiments: (1) close observation of primer and product sequences using the multiple sequence alignment software—CLC genomic workbench (Druml et al., 2016); (2) use of the online tool NCBI-BLAST to access regions of similarity and dissimilarity, sequence alignment using ClustalW and MEGA software, and construction of a phylogenetic tree; and (3) in vitro laboratory testing of primers with target and nontarget species (Asing et al., 2016a; Asing et al., 2016b). Meira et al. (2017) used the NCBI primer-BLAST tool to check their primer specificity in silico; Zhao, Xu, et al. (2019a) intentionally added mismatches at the penultimate base at the 3' end of their primer to improve its specificity.

6 | LIMITS OF DETECTION AND QUANTIFICATION

Limit of detection (LOD) and limit of quantitation (LOQ) are tests used to validate assays that focus on performance parameters. LOD can be defined as the smallest amount of an analyte (target species) that can be detected in a test sample (adulterated product) (Ahmad Nizar et al., 2019). In the meat detection test, LOD, often referred to as the sensitivity test, is observed in two ways. First, the DNA of the target sample is measured with a spectrophotometer and serially diluted with nuclease-free water. The test is performed on these samples until the smallest possible diluted DNA is detected (Wang, Hang, et al., 2019). Second, a mimic of an adulterated product is prepared in the laboratory with different proportions of the adulterant or target product. Similarly, the assay is tested with these products

to determine the lowest percentage at which the target species can be detected (Wang, Hang, et al., 2019). In the sensitivity tests by Asing et al. (2016), 10-fold serial dilutions of Malaysian box turtle's DNA were detected with a real-time duplex PCR assay at 0.00001 ng dilution, while binary and autoclaved meat mixtures of the same species were detected at 0.001% (w/w) in mixtures.

The LOQ, on the other hand, describes the lowest concentration of the analyte in a reaction that can be quantified with acceptable repeatability and accuracy. Kaltenbrunner et al. (2018c) define the LOQ of RT-PCR assay as the lowest amount of the target species that can be determined with a relative standard deviation of less than 25% (FAO, 2010). Therefore, in the study on quantification of sika deer in the food matrix, the limit of quantification of qPCR analysis was determined by analyzing DNA mixtures containing 1%, 0.5%, 0.4%, 0.3%, 0.2%, 0.1%, or 0.05% (w/w) sika deer and porcine DNA before setting the LOQ value at 0.5% (w/w) (Kaltenbrunner et al., 2018c). The purpose of these tests is to eliminate the possibility of detecting false positive or negative results when using these assays (Vaithyanathan et al., 2021).

7 | APPLICABILITY/VALIDITY TEST FOR ROUTINE USE

Testing the validity and/or applicability of an analytical method is critical to its general acceptance and adoption for routine use. When using molecular techniques to authenticate meat, it is critical that the assay developed be evaluated to ensure that it conforms to laboratory specifications and implementations (Veterian Key, 2017). Validity testing of assays for identification and/or quantification of wild meat products has been performed by numerous authors in the literature reviewed. Method validation can be used to assess the quality, reliability, and consistency of analytical results (Veterian Key, 2017). When Basanisi et al. (2020) applied the ddPCR technology they had developed to test 20 commercial meat products purchased from retailers, they found that the presence of DNA traces from other species in the samples tested could be due to accidental contamination or improper hygiene in cleaning the machines used to process the various meats.

Yu et al. (2021) confirmed the presence of adulteration of fur meat with conventional meat in their test to validate 16 different regular meat samples. The absence of wild boar meat (haram) in meat products sold in three Indonesian communities was verified using the developed RT-PCR assay by Guntarti et al. (2017). Compliance of meat producers with labeling was verified in tests of Meira et al. (2017) and Derz et al. (2021). Meira et al. (2017) detected the presence of horsemeat in two declared beef products (sausage

and hamburger) out of 65 tested. Derz et al. (2021), who developed a quantitative duplex PCR assay to verify the presence of declared chamois in 20 processed meat samples, found that more than half contained no chamois DNA and only three samples contained a very small amount of chamois meat. The applicability of the assay to meat products subjected to different processing steps or treatments (chemical, thermal, etc.) was also tested. A well-designed assay can perform equally well on raw, roasted, autoclaved, or cooked meat.

Thus, Druml et al. (2016) tested their reference RT-PCR assay for relative quantification of game meat on raw and cooked deer sausage, whereas the PCR-RFLP assay developed by Asing et al. (2016b) successfully amplified Malaysian box turtle products of all adulteration levels, including 1% (w/w) and autoclaved samples. Several other authors validated their assays to test their robustness and performance in the field (Kim et al., 2020; Wang et al., 2020; Wang, Hang, et al., 2019), to verify the authenticity of wildlife products offered for sale (quality control and traceability) (Szemethy et al., 2021), to ensure the submission of a credible analytical test report for forensic or law enforcement purposes (Vaithiyanathan et al., 2021), to ensure the efficiency of the method and its suitability for daily use or routine laboratory work (Iwobi et al., 2017; Szemethy et al., 2021), and to detect wildlife trafficking (Ali et al., 2017).

8 | DNA-BASED METHODS FOR WILDLIFE CONSERVATION

Wildlife conservation means protecting wild species and their habitats to maintain equilibrium in the ecosystem. Wildlife species can be an alternative source of meat for human consumption (Domínguez et al., 2019), but an appropriate balance must be found to ensure that animals are not overhunted, exploited, or abused. Wildlife trafficking is a very rich industry and provides the right incentives for unscrupulous individuals to break the law and hunt, kill, or maim protected and/or endangered species (Fajardo et al., 2010). Therefore, forensic wildlife research enables the apprehension of criminals involved in animal poaching, illegal trade, and adulterated wildlife products (Davitkov et al., 2017). Correct identification of wildlife species facilitates differentiation between legal and illegal wildlife products (Crerar et al., 2017).

Deer antlers, elephant ivory, shark fins, rhino horns, sea turtles, and cheetahs (Cardeñosa et al., 2017; Ewart et al., 2018; Jiang et al., 2018; Kitpipit et al., 2016; Magliolo et al., 2021; Pertiwi et al., 2020) are some animals and animal products illegally exploited in the literature reviewed. Samples for molecular analysis in wildlife preservation

testing differ slightly from the authentication of game meat products. Forensic scientists always attempt to collect non-invasive samples such as fecal pellets, hair, and so forth in these investigations. Powell et al. (2019) identified three deer species and their sexes with noninvasively collected tissues and fecal pellets. Muscle and blood samples were used in a genotyping test with multiple marker systems to distinguish 83 unrelated pigs, using a group of 164 wild pigs as the reference population in the study of Lorenzini et al. (2020). To protect the nearly extant three elephant species, Kitpipit et al. (2016) collected 113 ivory and blood samples from the forensic department of the Department of National Parks, Wildlife and Plant Conservation in Thailand. In an attempt to combat the illegal sale of shark fins and other shark byproducts, Cardeñosa et al. (2017) used a mini-barcoding PCR test to identify 200 processed samples of dried fins of unknown species from the Guangzhou dried seafood market in China.

In forensic research, the choice of DNA-based method for identifying wild species is highly dependent on the strength of the assay. The suitability of quantitative PCR for wildlife research was discussed by Powell et al. (2019) in their study, in which they found that quantitative RT-PCR requires minimal sample input and can analyze low-quality samples and no post-PCR analysis ensures that samples are free of contaminants. Muangkram et al. (2018) identified Asian tapir by sequencing a PCR-amplified 199-bp fragment of the cytochrome gene using the NCBI BLAST tool. They describe the test as a reliable and sensitive molecular assay that can be used to analyze low-quality DNA material, which may be useful in investigating wildlife crime in endangered species. The multiple PCR used by Kitpipit et al. (2016) to identify three elephant species based on their species-specific nucleotide polymorphisms was praised because it clearly distinguishes DNA from mixed elephant species, is sensitive, and provides easily interpretable results. DNA barcoding and mini-barcoding are techniques based on amplification and sequencing of the ~650-bp region of the mitochondrial COI region of a target species. Almerón-Souza et al. (2018) addressed a serious case of species mislabeling and the inadvertent trade and consumption of endangered shark species in Brazil. They examined 63 elasmobranchs traded in the local market and found that some specimens were on the national and global endangered species lists. They attribute the success of the study to the use of the barcoding technique COI DNA. Similarly, Cardeñosa et al. (2017) applied mini-barcoding to identify shark species and byproducts in processed products such as cosmetics and soups. Mini barcoding, in which a smaller fragment of the COI region is selected, was used because long DNA strands may be broken in highly processed target samples (Table 5).

Wildlife forensics has many challenges and limitations that complicate wildlife conservation. Evidence of criminal activity typically involves poor, low-quality material such as dried blood, hair, feces, dried bones, or processed products, making it difficult to identify exploited animals through simple, low-cost, low-precision testing. The lack of government initiatives to conserve some exotic species until they are overexploited and threatened with extinction contributes to the low sample sizes available for analysis and conservation efforts (Suntrarachun et al., 2018).

9 | CONCLUSION

Many authors in this review emphasized the nutritional and exotic qualities of game meat that make it very attractive to consumers, while others pointed out the importance of studying these species because they must be avoided for health reasons, for cultural and religious taboos, or to preserve wild species. There are several DNA-based methods that have been used to identify target species in food matrices. All these methods have their strengths and weaknesses. However, the choice of the ideal method for identification or quantification of target species in food is made by considering various factors such as sensitivity, specificity, accuracy, discriminatory power, reproducibility, cost, speed, availability of equipment, and availability of appropriately trained personnel (Izadpanah et al., 2018; Xue et al., 2017). Gene selection is an equally important factor in DNA-based tests for meat authentication. The authors spend time and resources to ensure proper selection of gene regions, and they also mention the characteristics that make them perfect for the assay. In almost all of the work, genes with high genetic variation between genera and low variation within species were selected as target genes for primer design. All designed assays were tested to ensure repeatability, reproducibility, and applicability of the assay. Many authors emphasized that this makes the assay acceptable and reliable. In addition, the results of the analysis can be reliably used for law enforcement purposes. In the wake of the European horsemeat scandal, consumers are more interested than ever in how the food they consume is authenticated. To maintain consumer confidence, many researchers have developed a variety of methods, ranging from complex, high-throughput procedures that require professional input to simple, inexpensive, on-site methods for verifying wild meat products. The DNA-based methods discussed in this review have demonstrated their effectiveness in combating the problem of some fraudulent meat producers and in detecting cases of unintentional contamination. Furthermore, the DNA-based methods for wildlife conservation highlighted in the review have demonstrated

their sensitivity and effectiveness in identifying overexploited and illegally traded wildlife species. Although some of these methods have limitations, such as high cost, complicated procedures, or limited applications, DNA-based methods for identification and quantification of target species still have the best potential to become the first choice for all meat origin molecular testing.

AUTHOR CONTRIBUTIONS

Bukola M. Adenuga: Writing—original draft; methodology; investigation; visualization. **Magdalena Montowska:** Conceptualization; funding acquisition; visualization; supervision; writing—review and editing; project administration.

ACKNOWLEDGMENTS

This work was supported by the Narodowe Centrum Nauki, Poland, project number 2020/37/B/NZ9/00082. Publication was co-financed within the framework of the Polish Ministry of Science and Higher Education's program: "Regional Excellence Initiative" in the years 2019–2023 (No. 005/RID/2018/19), financing amount 12 000 000,00 PLN.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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How to cite this article: Adenuga, B. M., & Montowska, M. (2023). A systematic review of DNA-based methods in authentication of game and less common meat species. *Comprehensive Reviews in Food Science and Food Safety*, 1–49. <https://doi.org/10.1111/1541-4337.13142>

Publication 3 (P3)

Adenuga, B. M., Biltres, R., Villa, C., Costa, J., Spychaj, A., Montowska, M., Mafra, I. (2025). Unravelling red deer (*Cervus elaphus*) meat adulteration in gourmet foods by quantitative real-time PCR. *Food Control*, 168, 110872.

<https://doi.org/10.1016/j.foodcont.2024.110872>



Unravelling red deer (*Cervus elaphus*) meat adulteration in gourmet foods by quantitative real-time PCR

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ARTICLE INFO

Keywords:

Food authentication

Red deer quantification

Cervus elaphus, meat products

Nuclear marker

ABSTRACT

Red deer (*Cervus elaphus*) meat is highly regarded as an exclusive gourmet food due to its sensory quality, nutritional value and premium price, thus being susceptible to adulteration practices by its substitution with inferior meat species. This study proposes a novel real-time PCR method targeting the troponin I gene for detecting and quantifying red deer meat in processed food products. The assay showed acceptable performance parameters, achieving absolute and relative limits of quantification of 0.01 ng of DNA and 0.5% (w/w) of red deer meat in both raw and thermally processed pâté. The assay parameters, using raw and autoclaved reference mixtures, regarding PCR efficiency (89.3% and 100.0%, respectively), slope of the calibration curve (−3.6067 and −3.3224, respectively) and R^2 (0.9889 and 0.9893, respectively) highlight the high performance of the calibration models for the determination of red deer meat. The method was successfully validated using blind mixtures and applied to 46 commercial meat products from Poland, Portugal and Spain. Data suggested the adulteration of 48% of the samples by the total or partial substitution of red deer species and the suitability of the method for the routine testing of game meat products in quality control laboratories.

1. Introduction

Gourmet food, characterised by its high quality, luxury and sophistication, entails the meticulous crafting of cuisine to showcase distinctive richness in aroma, flavour and taste. Red deer (*Cervus elaphus*) meat, among other game meats, is highly regarded as an exclusive gourmet item due to its rarity in the conventional meat market (Wiklund, Farouk, & Finstad, 2014). This exclusivity is underpinned by the high level of skills and creativity invested in its preparation. Researchers have consistently validated the sensory qualities of red deer meat, highlighting its nutritional value, including low-fat content, high protein, minerals and water-soluble nitrogen (Daszkiewicz et al., 2009; Milczarek et al., 2021). Additionally, red deer meat exhibits a low heavy metal content compared to other game species, making it relatively safe for human consumption (Milczarek et al., 2021).

The red deer holds significant economic importance in forest management in Poland and many other European countries, serving as a valuable game animal for venison production and a resource for the food industry. According to a study by Demartini et al. (2018), consumers

express a willingness to pay 12% more for red deer meat compared to beef. However, despite its premium price, challenges in the gourmet market for red deer meat stem from instances of adulteration, substitution of products with inferior species, or the undisclosed presence of certain species. These issues raise concerns for both consumers and the industry regarding product quality (Czarniecka-Skubina et al., 2022). The large difference in the price of raw meat may be one of the main causes of unfair behaviour and meat adulteration. For example, in Poland, the retail price of deer loin in 2024 is 5 times higher than pork loin and twice as expensive as beef steak (~34 € vs. ~7 € and ~16 €, respectively). As a response, it has become imperative to verify and confirm the authenticity of red deer gourmet products.

DNA-based methods provide a reliable means of verifying the species of origin in meat products, commonly employing the polymerase chain reaction (PCR) (Adenuga & Montowska, 2023; Amaral et al., 2016). PCR-based methods have been employed for red deer meat detection as species-specific PCR assays (Amaral et al., 2014; Rak et al., 2014), PCR with restriction fragment length polymorphisms (Fajardo et al., 2006; Nesvadbová et al., 2019) and multiplex PCR with capillary

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<https://doi.org/10.1016/j.foodcont.2024.110872>

Received 10 July 2024; Received in revised form 29 August 2024; Accepted 11 September 2024

Available online 12 September 2024

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electrophoresis (Kim et al., 2020). While these methods detect the presence of red deer in meat products, their qualitative approach falls short in accurately quantifying red deer meat, especially in complex matrices and highly processed products. Given that most red deer products consist of a mixture of species in proportions specified by producers, confirming not only the presence, but also the specified quantity of species is crucial. In this regard, real-time PCR stands up as the technique of choice in many laboratories for diagnostic tests and food analysis owing to its aptitude for quantification. Several applications in the authentication of meat products have been published (Amaral et al., 2017; Kaltenbrunner et al., 2018a; 2018c; Köppel et al., 2020; Li et al., 2023; Meira et al., 2017; Soares et al., 2013), including red deer products (Druml, Grandits, et al., 2015; 2015b, 2016; Fajardo et al., 2008; Kaltenbrunner et al., 2018b; Köppel et al., 2020; Liu et al., 2023). Fajardo et al. (2008) proposed a SYBR Green real-time PCR approach combining the use of cervid-specific primers targeting the mitochondrial 12S rRNA gene to detect red deer, fallow deer and roe deer. Druml et al. (2015a, 2015b, 2016) were able to detect and quantify deer species (red deer, fallow deer and sika deer) in model meat mixtures targeting the epidermal growth factor pseudogene (Druml et al., 2015a; 2015b) and the myostatin gene (Druml et al., 2016) by real-time PCR with specific probes. Afterwards, the same research group proposed a red deer specific real-time PCR assay targeting the protein kinase C ι gene, allowing to quantify red deer in model game sausages, but without any application to commercial samples (Kaltenbrunner et al., 2018b). Köppel et al. (2020) proposed a multiplex real-time PCR assay to detect and quantify DNA from chamois, roe deer, deer (including fallow deer, red deer and sika deer), pork and beef, targeting the same gene as Druml et al. (2015a), but without specifically identifying red deer. Recently, Liu et al. (2023) developed a multiplex real-time PCR method for the simultaneous detection of sika deer and red deer targeting the mitochondrial NADH dehydrogenase subunit 5 gene, but without validation and application data.

Distinguishing itself from existing approaches, the proposed quantitative real-time PCR assay targets the skeletal muscle troponin I nuclear gene for the first time to detect and quantify red deer (*C. elaphus*) in highly processed foods. By leveraging nuclear marker-based PCR, our assay seeks to enhance specificity and accuracy in the identification and quantification of red deer meat, addressing potential limitations observed in previous studies focused on different geographical regions and species compositions. The design of the novel method intends the construction of calibration models accounting with matrix and processing effects. Method validation involves testing the assay with blind samples and further analysis and quantification of commercially available products from three European countries (Poland, Portugal and Spain) containing varying compositions of the target species. This proposed method aims at expanding analytical possibilities and position our assay as a new valuable tool in combating adulteration and ensuring the authenticity of red deer gourmet products in the market.

2. Materials and methods

2.1. Samples

In order to evaluate the specificity of the developed method, meat samples of red deer and 16 other non-target animal species (roe deer, fallow deer, cattle, rabbit, pork, pigeon, duck, guinea fowl, quail, pheasant, chicken, turkey, lamb, wild boar, horse and goat) were tested. In addition, 20 plant species (soybean, pine nut, wheat, corn, rosemary, onion, garlic, parsley, bay leaf, chilli, mustard, coriander, sage, fennel, vine, olive, ginger, oregano, white pepper and sweet chilli) were cross-tested owing to their frequent occurrence in food products. Game meats and plant species were obtained at local stores in Poland and Portugal, including a shop at the Polish Hunting Association, Poznań District.

A total of 46 different commercial samples of meat products acquired at local markets in Poland and Portugal and online stores from Spain

were evaluated in this study. The samples included sausages, pâtés, canned meat and hams, mostly labelled as containing red deer in varying proportions with other meat and/or plant species. All commercial products were homogenised separately in a laboratory knife mill Grindomix GM200 (Retsch, Haan, Germany), using different materials and blender containers, previously treated with DNA decontamination solution to avoid contaminations and stored at $-20\text{ }^{\circ}\text{C}$ until DNA extraction.

2.2. Preparation of model mixtures

Model mixtures to simulate pâté were prepared in the laboratory with red deer (loin) and pork (loin and neck and liver) and spices acquired at local retail markets in Portugal. Immediately after purchase, all meats were cut and the outside portions were rejected. A blank mixture containing pork loin, neck, and liver at identical proportions was prepared and minced in a kitchen robot Bimby® (Vorwerk, Wuppertal, Germany) with the addition of 18 g of salt, pepper, rosemary, onion, coriander, garlic and ginger in order to simulate the pâté preparation. Afterwards, binary mixtures containing known proportions of red deer meat (0.01%, 0.05%, 0.1%, 0.5%, 1%, 5%, 10%, 50% (w/w) in pork were prepared. The first mixture containing 50% of red deer meat was prepared by adding 300 g of minced red deer meat to 300 g of the pork blank mixture in a kitchen robot Bimby® (Vorwerk, Wuppertal, Germany). The subsequent mixtures were prepared in a laboratory knife mill (Grindomix GM200, Retsch, Haan, Germany) as a serial dilution by successive additions of the pork blank mixture until the proportion of 0.01% red deer meat. Each model mixture was divided in two portions. One portion was immediately stored at $-20\text{ }^{\circ}\text{C}$, while a second portion was autoclaved at $115\text{ }^{\circ}\text{C}$ for 30 min to simulate the preparation of industrial canned/jarred pâté. All mixtures were homogenised separately, and all materials and different containers were previously treated with DNA decontamination solution. All raw and autoclaved mixtures were immediately stored at $-20\text{ }^{\circ}\text{C}$.

For method validation, two sets (raw and autoclaved) of blind model mixtures, containing 3%, 15%, 25% and 30% (w/w) of red deer meat in pork, were prepared similarly to the reference mixtures.

2.3. DNA isolation

The NucleoSpin food kit (Macherey-Nagel, Düren, Germany) was used to extract the DNA from model mixtures and commercial samples, according to the manufacturer's instructions, with minor modifications consisting in the addition of $2\text{ }\mu\text{L}$ of RNase (2 mg/mL) after the cell lysis step. The extractions were performed at least in duplicate assays using 200 mg of each sample. The extracts were kept at $-20\text{ }^{\circ}\text{C}$ until further analysis.

To evaluate the yield and purity of DNA extracts, UV spectrophotometric DNA quantification was performed on an absorbance microplate reader (SPECTROstar Nano, BMG Labtech, Ortenberg, Germany) using an LVis plate accessory of reduced volume (BMG Labtech, Ortenberg, Germany). The DNA content was assessed by the nucleic acid quantification protocol with the sample type defined for double-strand DNA and the absorbance data were evaluated with MARS data analysis software (BMG Labtech, Ortenberg, Germany).

2.4. Primers and probe design

Species-specific primers were designed based on a previous selection of nuclear genes with abundant nucleotide sequences for the specific and non-specific targets. Gene sequences were downloaded from the NCBI (National Center for Biotechnology Information) Genbank database into the Geneious Prime software (version 2023.2.1) where they were further examined for regions with highest amount of nucleotide dissimilarities among non-related species and high homology within the target species. Candidate sequences were input in the PrimerBlast tool of the NCBI page

(https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?GROUP_TARGET=on) to design primer pairs. Primers were designed in gene sequences coding for the troponin I, lactoferrin and endoglin. The nucleotide sequences were submitted to a basic local alignment search tool BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), which identifies regions of local similarity among homologue sequences of different species and calculates the statistical significance of the matches. Primer specificity was assessed using the Primer-BLAST tool that allows revealing homologies in relation to all sequences available at the database (Genbank). Primer properties and the absence of hairpins and self-hybridisation were assessed using the software Oligo calc (<http://www.basic.northwestern.edu/biotools/oligocalc.html>).

Primers targeting the lactoferrin and endoglin genes showed cross-reactivity with several species and with roe deer, respectively, being, therefore, discarded. From two primer pairs in the troponin gene, only one (TNNI2-F/TNNI2-R) exhibited minor cross-reactivity, being then designed a hydrolysis probe (TNNI2-P) using the Integrated DNA technologies tool Primer Quest (<https://eu.idtdna.com/PrimerQuest/Home/Index>). To assess the amplifiability of all DNA extracts, universal primers targeting a conserved eukaryotic nuclear 18S rRNA gene fragment were used (Costa et al., 2013). The primers and probe were synthesised by Eurofins MWG Operon (Ebersberg, Germany). The primers and probe sequences are presented in Table 1.

2.5. Qualitative PCR

PCR amplification was carried out in 25 µL of total reaction volume containing 2 µL of DNA extract (100 ng), ultrapure water (Sigma-Aldrich, Steinheim, Germany), 1 × PCR buffer E (67 mM Tris-HCL (pH 8.8), 16 mM (NH₄)₂SO₄, 0.01% Tween 20; Genaxxon Bioscience GmbH, Germany), 3.0 mM or 1.5 mM (TNNI2-F/TNNI2-R or 18SRG-F/18SRG-R, respectively) of MgCl₂, 200 µM of each dNTP (Bioron, Ludwigshafen, Germany), 1 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience GmbH, Germany) and 200 nM of each primer (Table 1). The reactions were performed in a thermal cycler MJ Mini (Bio-Rad, Hercules, CA, USA) using the following program: initial denaturation at 95 °C for 5 min; 40 or 33 cycles (TNNI2-F/TNNI2-R or 18SRG-F/18SRG-R, respectively) at 95 °C for 30 s, 59 °C or 65 °C (TNNI2-F/TNNI2-R or 18SRG-F/18SRG-R, respectively) for 30 s and 72 °C for 30 s; and a final extension at 72 °C for 5 min.

The amplified fragments were analysed by electrophoresis in a 2.0% agarose gel containing 1 × Gel Red (Biotium, CA, USA) for staining and carried out in 1 × SGTB buffer (GRISP, Porto, Portugal) for about 25–30 min at 140 V. The agarose gel was visualised under a UV light tray Gel Doc™ EZ System (Bio-Rad Laboratories, Hercules, CA, USA) and a digital image was obtained with Image Lab software version 5.2.1 (Bio-Rad Laboratories, Hercules, CA, USA).

2.6. Real-time PCR

For the real-time PCR amplifications, the reaction mixture of 20 µL included 1 × SsoFast Probes Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 240 nM of each primer (TNNI2-F/TNNI2-R), 160 nM of probe (TNNI2-P) (Table 1) and 2 µL of DNA extract (100 ng). The reactions were carried out in a fluorometric thermal cycler CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) with the

following conditions: 95 °C for 5 min, 50 cycles at 95 °C for 10 s, 59 °C for 10 s, and 72 °C for 30 s, with collection of fluorescence signal at the end of each cycle. The software Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories, Hercules, CA, USA) was used to evaluate the data from each real-time PCR run. Cycle of quantification (Cq), also known as cycle threshold (Ct), values were calculated using the software at user-defined threshold settings. Real-time PCR trials were repeated in three independent runs using $n = 4$ replicates in each one.

The MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines, as outlined by Bustin et al. (2009), and the criteria set by the European Network of GMO Laboratories, as detailed in the "Definition of minimum performance requirements for analytical methods of GMO testing" (ENGL, 2015) document, were followed for real-time PCR method development and validation. These criteria include demonstrating specificity exclusively towards the designated target species. The dynamic range should cover a minimum of four orders of magnitude, with a correlation coefficient (R^2) ≥ 0.98 . Acceptable PCR efficiency falls within the range of 90%–110%, with the slope ranging between -3.6 and -3.1 . The limit of detection (LOD) signifies the lowest amount or concentration of the analyte in a sample, detectable with a 95% confidence level, ensuring a maximum of 5% false-negative results. The limit of quantification (LOQ) should be equal to or lower than the minimum amount or concentration covered by the dynamic range (Bustin et al., 2009; ENGL, 2015).

2.7. Statistical analysis

GraphPad Prism version 8.0.2 software (GraphPad Software, San Diego, CA) was used for statistical analysis. Data following normal distribution, according to Shapiro-Wilk test, were further processed with one sample t -test, while data with a non-normal distribution were analysed by a non-parametric Mann-Whitney test. The significance of differences as evaluated for the Cq values of raw and processed reference mixtures at the same incurred level. Significant statistical differences were considered when $p < 0.05$.

3. Results and discussion

3.1. Primer specificity and sensitivity

In the present work, the selected primers targeted nuclear genes due to their fixed copy number in different cells and tissues, providing greater accuracy in species quantification compared to mitochondrial DNA (mtDNA), which exhibits varying copy numbers. The designed new primers targeting the troponin I gene were firstly confirmed to be red deer specific by *in silico* analysis, using the primer blast tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>), which did not identify similarities with non-target species. Following the optimisation of PCR conditions, the new primers were tested for their specificity for red deer using 16 non-target animal species, encompassing poultry birds, game birds, common and uncommon mammal species, other game meats, and 20 plants and spices commonly found in meat preparation. The PCR results revealed no reactivity with any tested species, except for the intended target red deer, roe deer (*Capreolus capreolus*) and fallow deer (*Dama dama*) (Table S1, supplementary material). To ensure the absence of any false negative result, the amplifiability of DNA

Table 1
Primers and probe used in this study.

Species	Target Gene	Primer	Sequence (5'-3')	Amplicon	NCBI accession/Reference
<i>Cervus elaphus</i>	Troponin I	TNNI2-F	AGCAGAACTCTTCCGGGTTG	76 bp	OU343091.1
		TNNI2-R	TGTCACGGCTTATGGTCCAC		
		TNNI2-P	FAM-AGAGTCTGTCTGTGCTGGTGAATGG-BHQ1		
Eukaryotes	18S rRNA	18SRG-F	CTGCCCTATCAACTTTCGATGGTA	113 bp	Costa et al. (2013)
		18SRG-R	TTGGATGTGGTAGCCGTTTCTCA		

extracts from each species was confirmed with universal primers (18SRG-F/18SRG-R), targeting a sequence of the nuclear 18S rRNA gene (Costa et al., 2013) (Table S1, supplementary material).

The sensitivity of the red deer-specific PCR assay was firstly evaluated utilising a 10-fold serial dilution of a red deer DNA extract, achieving a detectability of 0.01 ng (Fig. S1, supplementary material). This result is lower than the obtained in a PCR assay targeting a nuclear gene for meat species detection (0.05 ng of beef DNA) (Wang et al., 2019) and another assay targeting mtDNA (D-loop) for red deer detection (0.5 ng of red deer DNA). However, it did not reach the sensitivity of a cytochrome *b* gene assay (0.001 ng of red deer DNA) (Kim et al., 2020), which is expectable since it is a multicopy gene. Afterwards, the reference model mixtures simulating pâté with known proportions of red deer in pork were used raw and autoclaved to evaluate the relative sensitivity before and after thermal processing. The optimised PCR results reached a detectability of 0.01% of red deer in pâté mixtures, both raw and thermally treated (Figs. S2A and S2B, supplementary material). The obtained sensitivity is improved comparing with our previous report targeting the 12S rRNA gene that reached 0.1% of red deer in pork meat (Amaral et al., 2014). A lower detection (0.001% red deer in beef) was obtained by Rak et al. (2014) targeting the D-loop region in raw mixtures. However, in thermally processed mixtures, the obtained sensitivity was better than the reported by Rak et al. (2014) for red deer in beef treated for 40 min at 100 °C or 20 min at 121 °C (0.5% for both treatments). It is important to note that the assays of Amaral et al. (2014) and Rak et al. (2014) targeted mtDNA that possess multiple gene copy numbers per cell, allowing for much lower detection limits.

3.2. Real-time PCR method development

Following the development of a species-specific PCR assay to detect red deer DNA and considering the reactivity observed with fallow deer and roe deer, a hydrolysis probe was designed for real-time PCR maximising the specificity of the assay for red deer. For the same initial amount of DNA, the real-time PCR results showed high amplification delays (7.2 and 9.3 cycles) in relation to red deer (intended target), suggesting a negligible reactivity (>33 cycles) or practically no reactivity (>35 cycles) for fallow deer and roe deer, respectively (Fig. S3, supplementary material). These findings are justified by the two and three nucleotide mismatches in the probe region for fallow deer and roe deer, respectively, in addition to other mismatches in the primers for roe deer (Fig. S4, supplementary material). Difficulties in identifying a specific nuclear DNA marker for red deer were reported by other authors owing to the limited number of sequences deposited in the NCBI database and the high sequence similarity within deer species (Druml et al., 2015a; Kaltenbrunner et al., 2018b).

The absolute LOD and dynamic range of the real-time PCR assay were determined with the amplification of 10-fold serially diluted red deer DNA extracts, covering 7 concentration levels from 50 ng/μL to 0.00005 ng/μL. Fig. 1 displays the amplification curves (Fig. 1A) and respective calibration curve (Fig. 1B) of one real-time PCR example run. The absolute LOD reached the concentration of 0.005 ng/μL (0.01 ng) with a level of confidence above 95% since all 8 replicates from two real-time PCR runs were amplified. The absolute LOQ is also 0.005 ng/μL as it is the lowest level within the linearity of the calibration curve (ENGL, 2015). The dynamic range covered 4 magnitude orders of the target analyte, complying with the minimal requirements for real-time PCR assays (Bustin et al., 2009; ENGL, 2015). The obtained real-time PCR parameters of slope (−3.424), PCR efficiency (103.8%) and R^2 (0.995) were considered all acceptable for this type of assays (Bustin et al., 2009; ENGL, 2015).

The obtained sensitivity is similar to the reported by Kaltenbrunner et al. (2018b), who achieved 3.6 pg/μL of red deer DNA by real-time PCR targeting a nuclear gene. Liu et al. (2023) reached a much lower LOD (0.25–0.5 pg of red deer DNA) but targeting a mitochondrial gene (ND5).

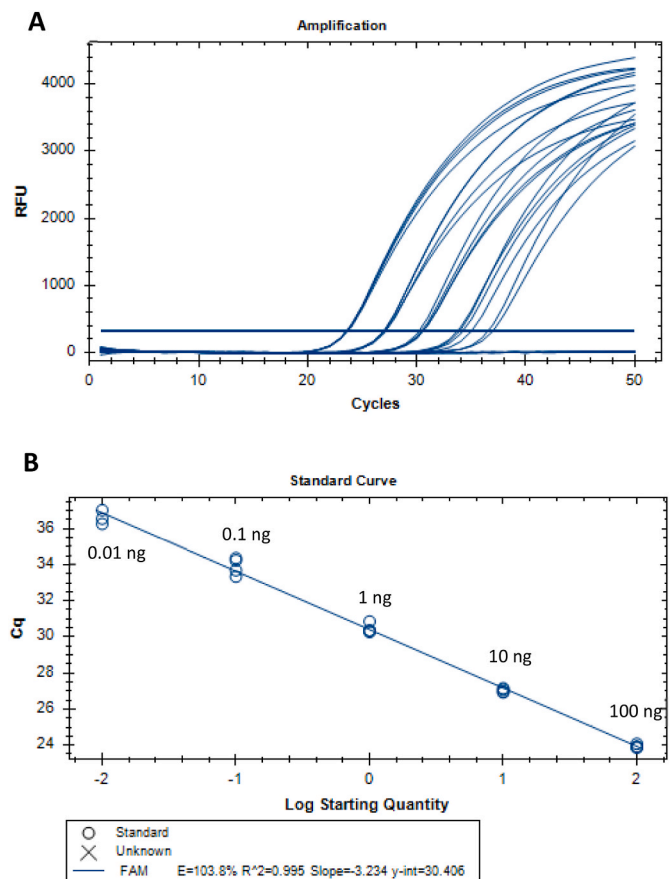


Fig. 1. Amplification and standard curves obtained by real-time PCR with a TaqMan probe targeting the troponin I gene using 10-fold serially diluted red deer DNA from 50 ng/μL to 0.00005 ng/μL ($n = 4$ replicates).

3.3. Construction of calibration curve

The DNA extracts of raw and autoclaved reference model mixtures, encompassing 8 concentration levels (0.01%, 0.05%, 0.1%, 0.5%, 1%, 5%, 10%, 50% w/w) of red deer meat in simulated pâté were amplified by real-time PCR for the construction of calibration models. Table 2 shows the real-time PCR results, successfully amplifying red deer meat down to 0.05% (w/w) of both raw and autoclaved mixtures, in all 12 replicates (Table 2). However, considering the cross-reactivity of fallow deer at 33.78 Cq using the same DNA extract concentration (50 ng/μL) as the reference mixtures (Fig. S3, supplementary material), the relative LOD was set at a level below that value, which is 0.5%. Accordingly, the calibration curves were set up in the dynamic range of 50–0.5%, covering four orders of magnitude for raw (Fig. 2A) and autoclaved (Fig. 2B) mixtures. The relative LOQ was set as 0.5% as it is the lowest level within the linearity of the calibration curve (ENGL, 2015). The real-time PCR assay parameters, using raw and autoclaved reference mixtures, regarding PCR efficiency (89.3% and 100.0%, respectively), slope of calibration curve (−3.6067 and −3.3224, respectively) and R^2 (0.9889 and 0.9893, respectively) highlight the high performance of the calibration models for the determination of red deer meat. The PCR efficiency was slightly below 90% in the case of raw mixtures; however, when dealing with complex food matrices, this finding can be considered acceptable due to PCR inhibiting compounds (ENGL, 2015). All reference model mixtures, before and after autoclaving, were significantly different ($p < 0.05$) for the same incurred level, except for the last tested level of 0.01%. Delays of 0.7–1.5 in the Cq values were observed for autoclaved model mixtures in relation to raw ones, except for the 0.01% level that revealed a smaller Cq difference (<0.26) (Table 2).

Table 2

Real-time PCR results of the amplification of reference model mixtures of red deer in pâté before and after thermal treatment.

Model mixtures containing red deer (% w/w)	Raw pâté mixtures		Autoclaved pâté mixtures	
	Cq $\pm$ SD ^a	Replicates (positive/total)	Cq $\pm$ SD ^a	Replicates (positive/total)
50	24.86 $\pm$ 0.06	(12/12)	26.18 $\pm$ 0.01 ^b	(12/12)
10	26.62 $\pm$ 0.06	(12/12)	28.14 $\pm$ 0.03 ^b	(12/12)
5	28.29 $\pm$ 0.35	(12/12)	29.23 $\pm$ 0.14 ^b	(12/12)
1	30.61 $\pm$ 0.15	(12/12)	31.31 $\pm$ 0.03 ^b	(12/12)
0.5	31.99 $\pm$ 0.30	(12/12)	32.99 $\pm$ 0.45 ^b	(12/12)
0.1	33.91 $\pm$ 0.38	(12/12)	35.05 $\pm$ 0.42 ^b	(12/12)
0.05	35.27 $\pm$ 0.38	(12/12)	36.40 $\pm$ 0.57 ^b	(12/12)
0.01	36.98 $\pm$ 1.15	(7/12)	37.24 $\pm$ 0.20	(8/12)
Correlation coefficient (R^2)	0.9944		0.9877	
Slope	-3.4031		-3.2034	
Intercept	30.553		31.587	
PCR efficiency (%)	96.7		105.2	

^a Mean cycle of quantification (Cq) $\pm$ standard deviation (SD).

^b Mean values statistical different ($p < 0.05$) from raw reference mixtures for the same spiking level, following one sample *t*-test or Mann-Whitney test, for normal or non-normal distribution, respectively.

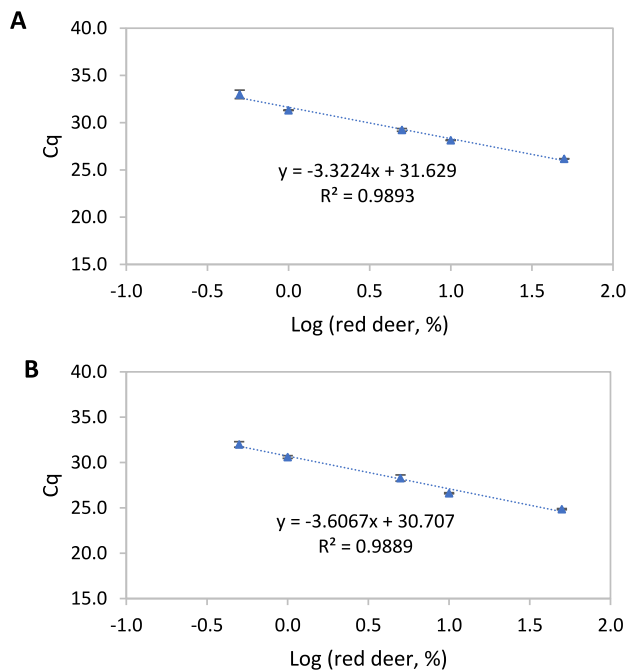


Fig. 2. Calibration curves obtained by real-time PCR with TaqMan probe targeting the troponin I gene using reference model mixtures of 50%–0.5% (w/w) of red deer meat in pork to simulate pâté, before (A) and after (B) thermal processing ($n = 12$ replicates).

Comparing with other species-specific real-time PCR assays for game meat quantification, the obtained relative LOQ is close to the achieved by Kaltenbrunner et al. (2018a, 2018b) for fallow deer and red deer (both 0.4%), targeting both nuclear genes. The same authors reached a

relative LOD of 0.01% since their cross-reactivity with other species was much delayed due to the use of minor groove binder (MGB) labelled probes that provide increased specificity. However, MGB probes are much more expensive than the common dual labelled TaqMan probes, which is a main drawback. Additionally, Kaltenbrunner et al. (2018a, 2018b) used mixtures of DNA extracts of single meat species as calibrants instead of using model mixtures of meats containing other ingredients simulating a real meat product, such as pâté. Therefore, in the present work, the LOD and LOQ of 0.5% was considered suitable for authentication purposes of processed game meat products, particularly relying on the use of more cost-effective reagents. The recent quantitative real-time PCR approach proposed by Liu et al. (2023) reached a relative LOQ of 0.1% for sika deer or red deer but targeting mtDNA. Other reports have also attempted to quantify deer species, though without discriminating red deer. Köppel et al. (2020) proposed a multiplex real-time PCR method to quantify deer species, pork and beef within the range 2–64% for deer species and using serial DNA dilutions as calibrants instead of DNA extracts of meat mixtures. Notably, their results are presented in terms of DNA contents rather than the weight composition of meat products, a critical distinction in the interpretation of LOD values. Several studies also utilised DNA dilutions to achieve specific concentrations in terms of weight/weight (w/w) percentage. Druml et al. (2015a) reached a LOQ of 0.5% for fallow deer and red deer. Later, the same research group was able to quantify deer species until 0.25% (Druml et al., 2016), in both cases using serially diluted DNA mixtures as calibrants instead of model mixtures of meat products to fully account the influence of matrix in the performance of assays.

In the present study, two calibration models that simulate real meat products, accounting both the effects of matrix and thermal processing, were proposed for the first time. These calibration models provide more realistic approaches than other reports since the complexity of matrix is accounted, as well as the loss of DNA integrity caused by autoclaving, which might decrease method sensitivity. Despite this scenario, a LOD and LOQ of 0.5% of red deer meat in simulated pâtés (thermally treated by autoclaving) was achieved with a calibration range of 50–0.5%, which is very suitable for authentication purposes. Therefore, the applicability of the developed method was assessed based on several key elements, such as stability in heat-treated or heavily processed products, handling complex food matrices involving more than one species, overcoming possible assay inhibitors, ensuring consistency through precision assessment, and verifying reliability through testing with blind sample.

3.4. Method validation

The fitness of the proposed method for quantification was evaluated based on the parameters of trueness, precision and repeatability (Bustin et al., 2009; ENGL, 2015). Therefore, two sets of blind mixtures, comprising 30%, 25%, 15% and 3% (w/w) of red deer meat in pâté mixture, raw and thermally treated, were tested. Table 3 summarises the actual and estimated concentrations of red deer meat together with precision and accuracy data. The precision was expressed by the coefficients of variation (CV), representing the relative standard deviation of results, which were obtained under repeatability conditions. The CV data were within 8.4–15.7% and 4.6–8.1% for raw and autoclaved blind mixtures, respectively, demonstrating the precision of the method ($\leq 25\%$) over the tested dynamic range (ENGL, 2015). The closeness of agreement between the actual and estimated red deer contents is evidenced by the calculation of bias, expressing the trueness of the assay. Bias data were mostly between -10.6 and 7.6%, thus within the acceptable criterion of $\pm 25\%$ (ENGL, 2015). The exception was for the level of 3% in raw mixture (D), which is the lowest tested level. It is important to refer that raw mixtures are very unstable due to the high level of enzymatic activity that might justify the superior variability in raw than in the processed mixtures, stabilised by autoclaving. According to the precision and accuracy data, both raw and processed calibration

Table 3

Validation results of the quantitative real-time PCR assay applied to raw and thermally processed blind meat mixtures.

Blind samples	Actual value (%)	Estimated value (%) ^a	SD ^b	CV (%) ^c	Bias (%) ^d
Raw mixtures					
A	30	31.86	4.40	13.80	5.80
B	25	24.00	2.41	10.00	-4.20
C	15	15.02	2.35	15.70	0.20
D	3	4.74	0.40	8.40	36.70
Processed mixtures					
E	30	29.11	2.35	8.10	-3.10
F	25	22.61	1.03	4.60	-10.6
G	15	16.24	0.96	5.91	7.60
H	3	2.69	0.24	8.00	-1.40

^a Values are the mean of replicate assays ($n = 12$).

^b SD – standard deviation.

^c CV – coefficient of variation.

^d Bias = ((mean estimated value-true value)/true value x 100).

models can be considered, generally, suitable for quantitative purposes.

3.5. Analysis of commercial samples

After the successful construction and validation of the calibration models, the following step was the assay application to detect and estimate the red deer content in processed game meat products. As earlier stated, the tested samples were 46 diversified gourmet game meat products, from which 28 declared red deer in varied proportions, some declared roe deer (14) or fallow deer (2), one declared pork and one wild boar and pork meat (Table 4). The samples were divided in two groups, namely uncooked or mildly cooked (up to 71 °C) and cured products such as sausages (samples #1 to #24) and thermally treated (115–121 °C) products such as pâtés (samples #25 to #46), for which the calibration models of raw and autoclaved mixtures were applied, respectively. About red deer meat products, the labelled species was detected and quantified in all samples except for #4 and #27, which declared 70% and 56% of red deer meat, respectively. In samples #1, #20 and #39, the estimated red deer contents were within 0.49–3.85%, more than 10-fold lower than the declared contents, while in samples #21–#23, #25, #26, #38, #40 and #45, the estimated contents were 2- to 6-fold inferior to the labelled ones. In opposition to that, the estimated red deer contents of samples #2, #3, #5–#10, #17–#18, #24, #28–#30 and #43 were according to the declared information. Therefore, these results suggest that, out of 28 red deer labelled samples, 13 were adulterated by the absence or reduction/substantial reduction of red deer amount of meat.

Concerning the roe deer labelled products, as expected, red deer was not detected in samples #11, #31–#34, #42 and #44. However, red deer was detected in samples #12–#16, #35 and #37 with contents mostly $\geq 20\%$, suggesting the partial or total substitution of roe deer with red deer. The two samples declaring fallow deer, namely #19 and #37, were estimated to contain $>50\%$ and 16% of red deer, also suggesting the total replacement of fallow deer with red deer based on the closeness of estimated contents and declared values. Considering the slight cross-reactivity of the applied real-time PCR assay with fallow deer, samples #19 and #37 were sequenced together with red deer and fallow deer. The aligned sequences confirm that both samples matched with red deer and not fallow deer (Fig. S4, supplementary data). Regarding the samples labelled with pork, namely #41 and #46 declaring wild boar and pork meat, respectively, as expected red deer was not detected.

In summary, out of 28 red deer labelled products, red deer meat was substantially reduced or absent in 13 samples. From the 14 roe deer labelled samples, red deer was present at proportions mostly above 20% in 7 samples, while in the 2 fallow deer samples, red deer was present at proportions close to the declared amount of fallow deer. Therefore, the

global results from 46 analysed game meat products suggest the adulteration of 48% of the samples by the total or partial substitution of other deer species. It is important to note that the sample survey included a range of game meat products from four countries of origin, covering geographical regions from northern/central to southern Europe. Accordingly, the results show that the highest number of adulterations and/or mislabelling were found in the samples from Poland, which accounted 15, while the remaining 7 were from Portugal (3) and Spain (4). These results emphasised the novelty of this study since most reports based on real-time PCR assays to quantify deer species do not demonstrate their true applicability in real meat products, being limited to model meat product analysis. Druml et al. (2015a) applied their deer-species real-time PCR assay to meat products from Austrian market, verifying that deer species (fallow deer, red deer and sika deer) were present in most of the samples at proportion equal or higher than 38%, but only few products declared the contents of game meats, from which 3 showed reduced deer amounts.

3.6. Comparative analysis with other real-time quantitative PCR assays

To comprehensively evaluate the relative performance of the developed method, a comparative analysis was performed with other published papers. For this purpose, some error metrics were calculated to assess the prediction accuracy: the mean absolute error (MAE), reflecting the average magnitude of the difference between predicted and actual values; the mean squared error (MSE) that highlights larger errors by squaring and then averaging the difference between predicted and actual values; and the mean absolute percentage error (MAPE) expressing the average size of the percentage difference between predicted and actual values. These metrics were calculated as follows:

$$Error_i = Predicted_i - Actual_i \quad (1)$$

$$MAE = \sum_{i=1}^n \frac{|Error_i|}{n} \quad (2)$$

$$MSE = \sum_{i=1}^n \frac{|Error_i|^2}{n} \quad (3)$$

$$MAPE = \sum_{i=1}^n \left(\frac{|Error_i|}{Actual_i} \right) \times 100 \quad (4)$$

$$Accuracy = 100\% - MAPE \quad (5)$$

The studies by Kaltenbrunner et al. (2018b) and Dolch et al. (2020) were selected for comparative analysis as their real-time PCR approaches were used for the determination of red deer and other meat species, respectively. In addition, the method proposed by Kaltenbrunner et al. (2018b) was used because, like in this study, a nuclear gene (encoding protein kinase C) was targeted for the quantification of red deer meat. The study of Dolch et al. (2020) was selected for a detailed comparison because they used a relative standard curve approach (method A) similar to the one proposed herein to quantify meat proportions from five poultry species in unknown emulsified type sausages prepared in two temperature conditions. Accordingly, relevant data on quantitative prediction results were extracted from these studies and the error metrics, namely MAE, MSE, MAPE and accuracy were calculated and compared with this study (Table 5).

In the present study, the errors and accuracy data of the assay in quantifying red deer meat in both raw and processed samples were evaluated, as presented in Table 5. For raw samples, the MAE was 1.16, the MSE was 1.87 and the MAPE was 17%, resulting in an overall accuracy of 83%. Processed samples demonstrated slightly higher accuracy with values of MAE, MSE and MAPE of 1.21, 2.03 and 8%, leading to an accuracy of 92%. The quantitative data obtained by Kaltenbrunner et al. (2018b) were diversified as the authors used both DNA mixtures

Table 4

Analysed game meat samples by quantitative real-time PCR targeting the troponin I gene of red deer.

Sample code	Sample description	Relevant label information	Origin	Real-time PCR		
				Cq (mean ± SD) ^a	Estimated red deer content (mean ± SD ^a , % w/w)	CV ^b (%)
Uncooked/cured						
#1	Salami with red deer	10% red deer meat	Poland	32.27 ± 0.10	0.49 ± 0.05	9.63
#2	Red deer and pork kabanos	51% red deer meat	Poland	26.61 ± 0.11*	>50	
#3	Dry myśliwska sausage with red deer	25% red deer meat	Poland	26.53 ± 0.04	20.37 ± 1.61	7.92
#4	Red deer sausage	70% red deer meat	Poland	ND ^c		
#5	Red deer sausage Wild grill	30.6% red deer meat	Poland	25.54 ± 0.11*	>50	
#6	Juniper sausage	>50% red deer meat	Poland	25.19 ± 1.09*	>50	
#7	‘Swojska’ sausage	<50% red deer meat	Poland	26.28 ± 0.12*	>50	
#8	Red deer salami	100% red deer meat	Poland	25.78 ± 0.10*	>50	
#9	Red deer ‘kindziuk’	70% red deer meat	Poland	25.65 ± 0.27*	>50	
#10	Tender red deer sausage	35.5% red deer meat	Poland	25.44 ± 0.14*	>50	
#11	Roe deer kabanos	100% roe deer meat	Poland	ND		
#12	Roe deer sausage	70% roe deer meat	Poland	26.57 ± 0.15	19.85 ± 0.98	4.96
#13	Roe deer sausage	>50% roe deer meat	Poland	27.02 ± 0.06*	44.55 ± 3.64	8.17
#14	Roe deer sausage	<50% roe deer meat	Poland	25.98 ± 0.05*	>50	
#15	Roe deer kabanos	100% roe deer meat	Poland	26.35 ± 0.06*	>50	
#16	Roe deer sausage	<50% roe deer meat	Poland	27.19 ± 0.13*	>50	
#17	Red deer sausage	60% red deer meat	Spain	26.98 ± 0.16*	>50	
#18	Red deer sausage	60% red deer meat	Spain	27.36 ± 0.33*	>50	
#19	Spicy fallow deer sausage	60% fallow deer meat	Spain	25.18 ± 0.83*	>50	
#20	Red deer sausage	60% red deer meat	Portugal	30.78 ± 0.09	3.85 ± 0.2	5.21
#21	Red deer sausage with fine herbs	60% red deer meat	Portugal	28.12 ± 1.29*	18.68 ± 2.22	11.9
#22	Red deer sausage with sesame seeds	60% red deer meat	Portugal	27.64 ± 0.09*	29.73 ± 0.91	3.07
#23	Red deer sausage with pepper	60% red deer meat	Portugal	28.94 ± 0.07*	7.52 ± 0.42	6.07
#24	Red deer loin	100% red deer meat	Spain	25.63 ± 0.29*	>50	
Thermally treated						
#25	Baked red deer polish pâté	70% red deer meat	Poland	27.2 ± 0.01	20.48 ± 0.15	0.73
#26	Red deer polish pâté (canned)	70% red deer meat	Poland	27.77 ± 0.15	13.84 ± 1.34	9.7
#27	Red deer sausage (canned)	56% red deer meat	Poland	ND		
#28	Baked red deer polish pâté	36.6 % red deer meat	Poland	25.74 ± 0.09	>50	
#29	Hunter’s polish pâté (canned; 3 types of meat: red deer, wild boar, roe deer)	>33% red deer meat	Poland	27.18 ± 0.09*	>50	
#30	Red deer ham	100% red deer meat	Poland	27.27 ± 0.03*	>50	
#31	Baked roe deer polish pâté	70% roe deer meat	Poland	ND		
#32	Roe deer polish pâté (canned)	70% roe deer meat	Poland	ND		
#33	Roe deer sausage (canned)	55% roe deer meat	Poland	ND		
#34	Baked polish pork pâté with roe deer and Armagnac	20% roe deer meat	Poland	ND		
#35	Baked roe deer polish pâté (baked)	51% roe deer meat	Poland	28.73 ± 0.39	7.21 ± 1.76	24.38
#36	Fallow deer pâté	25% fallow deer meat	Spain	27.67 ± 0.09	15.96 ± 3.00	18.79
#37	Roe deer pâté with port wine	7.2% roe deer meat	Spain	27.31 ± 0.09	18.95 ± 1.03	5.42
#38	Red deer pâté with boletus	20% red deer meat	Spain	28.43 ± 0.07	8.69 ± 0.44	5.1
#39	Red deer pâté	25% red deer meat	Portugal	31.75 ± 0.32	0.87 ± 0.21	23.85
#40	Red deer pâté with extra virgin olive oil	11.62% red deer meat	Spain	28.82 ± 0.26	6.68 ± 1.28	19.19
#41	Wild boar terrine with cognac	21% wild boar meat	France	ND		
#42	Roe deer terrine with cognac	21% roe deer meat	France	ND		
#43	Red deer terrine with cognac	21% red deer meat	France	27.09 ± 0.08	22.23 ± 1.33	5.96
#44	Roe deer terrine with juniper berries	21% roe deer meat	France	ND		
#45	Red deer pâté	30.52% red deer meat	Portugal	28.65 ± 0.16	7.47 ± 0.8	10.67
#46	Pork kabanos	100% pork meat	Poland	ND		

^a Values are the mean quantification cycles (Cq) of replicate assays ($n = 12$) $\pm$ standard deviation (SD).^b CV – coefficient of variation.^c ND, not detected; *Cq values obtained after an additional sample dilution to fit into the range of the calibration curves. The estimated red deer content was obtained after the application of the respective dilution factor.

Table 5

Comparison of error and accuracy metrics of the obtained real-time PCR quantitative analysis of red deer with other published works.

Target species	Type of calibrators ^a	Type of analysed sample ^a	Processing of analysed samples	MAE ^b	MSE ^c	MAPE ^d (%)	Accuracy (%)	References
Red deer	model pâté (0.05–50%)	blind pâté (3–30%)	Raw	1.16	1.87	17	83	This work
	model pâté (0.05–50%)	blind pâté (3–30%)	115 °C, 30 min	1.21	2.03	8	92	
Red deer	DNA mixtures (1–50%)	meat mixtures (5–50%)	Raw	2.61	16.02	16	84	Kaltenbrunner et al. (2018b)
	Meat mixtures (1–50%)	meat mixtures (5–50%)	Raw	5.31	67.37	41	59	
	DNA mixtures (25%)	model sausages (2–50%)	Raw	2.70	14.31	11	89	
	meat mixtures (25%)	model sausages (21%, 28.6%)	Raw	3.65	13.75	15	85	
	meat mixtures (25%)	model sausage (28.6%)	Raw	3.00	9.00	10	90	
	meat mixtures (25%)	model sausage (28.6%)	75–78 °C, 15 min	11.70	136.89	41	59	
	meat mixtures (25%)	model sausage (28.6%)	100 °C (water), 90 min	7.70	59.29	27	73	
	meat mixtures (25%)	model sausage (28.6%)	100 °C (salt water), 90 min	3.30	10.89	12	88	
	meat mixtures (25%)	model sausage (28.6%)	Microwave 15 s	2.80	7.84	10	90	
	meat mixtures (25%)	model sausage (28.6%)	Microwave 40 s	7.20	51.84	25	75	
	meat mixtures (25%)	model sausage (28.6%)	Microwave 2 min	10.10	102.01	35	65	
Chicken	meat mixture (0.5–69%)	model sausages (0.5–57.5%)	75 °C, 30 min	3.05	19.20	20	80	Dolch et al. (2020)
	meat mixture (0.5–69%)	model sausages (0.5–57.5%)	117 °C, 4 min	3.84	39.93	20	80	
Guinea Fowl	meat mixture (0.5–69%)	model sausages (0.5–57.5%)	75 °C, 30 min	3.62	32.77	21	79	
	meat mixture (0.5–69%)	model sausages (0.5–57.5%)	117 °C, 4 min	2.82	25.75	23	77	
Pheasant	meat mixture (0.5–69%)	model sausages (0.5–57.5%)	75 °C, 30 min	2.11	11.25	13	87	
	meat mixture (0.5–69%)	model sausages (0.5–57.5%)	117 °C, 4 min	1.43	4.47	26	74	
Quail	meat mixture (0.5–69%)	model sausages (0.5–57.5%)	75 °C, 30 min	2.10	12.32	6	94	
	meat mixture (0.5–69%)	model sausages (0.5–57.5%)	117 °C, 4 min	8.50	284.48	29	71	
Turkey	meat mixture (0.5–69%)	model sausages (0.5–57.5%)	75 °C, 30 min	2.88	16.68	16	84	
	meat mixture (0.5–69%)	model sausages (0.5–57.5%)	117 °C, 4 min	0.82	1.73	5	95	

^a Values in brackets refer to the % target species in the first column.^b MAE, mean absolute error.^c MSE, mean squared error.^d MAPE, mean absolute percentage error.

and meat mixtures as calibrators covering a dynamic range of 1–50% to estimate red deer contents in meat mixtures within 5–50%. While the accuracy data suggest acceptable estimates using DNA mixtures, with low absolute and relative errors leading to an accuracy of 84%, when using DNA mixtures, the accuracy was rather low (59%) mainly due to the high estimate errors. Then the same authors used a simplified strategy based on single calibrators (25%) of DNA or meat mixtures to estimate red deer contents in model sausages with increasing red deer meat content and different types of thermal processing. When estimating red deer content of raw sausages, the obtained accuracy data were generally high (85–90%) owing to the relatively low errors, regardless the use of DNA mixtures or meat mixtures as calibrators. However, when estimating the red deer contents of processed model sausages, the errors are generally very high with consequent poor accuracy data (59–90%). It is important to highlight that Kaltenbrunner et al. (2018b) only used raw calibrators, which can justify the high estimate errors in processed model sausages. In the present work, two calibration models were proposed to estimate red deer contents in raw/mild thermally processed and autoclaved/severally processed pâté mixtures, resulting in minor/small estimate errors, in opposition to Kaltenbrunner et al. (2018b), who did not use processed calibrators.

Dolch et al. (2020) used a similar real-time PCR approach to estimate the contents of five bird species in emulsified type sausages, though not displaying the respective calibration models. Generally, the results

reveal low/small errors for mild-processed sausages (75 °C, 30 min), with average acceptable accuracy of 85%, but slightly higher errors for more severely processed sausages (117 °C, 4 min), with consequent decrease in accuracy to 79%. Like Kaltenbrunner et al. (2018b), Dolch et al. (2020) used only raw calibrators to estimate meat species contents of mild and severely processed samples with consequent loss of accuracy.

Therefore, data of Table 5 highlight the overall high performance of the herein proposed method and the importance of developing appropriate calibrators mimicking as close as possible the samples to analyse, both in terms of matrix and thermal processing. The application of error metrics emphasised the high performance of the proposed model comparing with other reports and is very effective in helping decision-makers adopt the method for routine analysis. Additionally, when multiple methods are developed and validated, the use of error assessment metrics becomes crucial for a comprehensive evaluation.

4. Conclusions

This study presents a novel, species-specific, nuclear marker-based real-time PCR assay for detecting and quantifying red deer meat adulteration in gourmet foods. The method comprises two independent real-time PCR systems specifically designed for raw/mildly processed or severely treated samples in complex food matrices. The approach

achieved absolute and relative LOD of 0.01 ng of DNA and 0.5% red deer meat in raw or processed pâté, respectively, covering a dynamic range of 0.5–50%. The method was validated with blind samples, demonstrating acceptable parameters for trueness, precision, repeatability and robustness, which makes it suitable for routine analysis in control laboratories and other commercial settings. The applicability of the method was demonstrated analysing a range of commercial gourmet meat products, including those labelled as containing red deer, other deer or game species. The results revealed a surprisingly low abundance of red deer meat content in red deer-labelled products. Conversely, many roe deer-labelled products contained a significant, undeclared percentage of red deer meat. Additionally, the method exhibited a quantification accuracy of 83% and 92% for raw and thermally processed blind mixtures, respectively, which was comparable or higher than other reports, in the case of processed mixtures.

In summary, the method developed in this study proved to be a reliable and useful tool for meat inspection programmes, being suitable for the verification of red deer meat content in gourmet products.

Funding

This work received national funds from FCT (Fundação para a Ciência e Tecnologia), Portugal, through the strategic funding of UIDB/50006/2020 (DOI 10.54499/UIDB/50006/2020) from FCT/MCTES. C. Villa, J. Costa and I. Mafra thank FCT for funding through the Individual Call to Scientific Employment Stimulus (2023.07644.CEECIND, 2021.03583.CEECIND/CP1662/CT0012 and 2021.03670.CEECIND/CP1662/CT0011, respectively). This work was supported by the National Science Centre, Poland, project number 2020/37/B/NZ9/00082.

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Bukola M. Adenuga: Writing – original draft, Visualization, Investigation, Formal analysis. **Rita Biltés:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Caterina Villa:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization. **Joana Costa:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Anita Szychaj:** Methodology, Investigation. **Magdalena Montowska:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Isabel Mafra:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodcont.2024.110872>.

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Supplementary material

Table S1. Results of qualitative PCR targeting a universal eukaryotic region of the 18S rRNA gene and the troponin I gene of red deer using several relevant animal and plant species for cross-reactivity testing

Common name	Scientific name	Qualitative PCR	
		18SRG-F/18SRG-R	TNNI2-F/TNNI2-R
Red deer	<i>Cervus elaphus</i>	+	+
Roe deer	<i>Capreolus capreolus</i>	+	+
Fallow deer	<i>Dama dama</i>	+	+
Chicken	<i>Gallus gallus</i>	+	-
Beef	<i>Bos taurus</i>	+	-
Horse	<i>Equus ferus caballus</i>	+	-
Pheasant	<i>Phasianus colchicus</i>	+	-
Lamb	<i>Ovis aries</i>	+	-
Goat	<i>Capra hircus</i>	+	-
Duck	<i>Anas platyrhynchos</i>	+	-
Guinea fowl	<i>Numida meleagris</i>	+	-
Quail	<i>Coturnix coturnix</i>	+	-
Pig	<i>Sus scrofa domestica</i>	+	-
Rabbit	<i>Oryctolagus cuniculus</i>	+	-
Pigeon	<i>Columba livia</i>	+	-
Turkey	<i>Meleagris gallopavo</i>	+	-
Wild boar	<i>Sus scrofa</i>	+	-
Soybean	<i>Glycine max</i>	+	-
Pine nut	<i>Pinus species</i>	+	-
Wheat	<i>Triticum aestivum</i>	+	-
Rosemary	<i>Rosmarinus officinalis</i>	+	-
Mustard	<i>Brassica juncea</i>	+	-
Coriander	<i>Coriandrum sativum</i>	+	-
Onion	<i>Allium cepa</i>	+	-
Garlic	<i>Allium sativum</i>	+	-
Parsley	<i>Petroselinum crispum</i>	+	-
Bay leaves	<i>Laurus nobilis</i>	+	-
Sage	<i>Salvia officinalis</i>	+	-
Fennel	<i>Foeniculum vulgare</i>	+	-
Vine	<i>Vitis vinifera</i>	+	-
Olive	<i>Olea europaea</i>	+	-
Ginger	<i>Zingiber officinale</i>	+	-
Oregano	<i>Origanum vulgare</i>	+	-
White pepper	<i>Piper nigrum</i>	+	-
Sweet chilli	<i>Capsicum annuum</i>	+	-
Chilli	<i>Capsicum spp</i>	+	-
Corn	<i>Zea mays</i>	+	-

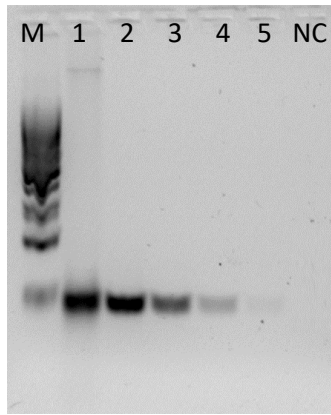


Fig. S1. Agarose gel electrophoresis of PCR products targeting the troponin I gene of red deer using serially diluted red deer DNA. Legend: M, 100 bp DNA Ladder; lane 1, 100 ng; lane 2, 10 ng; lane 3, 1 ng; lane 4, 0.1 ng; lane 5, 0.01 ng; NC, negative control.

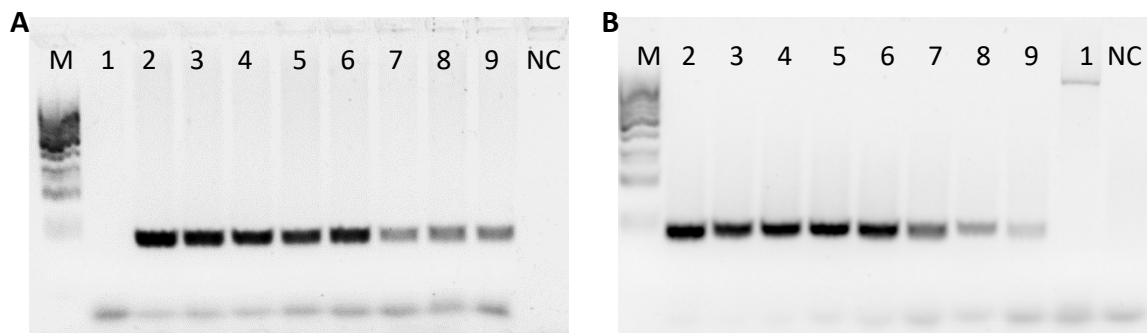


Fig. S2. Agarose gel electrophoresis of PCR products targeting the troponin I gene of red deer using red deer DNA extracts of model mixtures with known amounts of red deer in pork meat (simulated pâté), raw (A) and autoclaved (B). Legend: M, 100 bp DNA Ladder; lane 1, 0%; lane 2, 50%; lane 3, 10%; lane 4, 5%; lane 5, 1%; lane 6, 0.5%; lane 7, 0.1%; lane 8, 0.05%; lane 9, 0.01% (w/w); NC, negative control.

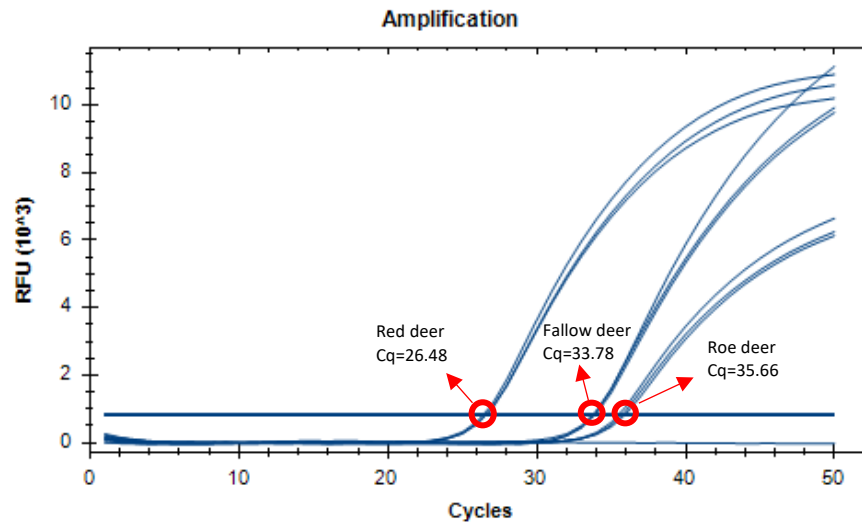


Fig. S3. Amplification curves obtained by real-time PCR with the TNNI2-P TaqMan probe targeting the troponin I gene using DNA extracts of red deer, fallow deer and roe deer at the same concentration (50 ng/ μ L).

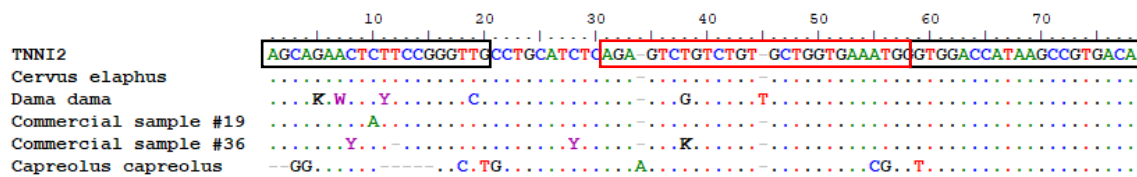


Fig. S4. Alignment of sequences of NCBI (TNNI2 – NCBI accession no. OU343091.1) and specimens of *C. elaphus*, *D. dama* and *C. capreolus*, together with samples #19 and #36 labelled with fallow deer, obtained by the amplification targeting the troponin I gene of red deer.

Publication 4 (P4)

Adenuga, B. M., Biltès, R., Villa, C., Costa, J., Spsychaj, A., Montowska, M., Mafra, I. (2024). A novel normalized quantitative real-time PCR approach for ensuring roe deer (*Capreolus capreolus*) meat authenticity in game meat foods. *Foods*, 13(23), 3728.

<https://doi.org/10.3390/foods13233728>

Article

A Novel Normalized Quantitative Real-Time PCR Approach for Ensuring Roe Deer (*Capreolus capreolus*) Meat Authenticity in Game Meat Foods

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Abstract: Roe deer meat is a prized game product in many European countries. However, concerns exist regarding the accuracy of the amount of declared roe deer in processed game meat foods. This study aimed to develop a reliable method for the detection and quantification of roe deer in commercialized game meat products. A TaqMan probe-based quantitative real-time PCR (qPCR) assay was designed, targeting a single-copy 120-bp region of the roe deer agouti signaling protein (ASIP) encoding gene. The method employed the normalized ΔC_q approach to establish a calibration curve for roe deer detection and quantification within 0.05–50% (*w/w*) in complex raw and processed matrices. The method proved to be specific for roe deer identification, achieving limits of detection and quantification of 0.04 ng of roe deer DNA and 0.05% (*w/w*) of roe deer in simulated pâté. Following validation with blind samples, highlighting the precision and trueness of the approach, the assay was applied to 46 market samples from four European origins (Poland, Portugal, France, and Spain). The analysis revealed significant discrepancies between declared roe deer content and actual levels in all roe deer labeled products. The global analysis of results, combining the previous survey on red deer species with present roe deer data, identified 61% of mislabeled/adulterated samples due to the absence of deer species, substitution of roe deer with red deer, substitution of fallow deer with other deer species and red deer with pork, and undeclared addition of roe deer. This study demonstrates the effectiveness of the developed qPCR method for accurate roe deer meat authentication in foods, showing its usefulness as a tool for routine food inspection to ensure labeling compliance.

Keywords: food authentication; game meat products; roe deer; nuclear marker; agouti signaling protein (ASIP) gene; qPCR

Citation: Adenuga, B.M.; Biltres, R.; Villa, C.; Costa, J.; Spychaj, A.; Montowska, M.; Mafra, I. A Novel Normalized Quantitative Real-Time PCR Approach for Ensuring Roe Deer (*Capreolus capreolus*) Meat Authenticity in Game Meat Foods. *Foods* **2024**, *13*, 3728. <https://doi.org/10.3390/foods13233728>

Academic Editor: Lorenzo Nissen

Received: 28 October 2024

Revised: 12 November 2024

Accepted: 19 November 2024

Published: 21 November 2024



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1. Introduction

Roe deer (*Capreolus capreolus*) is a highly valued game species in Europe, with an estimated population of 15 million individuals [1]. Its meat is prized for excellent flavor, texture, and nutritional value (134 calories, 21 g of protein, and 1.2 g of fat per 100 g serving portion) [2], contributing significantly to the economic value of the hunting industry in nations like Germany, the Czech Republic, Poland, France, Austria, Sweden, and Belgium [3]. In fact, roe deer hunting, combined with red deer and wild boar, results in a total economic value of more than 10 billion EUR, with substantial contributions from tourism, land management, and the sale of game meat [4]. Poland, in particular, is famous for its roe deer hunting season, in which hunters from European countries participate in

ensuring the availability of roe deer venison in the Polish meat market. Despite extensive research on other aspects of roe deer, such as meat quality [2,5], population dynamics [6], and predation [7], there is a concerning lack of recent studies to verify the authenticity of roe deer meat sold in Poland. The only existing study is qualitative and dates back several years [8].

Concerns about meat product adulteration are rising globally due to potential economic fraud, violation of ethical or religious dietary restrictions, and even health risks associated with consuming undeclared species [9]. A recent inspection conducted by the Polish food inspection authority, The Inspectorate for Commercial Quality of Agricultural and Food Products (IJHARS), revealed that over 80% of inspected establishments were guilty of irregularities in declared food items. Specifically, 54% of tested popular meat-based products, such as Kebab, contained unidentified meat species [10,11]. Consequently, it is crucial to establish a reliable species-specific method for accurate quantitative detection of game meat in ready-to-eat or processed commercial products.

To address the growing concerns about food authenticity, DNA-based methods have become the preferred analytical approach for species detection in animal products due to their accuracy and reliability [12,13]. DNA contains unique species-specific information, maintaining its stability under challenging technological conditions, and setting it apart from other food components like proteins or metabolites [14]. The polymerase chain reaction (PCR)-based methods have been extensively applied for identifying meat species such as goat, chicken, turkey, pork, beef, horse, and sheep utilizing techniques like species-specific PCR, PCR with restriction fragment length polymorphisms (RFLP), multiplex PCR, real-time PCR, and digital PCR, among others [8,9,14–21].

The past two decades have witnessed significant advancements in developing reliable methods for game meat verification. These advances have mainly focused on real-time PCR methods because of the advantages of high sensitivity, specificity, and aptitude for multiplex detection and quantification [12,13]. Game meat species, including deer species (red, fallow, roe, and sika) and chamois, have been detected by real-time PCR assays [21–31]. Roe deer meat was also detected and quantified by a TaqMan real-time PCR assay targeting the lactoferrin gene, reaching a limit of detection (LOD) of 0.03% (*w/w*) and a limit of quantification (LOQ) between 0.5 and 0.125%, using serially diluted DNA extracts from a model meat mixture with 2% roe deer in pork [31]. Similarly, the same gene was targeted to detect and quantify roe deer using serially diluted DNA to achieve an LOD and LOQ of 0.1% and 0.25% of targets in non-target DNA [23,24]. Afterward, a tetraplex real-time PCR assay with MGB probes targeting nuclear genes of four deer species (roe, red, fallow, and sika) was developed, achieving an LOD and LOQ of 0.1% and 0.5% (*w/w*), using DNA mixtures as calibrators [25]. Despite the progress made in quantifying roe deer meat using nuclear genes and TaqMan probes, limitations remain in the design of the quantitative approaches, particularly using serially diluted DNA extracts or DNA mixtures instead of real model mixtures as calibrators to simulate food matrices. Previous studies have successfully used reference model mixtures as calibrators to estimate red deer meat in foods by real-time PCR, simulating food matrix and thermal processing, as more realistic, and at the same time, more challenging approaches [27].

This work addresses these limitations by proposing a novel and efficient roe deer species-specific quantitative real-time PCR (qPCR) method. Compared to existing methods, it targets the nuclear gene coding for the agouti signaling protein (ASIP) and proposes a calibration model based on matrix-adapted standards for the first time. For this purpose, reference model mixtures, imitating scenarios where roe deer meat is mixed with other meat types and spices and accounting for the influence of thermal processing were prepared. Furthermore, to ensure its effectiveness in real-world situations, the method was validated using blind samples and applied to commercially available game meat products. The use of a relative standard curve method with reference materials produced under market-realistic conditions increases the practicality and reliability of

product quantification. Additionally, incorporating an endogenous reference gene amplified alongside the target gene allows method normalization and ensures accurate quantification by the real-time PCR system, minimizing potential variations in DNA extraction yield or degradation [9,19,20]. Therefore, this research aims to fill the existing gap by introducing a robust and specific quantification method for roe deer meat. The developed method will contribute to the integrity of the European meat market, having the potential to be applied for global food chain authentication. By ensuring accurate labeling and preventing meat product adulteration, this method can contribute to protecting consumers, upholding ethical and religious dietary practices, and promoting fair trade practices within the meat industry.

2. Materials and Methods

2.1. Samples

Fresh meat samples from roe deer, fallow deer, red deer, pork, chicken, cattle, lamb, goat, rabbit, turkey, duck, horse, guinea fowl, pigeon, pheasant, quail, and wild boar were obtained from reliable sources in markets across Poland and Portugal. Additionally, samples from twenty plant food species (wheat, soybean, corn, pine nut, garlic, rosemary, parsley, onion, chili, white pepper, coriander, bay leaf, mustard, olive, vine, sage, fennel, oregano, ginger, and sweet chili), commonly found in meat products, were included for specificity testing (Table S1, Supplementary Material).

Commercial meat products (46) were purchased at local markets in Portugal and Poland, as well as online stores in Spain, for evaluation in the present work. These samples comprised pâtés, sausages, hams, canned meat, and dumplings, primarily labeled with deer species in different amounts, mixed with other meats and herbs/spices. The samples underwent separate mincing/homogenization in a laboratory knife grinder Grindomix GM200 (Retsch, Haan, Germany), utilizing different materials and blender containers. Prior to processing, the equipment was treated with a DNA decontamination solution.

2.2. Reference Mixture Preparation

The reference mixtures were made to mimic roe deer pâté using meats of roe deer, pork muscles (loin), liver, and neck, as well as various spices/condiments, including salt, onion, garlic, pepper, coriander, ginger, and rosemary. The meat samples were finely chopped into smaller portions, with the outer layers discarded. Initially, a blank mixture was prepared with pork liver, neck, and loin, in equal proportions, followed by the addition of the referred spices/condiments. Subsequently, a reference mixture with 50% roe deer was prepared with the addition of 300 g of minced roe deer meat to the same amount (300 g) of the pork blank mixture. The meats were further ground and homogenized in a kitchen robot Bimby® (Vorwerk, Wuppertal, Germany), where the pâtés were produced. Serial additions of blank mixture were performed to prepare the following reference mixtures 10%, 5%, 1%, 0.5%, 0.1%, 0.05%, and 0.01% (*w/w*) of roe deer in pork pâté. To simulate the industrial preparation of pâtés in cans or bottles, a portion of each model mixture was autoclaved at 115 °C for 30 min.

To validate the method, blind samples were independently prepared. Two sets of blind mixtures, containing 0.3%, 3%, 15%, and 30% (*w/w*) of roe deer in pork pâté, were produced following a similar methodology to the reference mixtures.

Reference mixtures and samples were handled and homogenized separately, using materials and containers treated with a DNA decontamination solution. The raw set of mixtures was promptly stored at −20 °C to mitigate enzymatic activity and prevent microbial growth, while the set mixtures mimicking thermal processing were immediately autoclaved at 115 °C for 30 min and later stored at −20 °C.

2.3. DNA Extraction

DNA from raw meats, reference and validation mixtures, and commercial samples was extracted with the NucleoSpin food kit (Macherey-Nagel, Düren, Germany) with a slight modification: adding 2 µL of RNase (2 mg/mL) after the cell lysis step. The NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany) was used to extract plant DNA according to the manufacturer's instructions. DNA extractions were accomplished in duplicate (200 mg or 100 mg of each meat or plant sample, respectively) and stored at −20 °C.

The quality of DNA extracts was evaluated regarding purity and yield using a microplate UV spectrophotometry reader (SPECTROstar Nano, BMG Labtech, Ortenberg, Germany) and a reduced volume LVis plate accessory (BMG Labtech, Ortenberg, Germany). The DNA concentration was determined with the nucleic acid quantification protocol for double-strand DNA and absorbency data were assessed with the software MARS data analysis (BMG Labtech, Ortenberg, Germany).

2.4. Primers and Probe

Primers and probes were designed using publicly available resources and bioinformatic tools. The ASIP-encoding gene, which contained abundant sequences for both target and non-target species, was downloaded from the NCBI (National Center for Biotechnology Information) database for several species, including wild boar, pig, sheep, cattle, and red deer, and imported into Geneious Prime software (version 2023.2.1), where they were aligned and screened for regions with nucleotide dissimilarities among species. Primers were designed to amplify a 120-bp region of ASIP gene, tested for specificity using the PrimerBlast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?GROUP_TARGET=on, accessed on 18 September 2023) and short sequences Blast (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>, accessed on 18 September 2023) available on the NCBI resources page. The probe was designed with the Integrated DNA technologies tool, Primer Quest (<https://eu.idtdna.com/PrimerQuest/Home/Index>, accessed on 18 September 2023) and labeled with FAM and BHQ1 as reporter and quencher dyes, respectively. In addition, the OligoCalc software (<http://biotools.nubic.northwestern.edu/OligoCalc.html>, accessed on 18 September 2023) was used to verify the absence of hairpin formation, dimerization, and self-hybridization, as well as primer properties.

Universal primers targeting a conserved eukaryotic nuclear 18S rRNA gene fragment were used to assess the amplification capacity of DNA extracts. Additionally, the same primers and probe were used for real-time PCR method normalization [32].

The primers and probes were synthesized by Eurofins MWG Operon (Ebersberg, Germany), and their sequences are displayed in Table 1.

Table 1. Primers and probes used in this study.

Species	Target Gene	Primer	Sequence (5'-3')	Amplicon	NCBI Accession/Reference
<i>Capreolus capreolus</i>	Agouti signaling protein	ASIP-F	GAGTCCATTTTCCAGGGCCG	120 bp	NC_057828.1
		ASIP-R	GGGACTTAGGCCTGCAGTTT		
		ASIP-P	FAM-ACTCTCTGCTCTCTGTCTCTGGCT-BHQ1	113 bp	[32]
		18SRG-F	CTGCCCTATCAACTTTCGATGGTA		
Eukaryotes	18S rRNA	18SRG-R	TTGGATGTGGTAGCCGTTTCTCA		
		18SRG-P	FAM-ACGGGTAACGGGGAATCAGGGTT CGATT-BHQ1		

2.5. Qualitative PCR

PCR assays were run in a 25-µL total reaction volume containing 2 µL of DNA extract (100 ng), ultrapure water (Sigma-Aldrich, Steinheim, Germany), buffer (67 mM Tris-HCL (pH 8.8), 16 mM (NH₄)₂SO₄, 0.01% Tween 20), 200 µM dNTP (Bioron, Ludwigshafen, Germany), 3.0 mM (ASIP-F/R) or 1.5 mM (18SRG-F/R) of MgCl₂, 1 U of SuperHot Taq DNA

Polymerase (Genaxxon Bioscience GmbH, Ulm, Germany), and 200 nM (ASIP-F/R) or 240 nM (18SRG-F/R) of each primer (Table 1). The amplifications were carried out in a MJ Mini thermal cycler (Bio-Rad, Hercules, CA, USA) with the following program: initial denaturation at 95 °C for 5 min; 40 or 33 cycles (ASIP-F/R or 18SRG-F/R, respectively) at 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 5 min.

The PCR amplicons were analyzed by electrophoresis in a 1.5% agarose gel containing 1× Gel Red (Biotium, CA, USA) for staining and carried out in 1× SGTB buffer (GRISP, Porto, Portugal) for 25–30 min at 140 V. A digital image was obtained with under a UV light tray Gel Doc™ EZ System (Bio-Rad Laboratories, Hercules, CA, USA) and Image Lab software version 5.2.1 (Bio-Rad Laboratories, Hercules, CA, USA).

2.6. Real-Time PCR

Real-time PCR assays were conducted in 20-μL reaction mixtures composed of 2 μL (100 ng) DNA extract, 160 nM of each probe (ASIP-P or 18SRG-P), 240 or 280 nM of each primer (ASIP-F/R or 18SRG-F/R) (Table 1), and 1× SsoFast Probes Supermix (Bio-Rad Laboratories, Hercules, CA, USA). Each target gene (ASIP and 18S rRNA) was amplified in parallel reactions and ran simultaneously in the fluorometric thermal cycler CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA), following the temperature conditions of 95 °C for 5 min, 50 cycles at 95 °C for 10 s, and 65 °C for 45 s, with a fluorescence signal collection at the end of each cycle. The analysis output was processed using the Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories, Hercules, CA, USA). Real-time PCR assays were conducted in three independent runs, each with $n = 4$ replicates.

The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [33] were meticulously adhered to, complemented with the criteria established by the European Network of GMO Laboratories (ENGL) for real-time PCR method development and validation [34]. The acceptable criteria outlined by both MIQE and ENGL, including specificity, a dynamic range covering four orders of magnitude, a coefficient of regression value ≥ 0.98 , a slope ranging between -3.6 and -3.1 , and a PCR efficiency between 90% to 110%, were ensured throughout the experiment. The limit of detection (LOD) was considered as the lowest quantity of the analyte in a sample that should be detected with a confidence level of 95%, safeguarding no more than 5% of false-negative results. The limit of quantification (LOQ) was established as identical to or lesser than the lowest quantity within the dynamic range [33,34].

2.7. Statistical Analysis

GraphPad Prism version 8.0.2 software (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. The Shapiro–Wilk test was used to evaluate the normality of the data, meaning that data following normal distribution were analyzed by an unpaired t-test. In contrast, the non-parametric Mann–Whitney test was used for testing data following a non-normal distribution. The significance of differences was determined regarding the comparison of Cq values of raw and processed pâtés at the same concentration level for the normalized calibration method. Significant statistical differences were considered when $p < 0.05$.

3. Results and Discussions

3.1. Method Optimization and Specificity Evaluation

A critical aspect in method development lies in the design of species-specific primers and probes. In this work, the focus was on designing and optimizing novel species-specific primers targeting the ASIP-encoding gene, responsible for the pigmentation regulation in roe deer. This gene was selected considering the availability of sequences in the NCBI database. Moreover, it is a nuclear gene, thus having fixed copy number regardless of tissue and cell type and being more suitable for quantitative analysis, in opposition to mitochondrial DNA markers that have variable copy numbers. To enhance specificity and

uniqueness, several measures were applied. Firstly, a short product length of 120 bp was ensured to restrict the hybridization site, minimizing non-specific binding. Secondly, PCR temperature conditions were methodically optimized through repeated assays to identify the optimal parameters providing the highest product yield while avoiding cross-amplification.

The addition of TaqMan probes provided extra specificity by targeting a specific region within the amplified DNA sequence [34,35]. Additionally, the incorporation of the 18SRG primers [32] as a universal marker not only confirmed the presence of amplifiable DNA in all tested samples but it also allowed the normalization of the real-time PCR system by adopting the ΔCq (cycle quantification difference) approach for quantification [9,19,20]. This method is based on the construction of a calibration curve that involves measuring two specific DNA sequences, carefully selected with comparable efficiencies, the reference universal eukaryotic gene (nuclear 18S rRNA) and the species-specific target (ASIP), using the following expression:

$$\Delta Cq = Cq_{ASIP} - Cq_{euk}$$

where Cq_{ASIP} and Cq_{euk} represent the cycles of quantification for roe and eukaryotic systems, respectively.

Another significant component of the method development was obtaining high-quality DNA extracts with appropriate yield and high purity levels of 1.8 (A260/A280) and 2.3 (A260/A230) for roe deer. Generally, high-quality DNA was isolated for all species. The extracted DNA was used for specificity and sensitivity testing alongside developed and optimized primers and probes.

Prior to real-time PCR method development, the attainment of high-quality DNA extract with appropriate yield and purity level was assured for all mixtures and tested species. PCR optimization was crucial for achieving a high level of method specificity and sensitivity. Temperature gradients were tested with three annealing temperatures (60 °C, 62 °C, and 65 °C) and 65 °C was identified as the optimal temperature for enhancing target amplification while minimizing non-specific binding. Furthermore, slight adjustments to $MgCl_2$ concentration, cycle number, and stringent contamination prevention protocols were also essential for obtaining a clean specificity profile.

The method development progressed to the establishment of calibrators for quantitative analysis with two sets of reference model mixtures (raw and autoclaved pâtés) prepared by adding the target species from 50% to 0.01% of roe deer to pork pâté. Blind samples with varying proportions of roe deer meat was also produced to validate the established method before application to commercial samples.

To ensure method accuracy and avoid false positive results, primer specificity was assessed *in silico* against a broad range of potential cross-reactivity sources using tools such as NCBI Primer-Blast and Short Sequence Blast. Since no cross-reactivity was detected, the analysis shifted to *in vitro* testing of 16 animal species, namely pig and wild boar, other cervids (red deer and fallow deer), birds and poultry, cattle, rabbit, and others, as well as 20 plant species commonly found in processed meat products, including spices and crops such as soybean, wheat, corn, and herbs. The results show no reactivity with non-target species, except for red deer (*Cervus elaphus*) and fallow deer (*Dama dama*), producing the expected 120-bp fragment (Table S1, Supplementary Material). The amplification with 18SRG primers confirmed the absence of any false negative result (Table S1, Supplementary Material). Additionally, a hydrolysis probe was designed for real-time PCR to enhance the specificity of the assay for roe deer, avoiding the reactivity with red deer and fallow deer. Figure S1 (Supplementary Material) shows the real-time PCR amplification curves using a TaqMan probe targeting the ASIP gene and DNA extracts of roe deer, red deer, and fallow deer, thus confirming the specificity for the roe deer.

3.2. Sensitivity

The absolute sensitivity was determined using a 10-fold serial dilution of a roe deer DNA extract, ranging from 40 ng to 0.004 ng. The serially diluted DNA was first assayed by qualitative PCR targeting the ASIP gene of roe deer, producing the expected 120-bp fragment down to 0.004 ng of DNA (Figure S2, Supplementary Material). Afterward, the serially diluted roe deer DNA was amplified by real-time PCR with ASIP primers and a TaqMan probe. Figure 1 shows one example of a real-time PCR run, with the amplification curves (Figure 1A) and respective calibration curve (Figure 1B), with a dynamic range covering 4 orders of magnitude. As the level of 0.004 ng was not amplified in all replicates ($n = 12$), the absolute LOD was established as 0.04 ng to ensure a detection level of confidence above 95%. The absolute LOQ was equal to the LOD as it was within the calibration curve linearity [33]. The assay parameters of PCR efficiency (95.5%), slope (-3.424), and correlation coefficient (R^2) of 0.998 complied with the acceptable criteria for real-time PCR assays (Figure 1B) [33,34].

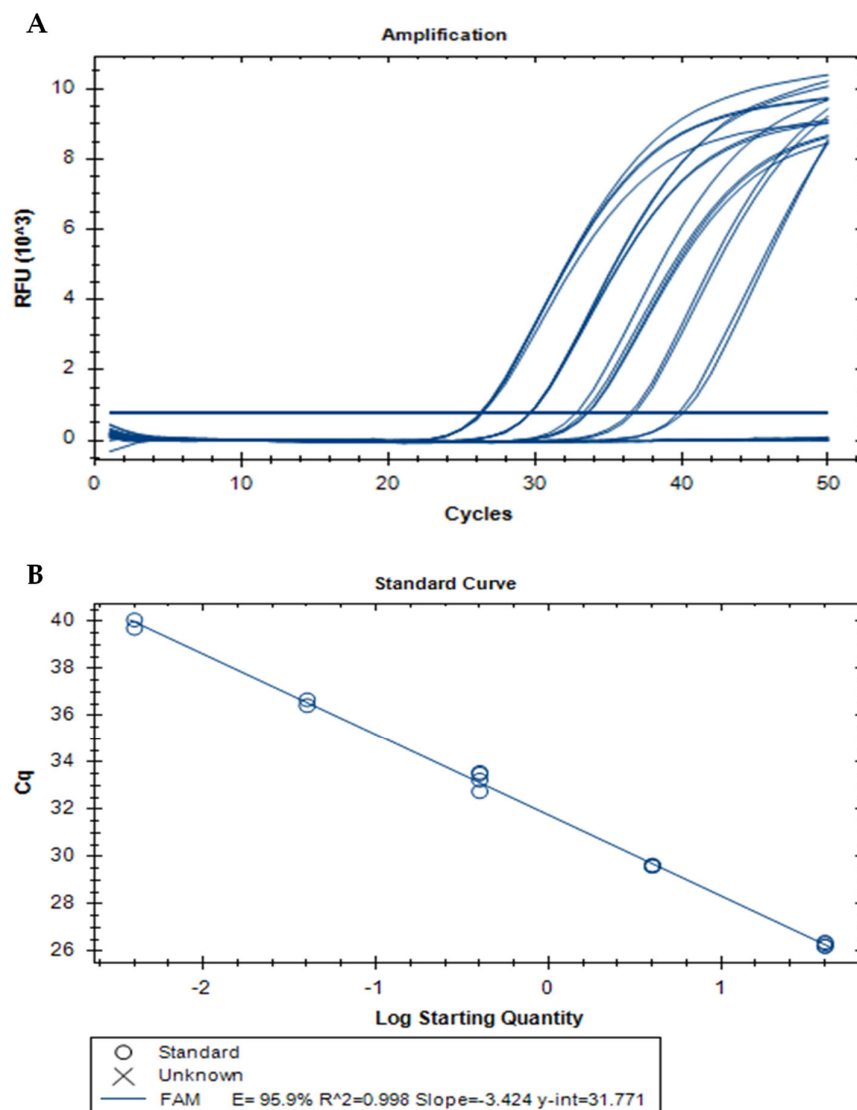


Figure 1. Amplification (A) and standard (B) curves obtained by real-time PCR with a TaqMan probe targeting the agouti signaling protein-encoding gene (roe deer DNA 40–0.004 ng, $n = 4$ replicates).

The obtained LOD (0.02 ng/ μ L) was slightly higher than the previously reached value for red deer targeting the troponin I gene (0.005 ng/ μ L) [27] and lower than that of other reports for roe deer targeting the lactoferrin gene (0.08 ng/ μ L) [31]. Therefore, the obtained LOD agrees with other reports targeting nuclear genes. In opposition, much lower sensitivities for deer species and other mammal or avian species were achieved by targeting mitochondrial genes, namely the ND5 gene for red deer (0.25–0.5 pg) [30] and the *cytb* gene for horse (0.1 pg) [9], pork (0.01 pg) [19], pigeon (0.1 pg), and chicken (0.1 pg) [18]. This is justified by the fact that mitochondrial genes are multicopy markers that allow much-increased sensitivity, but on the other hand, this is a drawback for accurate species quantification.

The relative LOD was evaluated using reference mixtures containing the target DNA (roe deer) at various proportions (50% to 0.01%, *w/w*) within a complex matrix simulating commercially available pâté (both raw and autoclaved). Both real-time PCR systems effectively detected the target species down to a concentration of 0.05% (*w/w*) in raw and heat-treated mixtures (Table 2). The LOD was established through three independent assays with four replicates each, ensuring less than 5% false-negative results.

Table 2. Real-time PCR results using reference mixtures of roe deer in simulated pâté (raw and autoclaved) targeting a reference gene (18S rRNA) and an agouti signaling protein (ASIP)-encoding gene of roe deer.

Model Mixtures Containing Roe Deer (% <i>w/w</i>)	Raw Pâté Mixtures			Autoclaved Pâté Mixtures		
	Cq $\pm$ SD ^a (18S rRNA)	Cq $\pm$ SD (ASIP)	Replicates (Positive/ Total)	Cq $\pm$ SD (18S rRNA)	Cq $\pm$ SD (ASIP)	Replicates (Positive/ Total)
50	16.46 $\pm$ 0.46	25.67 $\pm$ 0.10	(12/12)	17.74 $\pm$ 0.48	26.81 $\pm$ 0.42	(12/12)
10	16.49 $\pm$ 0.39	27.77 $\pm$ 0.08	(12/12)	18.34 $\pm$ 0.30	29.24 $\pm$ 0.38	(12/12)
5	16.74 $\pm$ 0.24	29.30 $\pm$ 0.06	(12/12)	18.20 $\pm$ 0.49	30.37 $\pm$ 0.47	(12/12)
1	16.97 $\pm$ 0.25	31.94 $\pm$ 0.17	(12/12)	19.09 $\pm$ 0.45	32.90 $\pm$ 0.51	(12/12)
0.5	16.62 $\pm$ 0.33	32.88 $\pm$ 0.21	(12/12)	19.08 $\pm$ 0.51	34.50 $\pm$ 0.47	(12/12)
0.1	16.81 $\pm$ 0.31	35.28 $\pm$ 0.21	(12/12)	18.40 $\pm$ 0.35	36.36 $\pm$ 0.53	(12/12)
0.05	16.60 $\pm$ 0.33	36.20 $\pm$ 0.37	(12/12)	18.48 $\pm$ 0.44	38.09 $\pm$ 1.10	(12/12)
0.01	NA	NA	(0/12)	NA	NA	(0/12)
R^2		0.9975			0.9964	
Slope		−3.5834			−3.7120	
Intercept		31.698			33.031	
PCR _{efficiency} (%)		90.1			86.0	

^a Mean quantification cycle (Cq) $\pm$ standard deviation (SD).

The relative LOD obtained in this study is lower than other previously reported for roe deer. An LOD within 0.32–0.64% (*w/w*) for roe deer was reported using a multiplex real-time PCR and DNA dilutions [21], while other authors achieved a relative LOD of 0.1% (*w/w*) also using serially diluted DNA or DNA mixtures [25]. Although an LOD of 0.03% (*w/w*) was obtained in another study [31], such a level was reached by analyzing serially diluted DNA extracts. Therefore, the present study reports, for the first time, a relative LOD accounting for the influence of food matrix and food processing, which is only comparable with the previously obtained LOD for red deer (0.5%, *w/w*) using reference model mixtures [27].

3.3. Normalized Calibration Curve

In the present work, a quantitative method based on reference model mixtures was proposed to determine the amount of roe deer in meat products. The method used two

sets of DNA extracts of reference model mixtures within 0.01–50% (*w/w*) of roe deer in simulated pâté, as raw and thermally processed. The real-time PCR amplification results targeting the ASIP gene of roe deer are displayed in Table 2, exhibiting very good linearity within 0.05–50% for both raw ($R^2 = 0.9975$) and thermally processed mixtures ($R^2 = 0.9964$). Regarding the slope and PCR efficiency of calibration curves, the obtained values are within the criteria of acceptance for the raw pâté calibrators (slope = -3.5834 and efficiency = 90.1%), but slightly below the recommended level for the autoclaved pâté calibrators (slope = -3.7120 and efficiency = 86.0%). Consequently, as an attempt to improve the real-time PCR quantitative performance, the parallel amplification of an endogenous reference gene was performed to normalize the calibration models (Table 2). This approach considers eventual amplification differences owing to variable DNA recovery, purity, and integrity because of food matrix and processing [9,19,20]. Figure 2 presents the normalized calibration curves using the ΔCq values of reference mixtures of 50% to 0.05% (*w/w*) of roe deer meat in simulated pâté, raw and after thermally processed. As verified, the obtained curve parameters met the acceptance criteria regarding the correlation coefficient ($R^2 > 0.99$), slope (-3.5099 and -3.4939 for raw and autoclaved mixtures, respectively) and PCR efficiency slope (92.7% and 93.3% for raw and autoclaved mixtures, respectively). Therefore, normalization improved considerably the performance of both calibration models as inferred by their enhanced PCR efficiency. Both calibration plots are very similar, particularly regarding slope, exhibiting a small intercept deviation of nearly 0.6 cycles (Figure 2). This difference was quite reduced with method normalization, shifting from 1.3 to 0.6 cycles (Table 2), indicating that this process effectively attenuated the influence of thermal processing. Nonetheless, statistical differences were observed between raw and processed model mixtures for all levels of amplification, except for the 5% and 0.05% of roe deer in pâté (Figure 2). Differences between raw and severely processed matrices, such as autoclaved meats, are expected to occur due to the loss of DNA integrity, with consequent delayed amplification [9,19]. Therefore, the construction of a calibration curve accounting for the influence of autoclaving is crucial in the case of pâté samples. However, the same is not expected to occur for cured or mildly thermally treated products owing to their stabilized enzymatic activity because of the low water activity or thermal treatment, respectively. This was previously observed for the quantification of soybean material in meat products, where the normalized calibration curves for raw and mildly processed mixtures (66–68 °C for 5 h) were practically overlapping [32]. The feasibility of method normalization using the ΔCq method was previously demonstrated in the quantification of other meat species such pork [19,20] and horse [9] in food products, as well as plant species such as soybean [34].

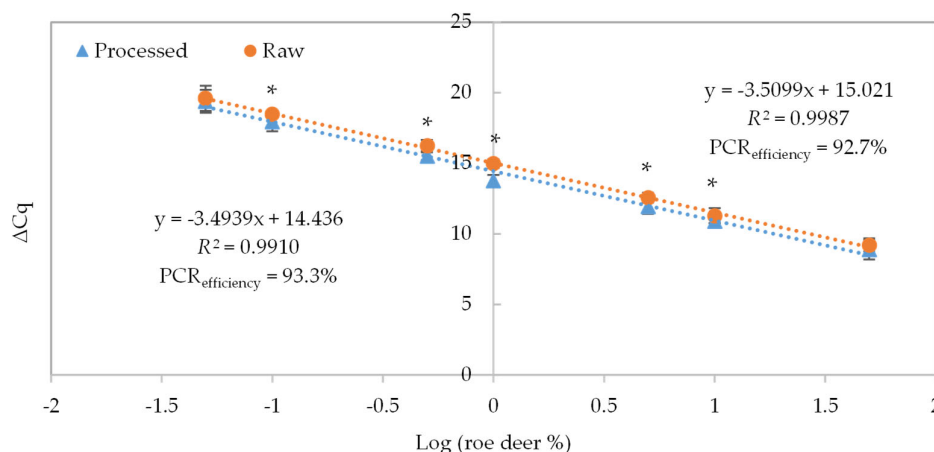


Figure 2. Normalized calibration curves achieved by TaqMan real-time PCR targeting the agouti signaling protein-encoding gene using the ΔCq method ($\Delta Cq = Cq_{ASIP} - Cq_{euk}$) and reference

mixtures of 50% to 0.05% (*w/w*) of roe deer meat in simulated pâté, raw (●), and thermally processed forms (▲) (*n* = 12 replicates). * Mean statistical differences (*p* < 0.05) following an unpaired t-test or Mann–Whitney test.

The LOQ, established as the lowest amount of the analyte that can be reliably measured, was established in this study at 0.05% (*w/w*) of roe deer meat in pork pâté for both raw and heat-treated mixtures. This study achieved a significant improvement compared to previous research attaining LOQ values between 0.125% and 0.5% (*w/w*) for roe deer [23,25,31]. Furthermore, an interlaboratory ring trial involving 14 European laboratories employed meat mixtures ranging from 2% to 49.4% for roe deer content [26], highlighting the widest quantitative range of the proposed method targeting roe deer herein.

The present study reports, for the first time, a normalized calibration curve prepared with matrix-adapted standards for the quantification of roe deer, accounting for the influence of food matrix and food processing, which is only comparable with the previously obtained calibration curve targeting red deer [27].

3.4. Validation

Method validation is required to establish the reliability, consistency, and accuracy of the developed novel method, confirming its fitness for quantification and its easy adoption in different laboratories [36]. Accordingly, four blind sample pairs were prepared with varying proportions (30%, 15%, 3%, and 0.3%, *w/w*) of roe deer meat incorporated into both raw and autoclaved pork pâté and used to assess the effectiveness of the quantitative real-time PCR assay. The roe deer contents of raw and thermally treated blind samples were estimated using the respective normalized calibration models of Figure 2 and compared with the actual values displayed in Table 3. Key parameters such as coefficient of variation (CV) and bias were considered in this evaluation to assess the method's precision and trueness, respectively. The CV data, expressing the relative standard deviation of the results obtained under repetitive conditions, were within 8.1–23.0% and 7.0–17.5% for raw and processed samples, respectively. The bias data, expressing the proximity between the actual and estimated roe deer contents, varied from −21.9 to 1.9% and from −12.1 to 13.6%, for raw and processed samples, respectively. Therefore, both CV and bias data meet the acceptance criteria (CV ≤ 25% and bias ±25%) for quantification by real-time PCR assays [34].

Table 3. Validation data determined by quantitative real-time PCR applied to blind mixtures.

Blind Samples	Actual Value (%)	Estimated Value (%) ^a	SD ^b	CV (%) ^c	Bias (%) ^d
Raw mixtures					
A	30	30.57	5.93	19.4	1.9
B	15	13.44	1.08	8.1	−11.6
C	3	2.46	0.22	8.8	−21.9
D	0.3	0.29	0.07	23.0	−2.2
Processed mixtures					
E	30	34.29	5.99	17.5	12.5
F	15	13.88	2.30	16.5	−8.0
G	3	3.47	0.24	7.0	13.6
H	0.3	0.27	0.05	17.9	−12.1

^a Mean values of replicate assays (*n* = 12). ^b SD—standard deviation. ^c CV—coefficient of variation. ^d Bias = ((mean estimated value − true value)/true value × 100).

3.5. Applicability of the Method to Commercial Products

In total, 46 marketed meat products originating from four European countries, Poland (28 samples), Spain (8 samples), Portugal (6 samples), and France (4 samples), were tested to verify labeling compliance. These products encompassed various types of processed foods, including loin, canned meat, ham, terrine, pâté, salami, and sausages. Product labeling indicated the following meat species: 28 red deer, 14 roe deer, 2 fallow deer, and the remaining 2 wild boar or pig meat (Table 4). This diverse selection of products and game species served three purposes: (i) validation—final method validation through its application to commercial deer-containing products; (ii) labeling compliance—to assess the accuracy of manufacturer claims and ensure product labeling reflects the actual ingredients; and (iii) adulteration survey—to estimate the level of mislabeling or adulterations in roe deer meat products in several European countries. The results of the roe deer determination in the 46 commercial samples are summarized in Table 4, complemented with the red deer contents obtained in our previous study [27].

To proceed with roe deer detection and quantification, the commercial products were split into two groups based on their production methods: uncooked/mildly cooked up to 71 °C/cured samples (sausages #1–#24) and thermally treated samples (pâtés #25–#46). The appropriate calibration models, for raw and autoclaved mixtures, respectively, were then employed to each group. All products underwent an initial test to detect amplifiable DNA by qualitative PCR targeting of a universal marker of the nuclear 18S rRNA gene and by discarding any false negative result. Subsequently, the roe deer-specific ASIP primers were used to qualitatively determine the presence of roe deer DNA (Table 4). As summarized in Table 4, of the 14 products labeled as containing roe deer meat, the target species was detected by both qualitative PCR and real-time PCR in 9 of them. The analysis of those samples, declaring roe deer contents of 7.2% to 100% (*w/w*), revealed a significant discrepancy between estimated and labeled values for roe deer. All of them contained a substantially lower amount of roe deer than what was declared, with estimated values falling between 0.07% and 19.23% (*w/w*) (Figure S3, Supplementary Material). Sample #34, with the least significant difference between declared and quantified roe deer content, only contained half the labeled amount. Sample #15 had the lowest quantified value at 0.07%, and roe deer DNA was undetectable or below the detection limit in samples #11–#14, and #37. Other samples (#16, #31–#33, #35, #42, and #44) suffered high reductions within 70–90%. These results suggest that all roe deer labeled samples were adulterated by total and substantial substitution in 6 and 8 samples, respectively. This is a serious issue of product mislabeling for consumers, particularly concerning a product that typically commands a higher price compared to regular meat products.

For samples labeled as containing other game species, namely red deer (28), fallow deer (2), and pork/wild boar (2), roe deer DNA was quantified in 8 red deer samples (#1, #2, #7, #17, #18, #25, #26, and #29) and one fallow deer/game product (#19). In samples #1, #2, and #29, the estimated trace levels of roe deer (0.10–0.36%) might be attributed to contamination rather than adulteration, though sample #29 also declares roe deer. The remaining samples showed contents within 1.40–6.53% of roe deer. Interestingly, all these samples, except for sample #1 with 0.49% of red deer, also exhibited high or considerable contents of red deer. This result suggests that undeclared roe deer addition was performed to increase the proportion of deer species rather than replacing red deer.

The complementary analysis of both roe deer and red deer contents highlights that in samples #1, #11, and #30, the deer species were probably fully substituted with pork. The contents of samples #12–#15 and #37 suggest full substitution of roe deer with red deer, while samples #16 and #35 indicate partial replacement of roe deer with red deer. Fallow deer meat was replaced with red deer/roe deer and red deer in samples #19 and #36, respectively. Samples #20–#23, #25–#26, #30–#34, #38–#40, and #42–#44 indicate reduced proportions of red or roe deer, suggesting its substitution with pork. Moreover, undeclared roe deer was added in samples #2, #7, #17, and #18. Therefore, the results of roe deer detection and quantification improved the previous survey on red deer in game meat products that indicated an adulteration/mislabeling level of 48% [27], which was

further complemented and increased to 61%. This increase was also due to considering the partial reduction in labeled species in the present analysis.

Table 4. Analyzed of commercial game meat products by qualitative PCR and quantitative real-time PCR using the normalized ΔCq method targeting a reference gene (18S rRNA) and the agouti signaling protein (ASIP) gene of roe deer.

Code	Sample Description	Relevant Label Information	Origin	Qualitative PCR ^a		Real-Time PCR				Estimated Red Deer Content (Mean ± SD, % <i>w/w</i>) [29]
				18SRG-F/18SRG-R	ASIP-F/ASIP-R	18S rRNA (Cq ± SD) ^b	ASIP (Cq ± SD) ^b	Estimated Roe Deer Content (mean ± SD, % <i>w/w</i>)	CV ^c (%)	
Uncooked/cured										
#1	Salami with red deer	10% red deer meat	Poland	+	+	18.08 ± 0.11	37.21 ± 0.43	0.10 ± 0.00	3.61	0.49 ± 0.05
#2	Red deer and pork kabanos	51% red deer meat	Poland	+	+	16.54 ± 0.26	33.69 ± 0.42	0.36 ± 0.02	5.53	>50
#3	Dry mysliwska sausage with red deer	25% red deer meat	Poland	+	–					20.37 ± 1.61
#4	Red deer sausage	70% red deer meat	Poland	+	–					>50
#5	Red deer sausage Wild grill	30.6% red deer meat	Poland	+	+	15.53 ± 0.09	35.99 ± 0.69	< LOD		>50
#6	Juniper sausage	>50% red deer meat	Poland	+	–					>50
#7	‘Swojska’ sausage	<50% red deer meat	Poland	+	+	15.73 ± 0.17	30.04 ± 0.16	2.18 ± 0.25	11.54	>50
#8	Red deer salami	100% red deer meat	Poland	+	–					>50
#9	Red deer ‘kindziuk’	70% red deer meat	Poland	+	–					>50
#10	Tender red deer sausage	35.5% red deer meat	Poland	+	–					>50
#11	Roe deer kabanos	100% roe deer meat	Poland	+	–					–
#12	Roe deer sausage	70% roe deer meat	Poland	+	+	16.46 ± 0.17	37.91 ± 1.65	< LOD		19.85 ± 0.98
#13	Roe deer sausage	>50% roe deer meat	Poland	+	–					44.55 ± 3.64
#14	Roe deer sausage	<50% roe deer meat	Poland	+	-					>50
#15	Roe deer kabanos	100% roe deer meat	Poland	+	+/-	16.62 ± 0.31	36.20 ± 0.82	0.07 ± 0.01	13.15	>50
#16	Roe deer sausage	<50% roe deer meat	Poland	+	+	16.32 ± 0.22	29.53 ± 0.21	4.29 ± 0.27	6.23	>50
#17	Red deer sausage	60% red deer meat	Spain	+	+	17.48 ± 0.15	30.07 ± 0.40	6.53 ± 0.65	9.92	>50
#18	Red deer sausage	60% red deer meat	Spain	+	+	16.92 ± 0.12	31.98 ± 0.49	1.40 ± 0.35	24.72	>50
#19	Spicy fallow deer sausage	60% game meat	Spain	+	+	17.16 ± 0.26	30.11 ± 0.41	5.17 ± 0.31	6.06	>50

#20	Red deer sausage	60% red deer meat	Portugal	+	–						3.85 ± 0.20
#21	Red deer sausage with fine herbs	60% red deer meat	Portugal	+	–						18.68 ± 2.22
#22	Red deer sausage with sesame seeds	60% red deer meat	Portugal	+	–						29.73 ± 0.91
#23	Red deer sausage with pepper	60% red deer meat	Portugal	+	–						7.52 ± 0.42
#24	Red deer loin	100% red deer meat	Spain	+	–						>50
Thermally treated											
#25	Baked red deer polish pâté	70% red deer meat	Poland	+	+	17.50 ± 0.32	30.31 ± 0.59	3.81 ± 0.14	3.58		20.48 ± 0.15
#26	Red deer polish pâté (canned)	70% red deer meat	Poland	+	+	17.15 ± 0.10	31.01 ± 0.42	2.50 ± 0.43	17.24		13.84 ± 1.34
#27	Red deer sausage (canned)	56% red deer meat	Poland	+	–						>50
#28	Baked red deer polish pâté	36.6% red deer meat	Poland	+	–						>50
#29	Hunter's polish pâté (canned; 3 types of meat: red deer, wild boar, roe deer)	>33% red deer meat	Poland	+	+	18.34 ± 0.24	37.27 ± 0.72	0.11 ± 0.01	12.15		>50
#30	Red deer ham	100% red deer meat	Poland	+	–						–
#31	Baked roe deer polish pâté	70% roe deer meat	Poland	+	+	17.67 ± 0.28	28.37 ± 0.44	19.23 ± 1.87	9.71		–
#32	Roe deer polish pâté (canned)	70% roe deer meat	Poland	+	+	17.74 ± 0.30	29.19 ± 0.46	12.11 ± 0.44	3.63		–
#33	Roe deer sausage (canned)	55% roe deer meat	Poland	+	+	18.17 ± 0.40	29.30 ± 0.35	14.95 ± 1.79	11.98		–
#34	Baked polish pork pâté with roe deer and Armagnac	20% roe deer meat	Poland	+	+	16.34 ± 0.17	27.33 ± 0.17	11.18 ± 1.23	11.04		–
#35	Baked roe deer polish pâté (baked)	51% roe deer meat	Poland	+	+	17.23 ± 0.18	28.15 ± 0.26	11.80 ± 0.08	0.71		7.21 ± 1.76

#36	Fallow deer pâté	25% fallow deer meat	Spain	+	–						15.96 ± 3.00
#37	Roe deer pâté with port wine	7.2% roe deer meat	Spain	+	–						18.95 ± 1.03
#38	Red deer pâté with boletus	20% red deer meat	Spain	+	–						8.69 ± 0.44
#39	Red deer pâté	25% red deer meat	Portugal	+	–						0.87 ± 0.21
#40	Red deer pâté with extra virgin olive oil	11.62% red deer meat	Spain	+	–						6.68 ± 1.28
#41	Wild boar terrine with cognac	21% wild boar meat	France	+	–						–
#42	Roe deer terrine with cognac	21% roe deer meat	France	+	+	16.15 ± 0.41	28.12 ± 0.24	5.86 ± 1.01	17.32		–
#43	Red deer terrine with cognac	21% red deer meat	France	+	–						22.23 ± 1.33
#44	Roe deer terrine with juniper berries	21% roe deer meat	France	+	+	19.30 ± 0.31	30.97 ± 0.38	7.12 ± 0.97	13.60		–
#45	Red deer pâté	30.52% red deer meat	Portugal	+	–						7.47 ± 0.8
#46	Pork kabanus	Pork meat	Poland	+	–						–

^a (+), positive amplification, (–), no detected amplification. ^b Mean quantification cycles (Cq) of replicate assays ($n = 8$) ± standard deviation (SD). ^c CV—coefficient of variation.

Summarizing the analysis of all 46 products by country of origin and type of mislabeling/adulteration, the data revealed that 100% of samples from Portugal had their red deer content reduced, suggesting its partial substitution with pork. The same type of adulteration was observed in 50% of samples from France. In 87.5% of samples from Spain, two types of mislabeling/adulterations were noted, namely the addition of undeclared roe deer and species substitution of fallow deer with other deer species and red deer with pork. The samples of Poland accounted for the most representative group, but they were also diverse regarding the identified mislabeling/adulterations (57%), which were due to the absence of deer species, reduced deer content, substitution of roe deer with red deer, and undeclared addition of roe deer.

The results underscore the importance of routine testing for meat products, particularly to identify adulteration through addition, substitution, and mislabeling. The results showed significant mislabeling across various game meat products. Roe deer meat was detected at trace levels in products labeled as containing other species, also suggesting contamination within the game industry. Furthermore, undeclared roe deer content in some products highlights major discrepancies in labeling, raising concerns about transparency and potential consumer deception. The reduced amount of the labeled roe deer species as well as the presence of undeclared red deer in roe deer-labeled products can indicate economically motivated adulteration practices. However, it is important to emphasize that these findings may not necessarily reflect the general food safety practices within these countries, but rather the specific samples included in this study. The mislabeling detected is likely caused by species misidentification due to their close evolutionary relationship belonging to the Cervidae family and, therefore, non-malicious mistakes made in the addition of meat from deer species by less skilled workers. The second highly probable reason may be differences in the price of deer meat sold by hunting districts and the fact that red deer meat is, in general, half the price of roe deer. Finally, the third reason may be the difference in the availability of fresh game meat throughout the year, i.e., roe deer meat is much more expensive but available for processing for more months of the year compared to red deer, approximately nine months versus six months.

Overall, the high prevalence of mislabeled or adulterated products underlines the need for stricter regulations and more rigorous monitoring to ensure consumer trust and adherence to labeling standards. The present results highlight the need to extend the study to a wider range of game meat products in EU-consuming countries, such as Italy, Germany, Czechia, the United Kingdom, and Sweden. The diversity of products should also be enlarged, including traditional products like stew/goulash and dumplings, as well as wild boar products considering their high relevance.

4. Conclusions

This research successfully addressed the issue of roe deer meat adulteration by developing a novel real-time quantitative PCR system targeting the roe deer ASIP-encoding gene. The method utilizes well-designed reference model mixtures that accurately reflect commercially available products. Two quantitative real-time PCR systems, one suitable for raw/mildly treated products and another for severely processed (autoclaving) meat products, were successfully developed using the normalized ΔC_q method. Both systems achieved absolute and relative LOD and LOQ of 0.04 ng of DNA and 0.05% (*w/w*) of roe deer in simulated pâté, respectively. Following validation with eight blind samples, which highlighted the precision and trueness of the approach, the method was applied to commercial products. The analysis of 46 game meat samples from Poland, Portugal, Spain, and France revealed a high level of discrepancies between manufacturers' claims and the actual presence of roe deer in some products. All the 14-roe deer labeled samples were mislabeled/adulterated by total or partial species substitution. A global analysis of all samples, combining the results of our previous study surveying red deer species, identified 61% of mislabeled/adulterated samples due to the absence of deer species, reduced deer content, substitution of roe deer with red deer, substitution of fallow deer with other deer

species and red deer with pork, and undeclared addition of roe deer. This highlights the effectiveness of the method in detecting/quantifying meat adulterations.

Therefore, the developed real-time PCR assay proved to be an effective tool for detecting and quantifying roe deer, demonstrating its potential as a reliable method for food authenticity testing. This method significantly contributes to the existing knowledge on roe deer meat authentication and demonstrates its usefulness as a tool for food inspection to ensure effective labeling compliance. Consequently, it will contribute to valorizing the game meat products, while promoting consumer trust and transparency, as well as fair trade among producers.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/foods13233728/s1>: Figure S1: Amplification curves obtained by real-time PCR with a TaqMan probe targeting the ASIP gene using DNA extracts of roe deer, red deer, and fallow deer at the same concentration (50 ng/μL); Figure S2: Agarose gel electrophoresis of PCR products targeting the agouti signaling protein (ASIP) gene of roe deer using serially diluted roe deer DNA. Legend: M, 100 bp DNA Ladder; lane 1, 40 ng; lane 2, 4 ng; lane 3, 0.4 ng; lane 4, 0.04 ng; lane 5, 0.004 ng; NC, negative control; Figure S3: Comparison of declared versus estimated roe deer contents by real-time PCR; Table S1: Results of qualitative PCR targeting a universal eukaryotic region of the 18S rRNA gene and the agouti signaling protein (ASIP) gene of roe deer using several relevant animal and plant species for cross-reactivity testing.

Author Contributions: Conceptualization, M.M. and I.M.; methodology, R.B., C.V., J.C., and A.S.; formal analysis, B.M.A., R.B., C.V. and J.C.; investigation, B.M.A., R.B., C.V., J.C. and A.S.; resources, M.M.; writing—original draft preparation, B.M.A.; writing—review and editing, C.V., M.M. and I.M.; visualization, B.M.A.; supervision, M.M. and I.M.; project administration, M.M. and I.M.; funding acquisition, M.M. and I.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research received national funds from FCT (Fundação para a Ciência e Tecnologia), Portugal, through the strategic funding of UIDB/50006/2020 from FCT/MCTES. C. Villa, J. Costa, and I. Mafrá thank FCT for funding through the Individual Call to Scientific Employment Stimulus (2023.07644.CEECIND, 2021.03583.CEECIND/CP1662/CT0012, and 2021.03670.CEECIND/CP1662/CT0011, respectively). This research was supported by the National Science Centre, Poland, project number 2020/37/B/NZ9/00082.

Data Availability Statement: The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author.

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

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A novel Normalized Quantitative Real-Time PCR Approach for Ensuring Roe Deer (*Capreolus capreolus*) Meat Authenticity in Game Meat Foods

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Table S1. Results of qualitative PCR targeting a universal eukaryotic region of the 18S rRNA gene and the agouti signaling protein (ASIP) gene of roe deer using several relevant animal and plant species for cross-reactivity testing.

Common name	Scientific name	Qualitative PCR	
		18SRG-F/18SRG-R	ASIP-F/ASIP-R
Roe deer	<i>Capreolus capreolus</i>	+	+
Red deer	<i>Cervus elaphus</i>	+	+
Fallow deer	<i>Dama dama</i>	+	+
Chicken	<i>Gallus gallus</i>	+	-
Beef	<i>Bos taurus</i>	+	-
Horse	<i>Equus ferus caballus</i>	+	-
Pheasant	<i>Phasianus colchicus</i>	+	-
Lamb	<i>Ovis aries</i>	+	-
Goat	<i>Capra hircus</i>	+	-
Duck	<i>Anas platyrhynchos</i>	+	-
Guinea fowl	<i>Numida meleagris</i>	+	-
Quail	<i>Coturnix coturnix</i>	+	-
Pig	<i>Sus scrofa domestica</i>	+	-
Rabbit	<i>Oryctolagus cuniculus</i>	+	-
Pigeon	<i>Columba livia</i>	+	-
Turkey	<i>Meleagris gallopavo</i>	+	-
Wild boar	<i>Sus scrofa</i>	+	-
Soybean	<i>Glycine max</i>	+	-
Pine nut	<i>Pinus species</i>	+	-
Wheat	<i>Triticum aestivum</i>	+	-
Rosemary	<i>Rosmarinus officinalis</i>	+	-
Mustard	<i>Brassica juncea</i>	+	-
Coriander	<i>Coriandrum sativum</i>	+	-
Onion	<i>Allium cepa</i>	+	-
Garlic	<i>Allium sativum</i>	+	-
Parsley	<i>Petroselinum crispum</i>	+	-
Bay leaves	<i>Laurus nobilis</i>	+	-
Sage	<i>Salvia officinalis</i>	+	-
Fennel	<i>Foeniculum vulgare</i>	+	-
Vine	<i>Vitis vinifera</i>	+	-
Olive	<i>Olea europaea</i>	+	-
Ginger	<i>Zingiber officinale</i>	+	-
Oregano	<i>Origanum vulgare</i>	+	-
White pepper	<i>Piper nigrum</i>	+	-
Sweet chilli	<i>Capsicum annuum</i>	+	-
Chilli	<i>Capsicum spp</i>	+	-
Corn	<i>Zea mays</i>	+	-

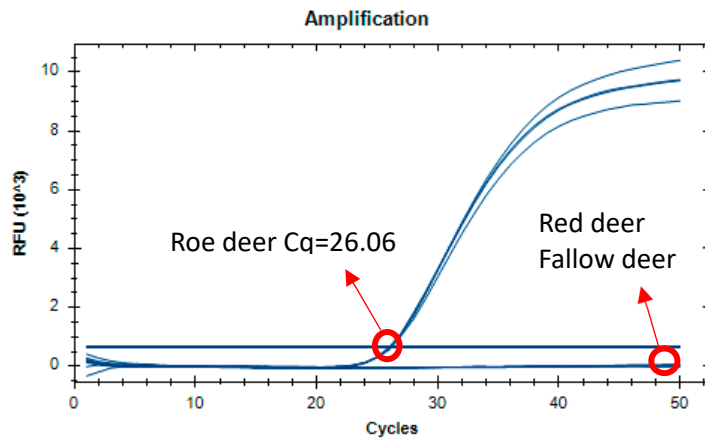


Fig. S1. Amplification curves obtained by real-time PCR with a TaqMan probe targeting the ASIP gene using DNA extracts of roe deer, red deer and fallow deer at the same concentration (50 ng/ μ L).

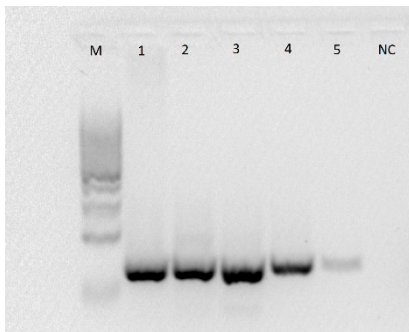


Fig. S2. Agarose gel electrophoresis of PCR products targeting agouti signalling protein (ASIP) gene of roe deer using serially diluted roe deer DNA. Legend: M, 100 bp DNA Ladder; lane 1, 40 ng; lane 2, 4 ng; lane 3, 0.4 ng; lane 4, 0.04 ng; lane 5, 0.004 ng; NC, negative control.

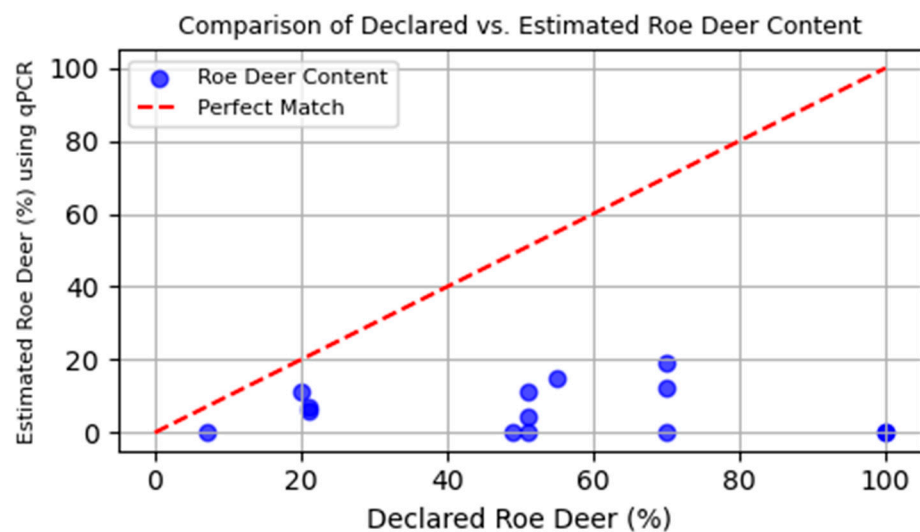


Fig. S3. Comparison of declared *versus* estimated roe deer contents by real-time PCR.

Publication 5 (P5)

Adenuga, B. M., Spychaj. A, Montowska, M. (2025). A new nuclear marker for quantitative analysis of wild boar and domestic pig meat in game meat products using PLAG1 zinc finger gene. *Scientific Reports*, 15, 20454. <https://doi.org/10.1038/s41598-025-05167-x>



OPEN

A new nuclear marker for quantitative analysis of wild boar and domestic pig meat in game meat products using PLAG1 zinc finger gene

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Wild boar meat and pork are commonly added to game products, either as forefront or background ingredients. Due to their lower cost and wider availability compared to deer species, there is a potential for producers to misrepresent the inclusion of these species in more expensive game products. Therefore, a novel, highly specific TaqMan qPCR method targeting a single-copy 127-bp region of the PLAG1 zinc finger gene was developed for quantifying these subspecies in processed foodstuff. The individual qPCR systems, adapted to different processing conditions (raw and thermally treated), were implemented using a matrix-adapted reference system. These systems were validated using laboratory-prepared wild boar-in-beef patties. The assay achieved satisfactory performance parameters for fraud detection, reaching a sensitivity of 0.1 ng of wild boar or pig DNA, and both detection and quantification limits (LOD and LOQ) at 0.1% for raw and 0.5% (w/w) for thermally processed samples, respectively. Subsequently, commercial game meat and pork products were analyzed to assess their compliance with labeling claims. The results revealed instances of undeclared species, likely intentional mislabeling, and cross-contamination. The developed method can serve as a valuable tool for ensuring label compliance, preventing food fraud, and upholding halal specifications in the game meat industry.

Keywords Food authentication, Meat products, Wild boar/Pork quantification, Nuclear marker, qPCR

Wild boars are omnivorous mammals with a rapidly expanding global population, making them prominent game animals, especially in Europe. Poland has experienced a particularly significant surge in wild boar numbers with more than 160,000 wild boars harvested during the 2022/2023 hunting season¹ substantially contributing to the Polish game meat market. Wild boar meat offers nutritional advantages over pork due to its leaner profile but one critical aspect of ensuring meat quality and safety is accurate species identification in game meat products. OPSON operations led by Europol and food market controls conducted by national inspections in individual countries underline the need for continuous control since meat products' adulteration and substitution counterfeiting techniques are going to continue in a situation of increasing inflation and pressure to reduce costs^{2,3}. The risk of irregularities is particularly high in developing countries where the meat sales system is unorganized, a legal framework for the prevention of meat products against fraud has loopholes and inspections do not have sufficiently advanced molecular methods⁴.

Food Crime Strategic Assessment² specifically highlights cases of quantitative adulteration of processed red meat products with chicken meat and high fat and sinew ingredients as lower-cost protein substitutes especially when the price of red meat increases. The correct labelling of wild boar meat or pork is crucial for protecting consumer choices, addressing allergies, adhering to religious dietary restrictions, upholding ethical standards, promoting fair trade, and ensuring product quality^{5,6}. Furthermore, the higher economic and nutritional value of game meat due to its limited availability in relation to common farm animals status necessitates the need for robust methods of ingredient identification, especially in processed meat products.

Over the last two decades, studies on wild boar meat identification have explored proteomics and DNA-based methodologies⁷. Comparative proteomic analysis revealed quantitative differences at the proteome level

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between wild boar and pork meat⁸. In many Muslim-majority countries, such as Indonesia, Iran, and Egypt, the detection of non-halal meat sources, including wild boar and pig, is crucial for ensuring food authenticity and religious compliance. Mitochondrial DNA markers^{9,10} such as D-loop, cytochrome b (*cyt-b*), NADH dehydrogenase subunit 5 (ND5), 12 S rRNA, COI and ATP synthase 6, have been commonly used for this purpose¹¹. Mitochondrial genes are abundant, with their DNA present in multiple copies per cell, making the detection of extremely low levels of mtDNA feasible (down to 1 pg)^{11,12}. However, the varying copy number of mitochondrial DNA across different tissues complicates accurate quantification, potentially leading to under- or overestimation¹³.

Microsatellites, mitochondrial DNA markers and single nucleotide polymorphisms (SNPs) are some of the DNA-based techniques that have been developed to distinguish wild boar meat from domestic pig. Microsatellite markers require a large reference dataset to enable accurate species assignment¹⁴. SNPs such as in the nuclear melanocortin receptor 1 (MC1R) and nuclear receptor 6 group A member 1 (NR6A1) genes have shown promising potential as a differentiation tool^{15–18}. However, these approaches have resulted in complex and expensive methods that often require a combination of multiple tests, and the risk of misclassification between these two subspecies remains high due to closely related ancestry, crossbreeding and the resulting hybrids between wild boars, domestic pigs and also invasive wild pigs^{19,20}. Overall, while these methods are promising, many of them reach their limits in terms of practicality or applicability, especially when analyzing complex meat mixtures.

Intercalation dyes in real-time PCR technique have been used previously to quantify pork or wild boar meat in food products, due to their ease of use, undemanding preparation, and high sensitivity^{10,21,22}. However, this approach is limited by the general non-specificity in amplicon detection, leading sometimes to erroneous positive results. Relatively few studies have quantified pork and wild boar meat using nuclear genes. Recently, Wang et al.⁶ adapted the β -actin gene in combination with a TaqMan probe²³ for pork quantitative studies in processed foods. The TaqMan probe approach offers the superior advantage of ensuring additional specificity through hybridization to the target gene, with the fluorophore only detected after successful binding to the target, reducing the likelihood of false positives. This mechanism enables higher sensitivity with TaqMan probes compared to intercalating dyes, due to reduced interference from non-specific binding in the background. Therefore, the TaqMan probe technology was employed in the present study to ensure both high specificity and sensitivity in the detection and quantification of wild boar meat.

To address the limitations of existing methods, this study proposes a novel approach using a nuclear gene-based primer targeting the PLAG1 zinc finger gene (previously named pleiomorphic adenoma gene 1). The PLAG1 gene has been associated (together with NR6A1 and LCORL genes) with the elongation of the back and an increased number of vertebrae in the domestic pig²⁴. Combined with TaqMan probe chemistry and matrix-adapted standards, this method can accurately detect and quantify wild boar meat in complex processed meat products, even under application of various processing treatments that can degrade DNA. This approach is particularly valuable for ensuring the authenticity and quality of game meat products, both in Europe and globally.

Materials and methods

Samples

European wild boar meat was collected from hunting activities carried out in hunting circuit No. 57, Sarbia Forest District, Greater Poland. Fallow deer meat was collected from animals hunted in the Puszcza Zielonka Landscape Park for experimental purposes conducted by the University of Life Sciences in Poznań. All animals were already hunted and samples were collected. Animals were not sacrificed for the study. Other meat samples (roe deer, red deer, cattle, rabbit, pork, pigeon, duck, guinea fowl, quail, pheasant, chicken, turkey, lamb, horse, and goat) were purchased from reliable sources (e.g., butcher shops, supermarkets) in Poznań, Poland. Other game meat was sourced from specialized local stores, including a shop at the Polish Hunting Association, Poznań District. The raw meat samples were prepared by removing the outer layers to eliminate contamination. Subsequently, they were cut into small pieces and stored in Eppendorf tubes at -80°C until use.

Seven plant-based ingredients (breadcrumbs/wheat, black pepper, rosemary, ginger, garlic, coriander, and onions) were purchased and analyzed in this experiment to test specificity. Thirty-eight commercial products evaluated in this study were acquired from local markets as well as specialized game meat stores in Poland. These samples were chopped into small pieces and homogenized in a Moulinex S 643.23 food processor (Moulinex, Paris, France) meat blender with separate containers. All equipment and surfaces were treated with a DNA sanitization solution before, during, and after homogenization to prevent contamination.

Preparation of reference mixtures

To simulate a complex, processed meat product (patty), reference binary mixtures were prepared in the laboratory. These mixtures contained wild boar meat and beef, along with additional ingredients commonly found in patties, such as salt, ice, breadcrumbs, pepper, rosemary, ginger, coriander, and onions. First, beef and wild boar meat were chopped into small pieces and then minced using TrinityPro Electrolux Professional food processor. The minced meat from the processor was then placed in separate stainless steel bowls. Measured quantities of the additional ingredients were added to each bowl, and the samples were thoroughly mixed for several minutes. The percentage composition of the mixtures was as follows: meat (84.35%), salt (1.52%), water (8.44%), breadcrumbs (5.06%), pepper (0.21%), rosemary (0.08%), ginger (0.08%), garlic (0.08%), coriander (0.08%), onion (0.08%). These mixtures were then set aside and refrigerated at 4°C .

Next, reference mixtures with varying ratios of wild boar meat in beef were prepared. One hundred grams of each unmixed meat (representing 0% and 100% wild boar content) were weighed out first, then subsequent mixtures were created by combining known proportions of beef and wild boar meat. Starting with 300 g each of

pure beef and pure wild boar meat, serial dilutions were performed to obtain mixtures containing 0.01%, 0.05%, 0.1%, 0.5%, 1%, 5%, and 10% (w/w) of wild boar meat in beef. This dilution was achieved by serially diluting the initial 50% mixture of wild boar meat in beef with specific amounts of pure beef patty. Each resulting mixture was then thoroughly homogenized in separate containers using the Moulinex S food processor. In addition to the reference mixtures, blind mixtures were prepared to validate the analytical method. These samples contained known concentrations of wild boar meat in beef patty (30%, 15%, 3%, and 0.3%). The preparation process for the blind mixtures followed the same steps as outlined for the reference mixtures.

Finally, three sets of both reference and blind samples were prepared for analysis: one set remained raw, one set was oven-baked at 160 °C, till a core temperature of 75 °C was reached, in a Rational Combi convection oven model SCC 61 (Landsbergam Lech, Germany), and finally, one set was autoclaved at 121 °C for 20 min. The weight of each heat-treated sample was kept similar to ensure even cooking throughout the process. All prepared heat-treated products were cooled down at room temperature before storage at – 80 °C prior to further analysis. The preparation method for reference and blind samples in this study aligns with previously established procedures used for red deer and roe deer authentication in processed game meat products^{25,26}.

Primers and probe

The PLAG1 reference sequences for *Suidae* species were downloaded from the NCBI nucleotides repository. These sequences were aligned using the Genious Prime Sequence alignment software to observe regions with high dissimilarities among species. The sequence of common warthog (*Phacochoerus africanus*), a wild member of the *Suidae* family, was selected for further proceedings due to the lack of a wild boar reference equivalent in the NCBI database. A region of interest was selected and transferred to the PrimerBlast tool available on the NCBI website for primer sequence design. Primers with a product length of 127 bp were designed, and their properties were assessed for single base runs, dinucleotide base runs, length, percent G-C, Tm (nearest neighbour), GC clamp, self-annealing, and hairpin formation using the Primer Stats tool (https://www.bioinformatics.org/sms2/pcr_primer_stats.html). Additionally, the uniqueness of each primer pair to the target was assessed by conducting a short sequence blast of each forward and reverse primer. A 25 bp length hydrolysis probe was designed using the Integrated DNA Technologies’ tool, Primer Quest (<https://www.idtdna.com/PrimerQuest/Home/>). The probe was designed in closer proximity to the forward primer to yield a higher signal and was labeled with FAM and BHQ1 fluorophores and quenchers. Myostatin (MSTN) gene primers and probes adapted from Druml et al.²⁷ were used as a positive control to establish the presence of DNA in all tested samples. Both, genes primers and probes details are listed in Table 1. The primers and probes were synthesised by Eurofins Genomics AT GmbH (Ebersberg, Germany).

DNA extraction

Meat samples were retrieved from – 80 °C storage and 200 mg of each sample was weighed out. DNA was isolated from all samples, including a blank for contamination checks, at least in triplicate using the Macherey-Nagel food DNA extraction kit, NucleoSpin® Food (Macherey-Nagel, Düren, Germany), following the protocol provided by the manufacturer with slight modifications. For plant samples 100 mg of each were weighed and was done using the Macherey-Nagel Plant DNA extraction kit, following the NucleoSpin® Plant II mini protocol. Extracts were measured for DNA concentration using the NanoDrop™ OneC microvolume UV-Vis spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) to assess UV absorbance at 260 nm (A260) and 280 nm (A280). The DNA was then stored at – 20 °C and later diluted to a concentration of 10 ng/ µL and stored at 4 °C for regular use.

Qualitative PCR

To establish optimal reaction conditions for the subsequent quantitative real-time PCR (qPCR) assays using the PLAG1 primers, qualitative PCR experiments were initially performed. Gradient PCR was employed to evaluate a range of annealing temperatures (50 °C to 70 °C) and varying extension times. This optimization process identified the most suitable amplification conditions as follows: 95 °C for 5 min for initial denaturation, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 50 s, extension at 72 °C for 30 s, and a final extension step at 72 °C for 5 min. For the MSTN primers, conventional PCR conditions were: 95 °C for 5 min for initial denaturation, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, extension at 72 °C for 30 s, and a final extension step at 72 °C for 5 min.

Each reaction mixture for endpoint analysis contained a total volume of 25 µL and comprised the following components: 10 ng of template DNA, 1 unit of Gold Taq DNA polymerase, 1 x Buffer Gold, 2 mM MgCl₂ (Syngen Biotech, Poland), 160 µM dNTP mix (Sigma-Aldrich, Schnelldorf, Germany), and 0.1 µM concentration of

Target species	Target gene	Primer	Sequence (5'-3')	Amplicon	NCBI accession/ Reference
Phacochoerus africanus	PLAG1 zinc finger	PLAG1-F	ACGCCATCTCTATTCCCGAG	127 bp	XM_047783901.1 (LOC125129313)
		PLAG1-R	AGCCCCGAGCTTAGATGACGA		
		PLAG1-P	5'-FAM-TCGAGAGCTATCTGATGGAGCTGCA- BHQ1-3'		
Mammals	Myostatin	MSTN-F	TTGTGCAAATCCTGAGACTCAT	70 bp	Druml et al., 2016 ²⁷
		MSTN-R	TTCAGAGATCGGATTCCAGTATA		
		MSTN -P	5'FAM-CCCATGAAAGACGGTACAAG-BHQ1-3'		

Table 1. Primers and probes used in this study.

each primer (PLAG1-F/PLAG1-R or MSTN-F/MSTN-R). Sterile water for molecular biology (Merck Millipore, Darmstadt, Germany) was added to reach the final reaction volume. Amplification was performed in an Azure Cielo™ 6 Real-Time PCR Systems thermal cycler (Azure Biosystems Dublin, CA, USA).

Amplification products were visualized on a 2% agarose gel (Sigma-Aldrich, Schnellendorf, Germany) containing SimplySafe™ stain (EURx Ltd., Gdańsk Poland) and submerged in 1x TBE buffer (tris-borate-EDTA). Electrophoresis was carried out in a SUB15 gel unit (Hoefer, Inc., Holliston, MA, USA) at 125 V for 45 min. A PCR Low Ladder (Sigma-Aldrich) was used to determine the size of the amplified fragments. Gels were visualized under UV light using a Gel Doc™ XR + System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for documentation and analysis.

Real-time PCR

For each real-time PCR analysis run, each reaction volume of 20 µL contained 10 ng of template DNA, 1 unit of Gold Taq DNA polymerase, 1 x Buffer Gold, 2.5 mM MgCl₂, 200 µM dNTP mix, 0.16 µM concentration of each primer, and 0.8 µM TaqMan Probe. Sterile water for molecular biology (Merck Millipore, Darmstadt, Germany) was added to reach the final reaction volume. The temperature conditions were as follows for PLAG1: 95 °C for 5 min, 37 or 45 cycles of 95 °C for 1 min, and 60 °C for 50 s. Fluorescence were captured at the start of annealing step of every cycle while the MSTN conditions were: 95 °C for 5 min, 40 cycles of 95 °C for 30 s, and 52 °C for 1 min. Amplification was performed in an Azure Cielo™ 6 Real-Time PCR Systems thermal cycler (Azure Biosystems Dublin, CA, USA). A No Template Control (NTC) was used in all experiments.

Quantification and validation

DNA was extracted from reference samples containing 0.01–50% (w/w) wild boar meat in beef patty, prepared both raw and thermally treated. DNA samples were diluted to a final concentration of 10 ng/µL for real-time TaqMan probe-based amplification. For each reference sample, four technical replicates and three assay replicates were analyzed. The resulting cycle threshold (Cq) values were averaged to obtain mean Cq values for each standard. These mean Cq values were plotted against the logarithmic (w/w) % concentrations of the target species in the reference samples. Data were processed and analysed using the Azure Cielo™ Real-Time PCR System Manager Software (Azure Biosystems Dublin, CA, USA). Cycle threshold values were calculated using the software's manual option that allowed user-defined threshold settings. Assay efficiencies calculated from the standard curve slope met the acceptance criteria outlined by Bustin et al.²⁸ and FAO CAC/GL²⁹. PCR efficiencies ranged from 90 to 110%, with R² values exceeding 98% and slopes between −3.1 and −3.6. Each of the four blind samples prepared for each reference standard was analyzed using the developed standard curves through value interpolation based on the established linear relationship between the standard's Cq values and the target weight/weight concentration logarithm values.

Statistical analysis

Statistical analysis of qRT-PCR results was performed using Statistica 13.1 (StatSoft Inc., Tulsa, OK, USA). One-way analysis of variance (ANOVA) and Tukey's Honestly Significant Difference (HSD) was used to determine significant differences between mean values ($P < 0.05$). The effect of treatment and wild boar meat concentration was evaluated. The results were expressed as mean ± standard deviation.

Results and discussion

Specificity

The PLAG1 zinc finger gene was selected for its association with meat production and physical traits, emphasizing its importance for domestication and selective breeding²⁴. Given the close similarity between *Suidae* species, the common warthog (*Phacochoerus africanus*), a wild family member, was chosen as a model organism for primer development. An in silico analysis was performed to compare the nucleotide sequences of the PLAG1 gene of 16 animal species: common warthog (*Phacochoerus africanus*), domestic pig (*Sus scrofa*), buffalo (*Bubalus bubalis*), sheep (*Ovis aries*), goat (*Capra hircus*), red deer (*Cervus elaphus*), wild yak (*Bos mutus*), domestic cattle (*Bos taurus*), gray wolf (*Canis lupus*), common rat (*Rattus norvegicus*), horse (*Equus caballus*), human (*Homo sapiens*), chicken (*Gallus gallus*), duck (*Anas platyrhynchos*), turkey (*Meleagris gallopavo*) and goose (*Anser cygnoides*). The results revealed multiple nucleotide mismatches for all non-target species, except for domestic pig, which exhibited only one mismatch with the anchored query nucleotide sequences (Fig. 1).

The in silico analysis was subsequently validated experimentally through in vitro cross-testing. DNA samples from 17 animal species, including various game animals, birds and common slaughter animals, were subjected to analysis. Qualitative PCR amplification yielded visible bands on agarose gel electrophoresis exclusively for wild boar and pig (Fig. 2). This could support the applicability of the method developed here to a wider range of *Suidae*.

TaqMan probe-based real-time PCR analysis detected no amplification (Cq value) for most species, with the exception of rabbit and turkey, which exhibited minimal fluorescence signals at cycle 30. Since the quantification analysis in this study used a user-defined threshold, this cross-reactivity fell below detectable threshold and had no effect on the analysis. To assess potential cross-reactivity with non-animal components, seven plant species commonly used in meat processing were tested against the PLAG1 primers (Supplementary information Table S1). No cross-reactivity was observed in these samples. A universal PCR system using the myostatin primer/probe combinations^{27,30} confirmed the presence of amplifiable DNA in all mammalian species (Supplementary Material, Table S1).

Query	1	ACGCCATCTCTATTCCCGAGAAAGAACGCCGCTGAAGGGGGAGATCGAGAGCTATCTGATGGAGCTGCAGGGCGGCCGCCCTCGTCTCT	90
XM_047783901.1	1344		1433
XM_013996756.2	1237		1326
XM_044928757.2	4700	A.....CT.....T.....C.....T.....C.....	4789
XM_042254231.1	1062	A.....CT.....T.....C.....T.....C.....	1151
XM_043880362.1	1522	A.....CT.....T.....C.....AT.....C.....	1611
XM_013969222.2	1427	A.....CT.....T.....C.....AT.....C.....	1516
XM_005889161.1	1376	A.....CT.....T.....C.....T.....C.....	1463
XM_005215434.4	1092	A.....CT.A.....T.....C.....T.....C.....	1181
XM_038441198.1	1555	T.....T.....A.....A.....T.....T.....A.....A.....T.....T.....G.....	1642
XM_063287274.1	8810	A.....CT.A.....A.....T.....T.....A.....A.....T.....A.....A.....T.....A.....	8899
XM_023648589.1	1577	A.....T.....A.....AT.A.....A.....T.....T.....A.....T.....T.....T.....A.....	1664
XM_047421866.1	7674	A.....T.....A.....AT.A.....A.....T.....C.....T.....A.....A.....T.....T.....A.....	7761
XM_046911732.1	1648	AG.T.....G.....T.....A.....TT.....A.....A.....A.....T.....CT.A.....T.....AA.T.....TAT.....T.....G.....	1737
XM_038175435.1	1836	AG.T.....G.....T.....A.....AT.....A.....A.....A.....T.....CT.A.....T.....AA.T.....TAT.....T.....T.....A.....	1925
XM_019614040.2	13558	G.....T.....A.....TT.....A.....A.....A.....T.....CT.A.....T.....AA.T.....TAT.....T.....T.....A.....	13639
XM_066993005.1	1366	AG.T.....G.....T.....A.....AT.....A.....A.....A.....T.....CT.A.....T.....AA.T.....TAT.....T.....T.....A.....	1455

Query	91	CGCAGGACTCTC---CAGCGTCGTCATCTAAGCTCGGGCT	127
XM_047783901.1	1434		1470
XM_013996756.2	1327	G.....	1363
XM_044928757.2	4790	.C.....C..G..C..A..G...	4824
XM_042254231.1	1152	.C.....C..G..C..A..G...	1186
XM_043880362.1	1612	.C.....C..G..C..A..G...	1646
XM_013969222.2	1517	.C.....C..G..C..A..G...	1551
XM_005889161.1	1464	.C.....A..C..G..C..A..G...	1498
XM_005215434.4	1182	.C.....A..C..G..C..A..G...	1216
XM_038441198.1	1643	.C....T..C---A..A.....A...	1677
XM_063287274.1	8900	.C....T....---A..A..A..G.....T.A...	8934
XM_023648589.1	1665	.C.....T....---A..A.....A..A...	1699
XM_047421866.1	7762	.C..A..T...AAG..T...A.....A...	7799
XM_046911732.1	1738	.C....T....---A..A..T....A..A..A....	1774
XM_038175435.1	1926	.C....T....---A..A..T....A..A..A....	1962
XM_019614040.2	13640	.C....T....---A..A..T....A..A..A....	13676
XM_066993005.1	1456	.C....T....---A..A..T....A..A..A....	1492

Fig. 1. BLAST nucleotide sequence alignment of the PLAG1 gene of common warthog and pig-specific sequences against 14 other non-target species.

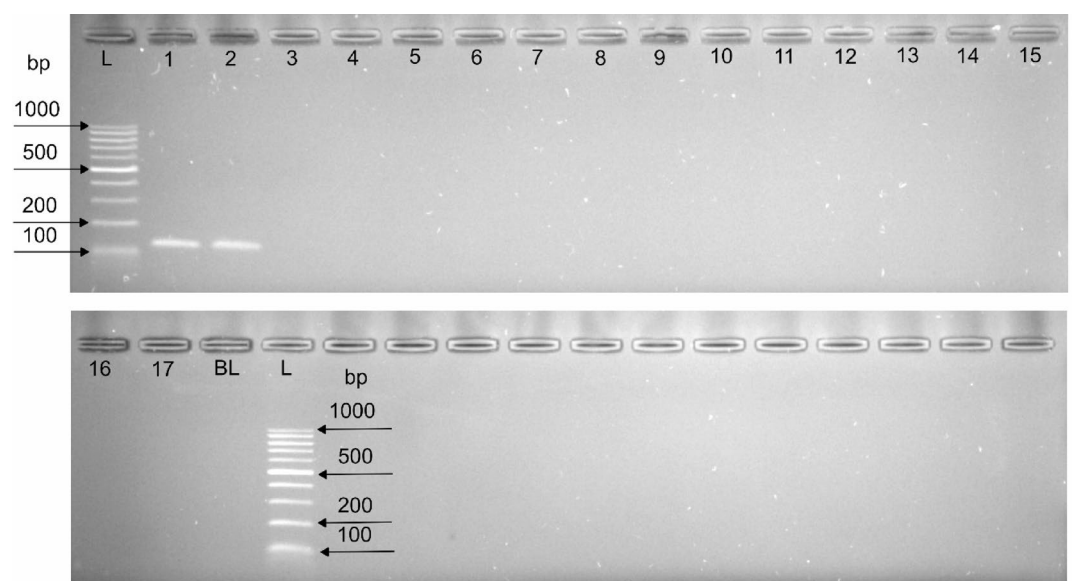


Fig. 2. PLAG1 gene primers' product in a species cross-reactivity experiment. Lanes: (L) molecular ladder; (1) wild boar; (2) pig; (3) red deer; (4) roe deer; (5) cattle; (6) goat; (7) chicken; (8) pigeon; (9) horse; (10) pheasant; (11) lamb; (12) duck; (13) guinea fowl; (14) quail; (15) rabbit; (16) turkey; (17) fallow deer; (BL) negative control.

Sensitivity

To evaluate the sensitivity of the assay, DNA extracted from raw wild boar meat was serially diluted 10-fold from 50 ng to 0.05 ng. An absolute sensitivity of 0.5 ng was determined, as this is the lowest concentration at which a weak band can be qualitatively detected (Figure S1). A further 10-fold serial dilution series of the same wild boar DNA (100 ng to 0.01 ng) was then amplified over three days with 3 replicates each using real-time PCR with PLAG1 primers and a TaqMan probe. The lowest detectable amount with 95% confidence was 0.1 ng. The generated standard curve, as shown in Fig. 3, showed a strong linear relationship ($R^2 = 0.992$) between the log of starting amount and C_q values, with an efficiency of 105.42% and a slope of -3.198 , all within acceptable ranges for quantitative PCR^{28,29}. A similar sensitivity was obtained for porcine DNA (Figure S2), also indicating a high amplification fidelity that meets the minimum requirements for a real-time PCR assay.

To determine the relative limit of detection (LOD) and the limit of quantification (LOQ) for the new qPCR system, serially diluted binary reference mixtures with wild boar meat in proportions of 0.01–50% (w/w) were analysed. The LOD, defined as the lowest weight% at which the target species (wild boar) can be detected, was set at 0.1% for raw and 0.5% for thermally treated (oven-cooked) samples. However, in our experiment, we obtained an unsatisfactory LOD value for autoclaved samples of 5% (w/w). The LOQ, defined as the lowest concentration at which the target species can be quantified with a relative standard deviation of no more than 25%, was also determined for the three sample types (raw, oven-cooked, autoclaved) based on 12 replicate analyses at 0.1%, 0.5% and 5% (w/w). Although the PLAG1 gene product was found to be relatively sensitive to autoclaving, our procedure applies to the identification of adulterants in game products because such products present on the market are mainly preserved by pasteurization.

The above obtained parameters are comparable to or even better than those previously described in the literature. Mayer & Hoehgegger³¹ reported LOD values of 2% and 5% for wild boar and pig respectively, by targeting sequence in the MCR1 gene. In the study reported by Kaltenbrunner et al.¹⁷ which aimed to differentiate wild boar and pig based on SNPs on chromosomes 1 and 9, the LOD for either wild boar or domestic pig

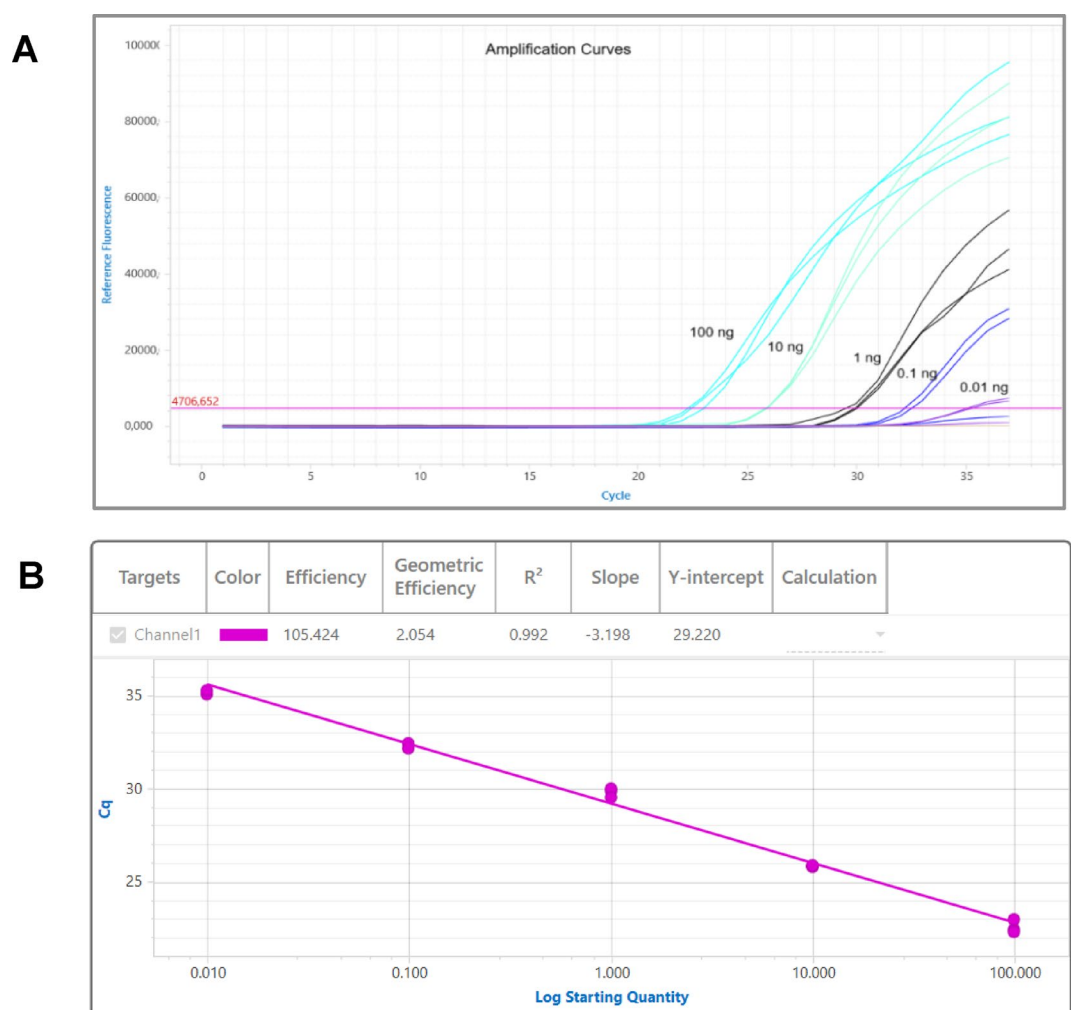


Fig. 3. Amplification (A) and standard (B) curves obtained by real-time PCR with a TaqMan probe targeting the PLAG1 zinc finger gene using 10-fold serially diluted wild boar DNA from 100 ng/ μ L to 0.01 ng/ μ L ($n = 3$ replicates).

DNA, determined by analysing DNA mixtures in herring sperm DNA, was 0.2% (w/w), however, the LOD determination for mixtures containing wild boar DNA in domestic pig DNA and vice versa failed due to cross-reactivity and signal suppression between these subspecies. Better detection limits were achieved when the assay targeted the pork *cyt-b* gene coupled with EvaGreen dye²¹. The LOD value reported in this study for raw pork mixed with beef was down to 0.0001% (w/w), although the reported sensitivity was reduced by two orders of magnitude (to 0.01%) after autoclaving meat mixtures. In another study using *cyt-b* mt-DNA, the LOD value for wild boar meat in reference beef meatballs was 0.1% (w/w)³². Xu et al.³³ using the nuclear β -actin gene and droplet digital PCR (ddPCR) method reported LOD 0.1% (w/w) and LOQ 1% (w/w) for mixtures of pork in beef made of drying oven meat. However, other researchers have previously described the application of a similar combination of the β -actin gene with ddPCR with estimated LOD at 0.01% (w/w) and LOQ at 0.1% (w/w) for pork in beef mixtures³⁴.

Quantitative detection of target species

The complex nature of meat products, characterized by the presence of multiple animal species, fats, connective tissues, and additives, necessitates a robust species quantification approach. Real-time PCR was selected for this study due to its high sensitivity, specificity, and ability to quantify DNA in real-time, making it suitable for analyzing complex food matrices. To establish calibration curves, a binary meat mixture simulating a real-world product was created by combining wild boar and beef with herbal ingredients and spices, followed by mechanical mixing and thermal treatment. DNA was extracted from all binary meat mixtures containing 0.01–50% (w/w) wild boar meat in beef, and these samples were processed in three states: raw, oven-cooked, and autoclaved. Four technical replicates and three assay replicates were analyzed for each reference sample. The resulting cycle threshold (Cq) values were averaged, and the mean Cq values were plotted against the logarithmic (w/w) % concentrations of the target species (Table 2).

The ANOVA results revealed significant differences in mean Cq values observed across different treatments for concentration levels from 50 to 1%, with the exception of the following pairs: 50% of wild boar in oven-cooked and autoclaved mixtures, 5%, 1% and 0.1% of wild boar in raw and oven-cooked mixtures (Table 2). Post-hoc Tukey’s test indicated that for higher inclusion levels ($\geq 1\%$), autoclaved samples had significantly higher Cq values (lower DNA recovery) compared to raw and oven-cooked samples ($P < 0.05$). At the 1% and 0.5% levels autoclaved samples showed a substantial reduction in positive detection rates (3/12), whereas raw and oven-treated samples maintained detectability. The delay in amplification observed in autoclaved samples was likely due to a deterioration in DNA integrity reducing its integrity and amplifiability.

The obtained standard curves are depicted in Fig. 4; Table 2 showed good linearity and repeatability with R^2 values exceeding 0.98, slope values between -3.12 and -3.25 and PCR efficiencies between 102.99% and 108.99%. The curve parameters met the acceptance criteria of MIQE²⁶ and FAO/CAC guidelines²⁷. Based on these robust calibration curves, the real-time PCR developed here was subsequently used for the quantitative detection of the target species, first in three sets of validation samples and then in a series of commercial samples.

In-lab samples validation

To evaluate the efficacy of the novel quantitative real-time PCR assay, a validation study was conducted using laboratory-prepared beef patties. Twelve samples, containing 30%, 15%, 3%, and 0.3% (w/w) wild boar meat, were processed in three different conditions: raw (samples A - D), oven-cooked (samples E - H), and autoclaved (samples I - L). The wild boar content in these samples was determined using the previously established calibration curves for each processing condition. Key performance parameters, including precision (coefficient

Model mixtures containing wild boar deer (% w/w)	Raw mixtures		Oven-cooked mixtures		Autoclaved mixtures	
	Cq $\pm$ SD*	Replicates (positive/total)	Cq $\pm$ SD	Replicates (positive/total)	Cq $\pm$ SD	Replicates (positive/total)
50	25.74 ^a $\pm$ 1.27	(12/12)	25.42 ^b $\pm$ 0.15	(12/12)	27.46 ^b $\pm$ 0.36	(12/12)
10	27.69 ^b $\pm$ 1.38	(12/12)	26.60 ^c $\pm$ 0.28	(12/12)	30.16 ^a $\pm$ 0.43	(12/12)
5	29.04 ^b $\pm$ 1.44	(12/12)	29.03 ^b $\pm$ 0.59	(11/12)	30.85 ^a $\pm$ 0.51	(11/12)
1	30.74 ^b $\pm$ 1.45	(12/12)	30.46 ^b $\pm$ 0.33	(12/12)	33.33 ^a $\pm$ 0.35	(3/12)
0.5	32.25 ^{ab} $\pm$ 1.55	(12/12)	31.45 ^b $\pm$ 0.14	(12/12)	33.75 ^a $\pm$ 0.15	(3/12)
0.1	34.06 $\pm$ 1.53	(11/12)	33.79 $\pm$ 0.41	(10/12)	NA	(0/12)
0.05	NA	(0/12)	35.14 $\pm$ 0.88	(10/12)	NA	(0/12)
0.01	NA	(0/12)	NA	(0/12)	NA	(0/12)
Correlation coefficient (R^2)	0.9945		0.9829		0.9899	
Slope	-3.1237		-3.2524		-3.1913	
Intercept	31.011		30.639		33.088	
PCR efficiency (%)	108.99		102.99		105.76	

Table 2. Real-time PCR results of the amplification of reference model mixtures of wild boar meat in beef to simulate raw and thermally processed (oven-cooked and autoclaved) patties targeting the PLAG 1 zinc finger gene of wild boar. *Mean cycle of quantification (Cq) $\pm$ standard deviation (SD); a,b, c – mean with different letters in a row indicate a significant effect of treatment ($P < 0.05$).

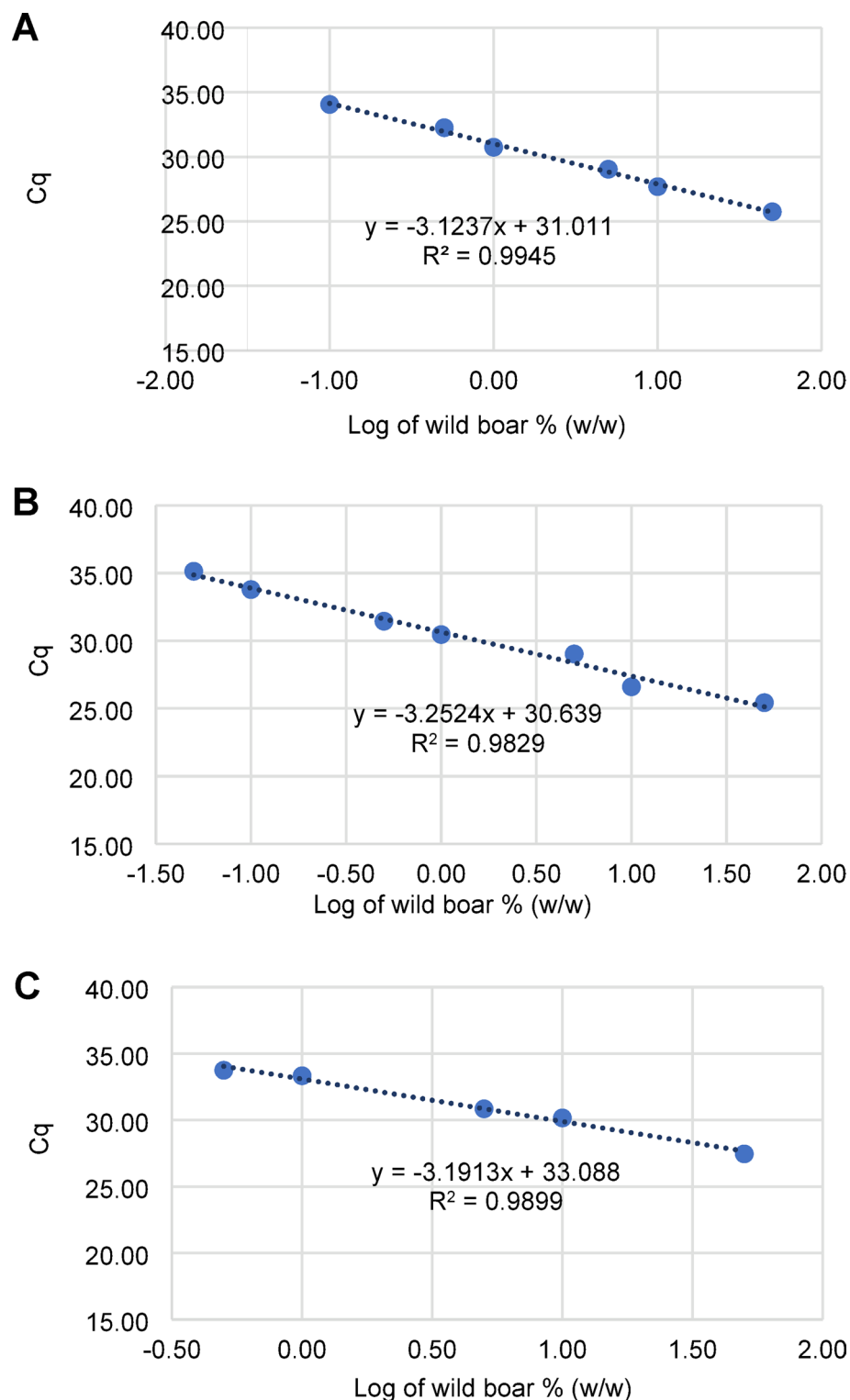


Fig. 4. Calibration curves obtained by real-time PCR with TaqMan probe targeting the PLAG1 zinc finger gene using the reference model mixtures of 50–0.01% (w/w) of wild boar meat in beef to simulate (A) raw, (B) thermally processed – oven cooked and (C) autoclaved patties ($n = 12$ replicates).

of variation, CV) and accuracy (bias), were assessed to evaluate the method's reliability. The results shown in Table 3 adhered to the CAC-GL guidelines for quantitative real-time PCR assays, with CV values below 25% and bias values within $\pm 25\%$ ²⁹.

For raw meat mixtures, the estimated wild boar content was generally accurate, with bias values ranging from –25 to 19%. The coefficient of variation ranged from 7.52 to 17.61%, indicating acceptable repeatability

Blind samples	Actual value (%)	Estimated value (%) ^a	SD ^b	CV (%) ^c	Bias (%) ^d
Raw mixtures					
A	30	27.07	2.68	9.90	−10
B	15	17.86	3.15	17.61	19
C	3	2.56	0.19	7.52	−15
D	0.3	0.23	0.03	15.11	−25
Oven-cooked mixtures					
E	30	39.30	2.70	6.87	31
F	15	11.20	0.59	5.26	−25
G	3	2.28	0.42	18.43	−24
H	0.3	0.26	0.03	10.19	−12
Autoclaved mixtures					
I	30	26.34	2.28	10.89	−12
J	15	11.97	0.86	7.23	−20
K	3	2.33	0.63	31.46	−22
L	0.3	> LOD	-	-	-

Table 3. Validation results of the quantitative real-time PCR assay applied to raw, oven-cooked and autoclaved blind meat mixtures. ^aValues are the mean of replicate assays ($n = 12$). ^bSD – standard deviation. ^cCV – coefficient of variation. ^dBias = ((mean estimated value-true value)/true value x 100).

across all concentration levels. For oven-cooked meat mixtures, the 3% target concentration (sample G) showed the highest variability with a CV of 18.43% and a bias of −24%. This indicates that the DNA was degraded by the heat treatment, which could affect the accuracy and precision of the test. In addition, sample E (30% target concentration) showed a bias of 31%, possibly due to uneven distribution of ingredients. The higher concentrations of the autoclaved mixtures showed acceptable values for bias and precision, while the lowest concentrations (0.3%) were below the detection and quantification limits (> 5% w/w LOD/LOQ).

Evaluation of commercial game meat products

To assess the accuracy of product labeling, thirty-eight commercially available game meat products (P1-P38) were sourced. These products were declared to contain wild boar or pig, with the exception of four products primarily composed of other game species (Table 4). The developed method was used to analyze these products and verify the declared proportions of wild boar meat or pork. The products were categorized into four groups based on their level of processing, i.e. raw-cured (3 products), smoked, cured, and cooked (22 products), baked/oven-cooked (8), and pasteurized (5). The DNA extracted from each product was first universally tested for amplifiable DNA with the mammalian myostatin marker (MSTN) before being analyzed in triplicate on three separate days using the PLAG1 TaqMan probe-based qPCR. Raw calibrators were used for raw-cured and air-dried products, while oven-cooked calibrators were used for baked/oven-cooked and pasteurized products. The quantitative determination range of the newly developed method was effectively demonstrated by the analysis of products with varying levels of wild boar/pork content, ranging from 91.33 to 1.91%. This highlights the method's high sensitivity, even under application of processing methods that can degrade DNA and inhibit PCR efficiency.

Within the raw-cured product category, the estimated pork content was significantly lower than the declared values, which may suggest a higher than declared fat content or replacing a type of meat in P1 and P2. One-third of the declared pork content was estimated in P3 but in this case, the addition of curing salt and water could have reduced the percentage of meat in the final product. In the category of smoked, cured, and cooked products, many samples exhibited high estimated wild boar or pig content, consistent with the declared ingredients. For example, samples P6-P8, P11, P17-P21 and P23 showed good agreement between estimated and declared values. In other samples, such as P9, P10, and P16, where the producer did not declare a specific content, the estimated wild boar content was 39.79–63.53%, with a low coefficients of variation (CV), indicating good consistency and accuracy in the distribution of wild boar and pork within the product.

In the baked/oven-cooked products, the estimated wild boar/pig content was lower than the declared values for P27, P28, and P33, indicating that, likely, these products contained a higher content of non-meat additives or poultry offal. Two products, i.e. P30 and P32, showed good agreement between estimated and declared values. All pasteurized sausages and pâtés exhibited low estimated wild boar/pig content and high variability, with CV values ranging from 14.04 to 24.94%. This suggests, on the one hand, uneven distribution or degradation of DNA during the pasteurization process, and on the other hand, a higher addition of substitutes which may translate into lower-quality products in this category. Only P36 contained an estimated content of less than half (30%) of the declared composition.

Additionally, the game products that should contain only red deer or roe deer meat (P4, P12, P26, P29) tested positive for wild boar/pig DNA, with inclusion level ranges from 3 to 14%. Cross-contamination due to inadequate sanitation of processing equipment, such as grinders and mixers, has been experimentally investigated by Chung and Hellberg³⁵. Their study demonstrated that improperly cleaned equipment can result in species carryover at levels typically below 1%, although the initial sample processed through an uncleaned

Product No.	Product	Declared ingredients of animal origin	Declared target wild boar or pig species	Qualitative PCR	Real-time PCR		
					Cq (mean ± SD) ^a	Estimated wild boar meat /pork content (mean ± SD ^a , % w/w)	CV ^b (%)
Raw-cured products							
1	Salami with venison, medium minced, smoked, cured, air-dried	Pork meat, pork fat, 10% red deer meat, pork rinds	Pig	+	28.04 ± 0.55	4.17 ± 0.33	7.98
2	Red deer sausage with pork, finely minced, raw, smoked, dried, ripened in an inedible protein casing	Red deer meat 98 g, pork 42 g, haemoglobin	Pig	+	27.14 ± 0.36	6.29 ± 0.28	4.51
3	Pork loin, smoked, raw	Pork (Złotnicka White breed) 100%	Pig	+	24.59 ± 1.00	35.71 ± 5.71	15.98
Smoked, cured and cooked products							
4	Red deer sausage, medium minced, smoked, cooked, dried	Red deer meat 100%	Not declared	+	26.35 ± 0.54	14.20 ± 4.06	28.56
5	Red deer and pork kabanos, medium minced, smoked, steamed, dried	Red deer meat 71 g, pork meat 66 g	Pig	+	25.96 ± 0.61	18.46 ± 3.38	18.30
6	Pork sausage with venison, medium minced, smoked, steamed, dried	Pork 99 g, red deer meat 33 g	Pig	+	24.34 ± 0.52	59.35 ± 17.83	30.04
7	Red deer sausage, smoked, cooked	Red deer meat 70%, wild boar meat 30%	Wild boar	+	24.84 ± 0.59	40.92 ± 7.90	19.30
8	Pork sausage with red deer meat, medium minced, smoked, steamed	Pork meat 62 g, red deer meat 30.6 g, pork fat	Pig	+	24.75 ± 0.46	42.84 ± 4.62	10.78
9	Sausage, smoked, steamed, medium-ground, dried	Red deer meat, wild boar meat	Wild boar	+	24.88 ± 0.31	39.79 ± 0.49	1.24
10	Sausage, smoked sausage, steamed, medium-minced	Wild boar meat, deer meat	Wild boar	+	24.43 ± 0.30	53.66 ± 1.85	3.44
11	Venison sausage, smoked, steamed, medium minced, dried	Wild boar meat 82.9 g, red deer meat 35.5 g	Wild boar	+	24.29 ± 0.26	54.25 ± 4.36	8.04
12	Roe deer kabanos, medium minced, smoked, cooked, dried	Roe deer meat 100%	Not declared	+	27.02 ± 0.41	6.95 ± 0.62	8.97
13	Roe deer sausage, smoked, cooked	Roe deer meat 70%, wild boar meat 30%	Wild boar	+	24.47 ± 0.53	47.99 ± 7.22	15.04
14	Roe deer sausage, smoked, cooked, medium-minced, dried	Roe deer meat, wild boar meat	Wild boar	+	24.06 ± 0.39	64.47 ± 3.60	5.58
15	Venison frankfurter, smoked, cooked, homogenized	Pig meat, wild boar meat, roe deer meat, pasteurized cow's milk	Pig and wild boar	+	25.39 ± 0.15	23.78 ± 3.74	15.72
16	Venison sausage, smoked, cooked, homogenized	Pig meat, wild boar meat, roe deer meat, pasteurized cow's milk	Pig and wild boar	+	24.08 ± 0.26	63.53 ± 3.86	6.07
17	'Old Polish' kabanos (TSG EU certificate), medium minced, smoked, roasted, dried	Pork 170 g	Pig	+	23.63 ± 0.45	91.33 ± 9.85	10.78
18	Wild boar sausage, medium minced, smoked, cooked	Wild boar 100%	Wild boar	+	24.58 ± 1.04	36.06 ± 8.17	22.65
19	Frankfurter sausage, finely chopped, smoked, cooked	Pork (Złotnicka White breed) 100%	Pig	+	24.07 ± 1.16	52.20 ± 11.07	21.20
20	Garlic sausage, medium minced, smoked, cooked	Pork (Złotnicka White breed) 100%	Pig	+	24.29 ± 1.04	43.26 ± 7.43	17.17
21	Roasted Polish sausage, medium minced, smoked	Pork (Złotnicka White breed) 100%	Pig	+	23.80 ± 1.34	62.14 ± 8.61	13.86
22	Dried wild boar sausage, medium minced, smoked, cooked, dried	Wild boar 100%	Wild boar	+	25.92 ± 1.25	13.70 ± 2.15	15.65
23	Wild boar kabanos, medium minced, smoked, cooked, dried	Wild boar 100%	Wild boar	+	24.14 ± 0.97	47.98 ± 5.26	10.97
24	Game and pork kabanos, medium minced, smoked, cooked, dried	Wild boar meat 72 g, pork meat 67 g	Pig and wild boar	+	24.60 ± 1.06	39.06 ± 4.54	11.63
25	Dumplings (pelmeni)	Red deer meat, wild boar fat	Wild boar	+	24.52 ± 0.07	53.56 ± 2.86	5.33
Baked/Oven-cooked products							
26	Baked red deer pâté	Red deer meat 70%, turkey liver 17%, deer stock (broth) 10%	Not declared	+	28.63 ± 0.40	3.04 ± 0.12	3.90
27	Venison pâté with pork liver, baked	Red deer meat 36.6 g, pork fat 32.1 g, wild boar tongue 30.5 g, chicken liver 12.8 g	Pig and wild boar	+	26.69 ± 0.46	12.03 ± 0.68	5.65
28	Game pâté, baked	Red deer meat, wild boar meat, roe deer meat, wild boar fat, wild boar offal	Wild boar	+	29.29 ± 0.52	1.89 ± 0.21	10.93
29	Baked roe deer pâté	Roe deer meat 70%, turkey liver 17%, roe deer broth 10%	Not declared	+	27.01 ± 0.43	9.57 ± 0.32	3.30
30	Pork liver pâté with venison & Armagnac	Pork liver 25%, pork meat 23%, roe deer meat 20%, pork fat, pork connective tissue, milk proteins	Pig	+	24.25 ± 0.46	68.38 ± 3.79	5.55
31	Wild boar loin, baked	Wild boar 100%	Wild boar	+	25.09 ± 0.29	37.99 ± 5.17	13.60
32	Venison pâté, baked	Roe deer meat 51 g, pork offal 37 g, wild boar meat 5 g	Pig and wild boar	+	24.99 ± 0.51	40.33 ± 4.34	10.77
Continued							

Product No.	Product	Declared ingredients of animal origin	Declared target wild boar or pig species	Qualitative PCR	Real-time PCR		
					Cq (mean $\pm$ SD) ^a	Estimated wild boar meat /pork content (mean $\pm$ SD ^a , % w/w)	CV ^b (%)
33	Baked wild boar pâté	Wild boar meat 80%, turkey liver 7%, wild boar stock (broth) 10%, eggs	Wild boar	+	27.95 $\pm$ 0.48	4.95 $\pm$ 0.37	7.56
Pasteurized products in a jar							
34	Red deer sausage in a jar, medium mince, no added preservatives, pasteurized	Red deer meat 56%, pork 21%, pork gelatin	Pig	+	27.27 $\pm$ 0.47	11.39 $\pm$ 1.60	14.04
35	Canned game meat with pork, medium mince, no added preservatives, pasteurized	Roe deer meat 55%, pork meat 27%, pork gelatin	Pig	+	27.87 $\pm$ 0.60	7.49 $\pm$ 1.79	23.87
36	Wild boar pâté	Wild boar meat 80%, turkey liver 7%, wild boar stock (broth) 10%	Wild boar	+	25.93 $\pm$ 0.55	30.08 $\pm$ 5.43	18.06
37	Wild boar sausage in a jar, medium mince, no added preservatives, pasteurized	Wild boar meat 55%, pork meat 27%, pork gelatin	Pig and wild boar	+	28.26 $\pm$ 0.64	5.58 $\pm$ 1.39	24.94
38	Wild boar pâté with mushrooms	Stewed wild boar meat 30%, water and wild boar meat 38%, pork liver, pork belly, skimmed milk powder, powdered eggs	Pig and wild boar	+	29.72 $\pm$ 0.52	1.91 $\pm$ 0.30	15.57

Table 4. Analysis of game meat samples by quantitative real-time PCR targeting the PLAG1 zinc finger gene.

^aValues are the mean quantification cycles (Cq) of replicate assays ($n=9$) $\pm$ standard deviation (SD); ^bCV – coefficient of variation.

grinder contained up to 24% of the contaminant. Therefore, the presence of undeclared wild boar/pork at 3% could suggest either unintentional contamination or minor processing errors. However, higher levels of undeclared wild boar/pork (7%, 9.5%, and 14%) are unlikely to be attributed to accidental carryover. Instead, these levels strongly indicate deliberate mislabeling or economically motivated adulteration, where cheaper meat is substituted for more expensive varieties. This intentional action would therefore represent a food fraud. One of the tested products was a Traditional Specialty Guaranteed (TSG) kabanos (P17), which must adhere to specific traditional composition and production methods to qualify for this designation (Council Regulation (EC) No 509/2006). The analysis of this product revealed a 91% match between the estimated and declared content, confirming compliance with labeling requirements and EU regulations.

Comparing the results obtained here with those from our previous studies where the content of red deer or roe deer was quantitatively analyzed^{25,26} it is clear that producers frequently under-declare meat content. For example, in sample P1, only 4% of the declared pork was detected, while less than 1% of declared red deer²⁶ and undeclared roe deer²⁵ meat was found. Conversely, in P13 and P32, the estimated wild boar/pork content exceeded that of the main deer ingredients. These discrepancies suggest that producers may use excessive amounts of declared but cheaper meat or add undeclared, cheaper substitutes to reduce production costs. Cross-contamination during production, due to inadequate cleaning of equipment like grinders or mixers, can also lead to the presence of unintended meat species in products but in such cases rather in trace amounts up to 1%.

Kaltenbrunner et al.³⁶ reported the detection of domestic pig DNA in all tested samples of commercial products with one exception (14/15 meat products with the declared content of wild boar meat solely) although their method analyzing three genome loci carried the risk of misclassification. Basasini et al.¹² applied *cyt-b* sequences for droplet digital PCR assay and revealed undeclared pork DNA in three out of 13 samples positively tested for pork presence, i.e. turkey/chicken sausage, turkey burger and horse sausage. Xu et al.³³ testing the β -actin gene and ddPCR method found a higher amount of pork (31.67%) than declared (19%) in one beef ball of the 16 tested samples. Overall, the present results confirm that more frequent checks of the quantitative composition of meat products on the market are necessary to ensure product compliance with the label.

Conclusions

Wild boar and pork meat are frequently found in game products, either as primary or secondary ingredients. Due to their lower cost and wider availability compared to other game species such as red deer or roe deer, there is a potential for producers to misrepresent the inclusion of these species in more expensive game products. To address this issue, a novel, highly specific, sensitive, and precise TaqMan qPCR method targeting the PLAG1 zinc finger gene of wild boar and pig was developed. The individual qPCR systems, adapted to different processing conditions (raw and thermally processed), were developed using a matrix-adapted reference system. These systems were validated using laboratory-prepared beef-wild boar patties. Subsequently, thirty-eight commercial game meat products were analyzed to assess their compliance with labeling claims. The results revealed instances of undeclared species, intentional mislabeling, and likely cross-contamination. The developed method can serve as a valuable tool for ensuring label compliance, preventing food fraud, and upholding halal specifications in the game meat industry.

Data availability

Relevant data can be found here: <https://reprod.icm.edu.pl/dataset.xhtml?persistentId=doi:10.18150/W2T8QQ>.

Received: 17 January 2025; Accepted: 30 May 2025

Published online: 01 July 2025

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Acknowledgements

This work was supported by the National Science Centre, Poland, project number 2020/37/B/NZ9/00082.

Author contributions

BMA: Methodology, Investigation, Formal analysis, Visualization, Writing - Original Draft. AS: Methodology, Investigation, Writing - Review & Editing. MM: Conceptualisation, Methodology, Writing - Review & Editing, Funding acquisition, Project administration, Supervision.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-025-05167-x>.

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Supplementary material

A new nuclear marker for quantitative analysis of wild boar and domestic pig meat in game meat products using PLAG1 zinc finger gene

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Table S1. Results of qualitative PCR targeting a myostatin and PLAG1 zinc finger gene using several relevant animal and plant species for cross-reactivity testing.

Species	Scientific name	MSTN	PLAG1
Wild boar	<i>Sus scrofa scrofa</i>	+	+
Pig	<i>Sus scrofa domestica</i>	+	+
Red deer	<i>Cervus elaphus</i>	+	-
Roe deer	<i>Capreolus capreolus</i>	+	-
Fallow deer	<i>Dama dama</i>	+	-
Chicken	<i>Gallus gallus</i>	+	-
Beef	<i>Bos taurus</i>	+	-
Horse	<i>Equus caballus</i>	+	-
Pheasant	<i>Phasianus colchicus</i>	+	-
Lamb	<i>Ovis aries</i>	+	-
Goat	<i>Capra hircus</i>	+	-
Duck	<i>Anas platyrhynchos</i>	+	-
Guinea fowl	<i>Numida meleagris</i>	+	-
Quail	<i>Coturnix coturnix</i>	+	-
Rabbit	<i>Oryctolagus cuniculus</i>	+	-
Pigeon	<i>Columba livia</i>	+	-
Turkey	<i>Meleagris gallopavo</i>	+	-
Rosemary	<i>Rosmarinus officinalis</i>	-	-
Onion	<i>Allium cepa</i>	-	-
Garlic	<i>Allium sativum</i>	-	-
Ginger	<i>Zingiber officinale</i>	-	-
Black pepper	<i>Capsicum spp.</i>	-	-
Wheat	<i>Triticum spp</i>	-	-
Cilantro	<i>Coriandrum sativum</i>	-	-

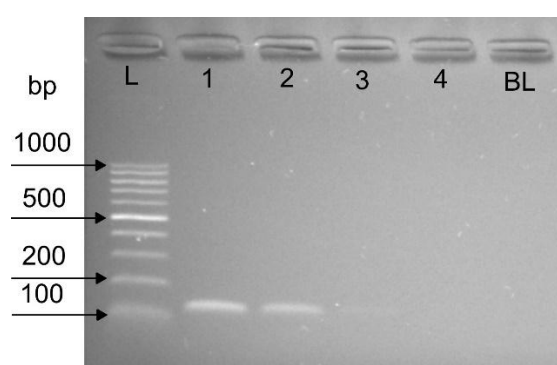


Figure S1. Agarose gel electrophoresis of PCR products targeting the PLAG1 gene using serially diluted wild boar DNA. Lanes: (L) molecular ladder; (1) 50 ng; (2) 5 ng; (3) 0.5 ng; (4) 0.05 ng; (BL) negative control.

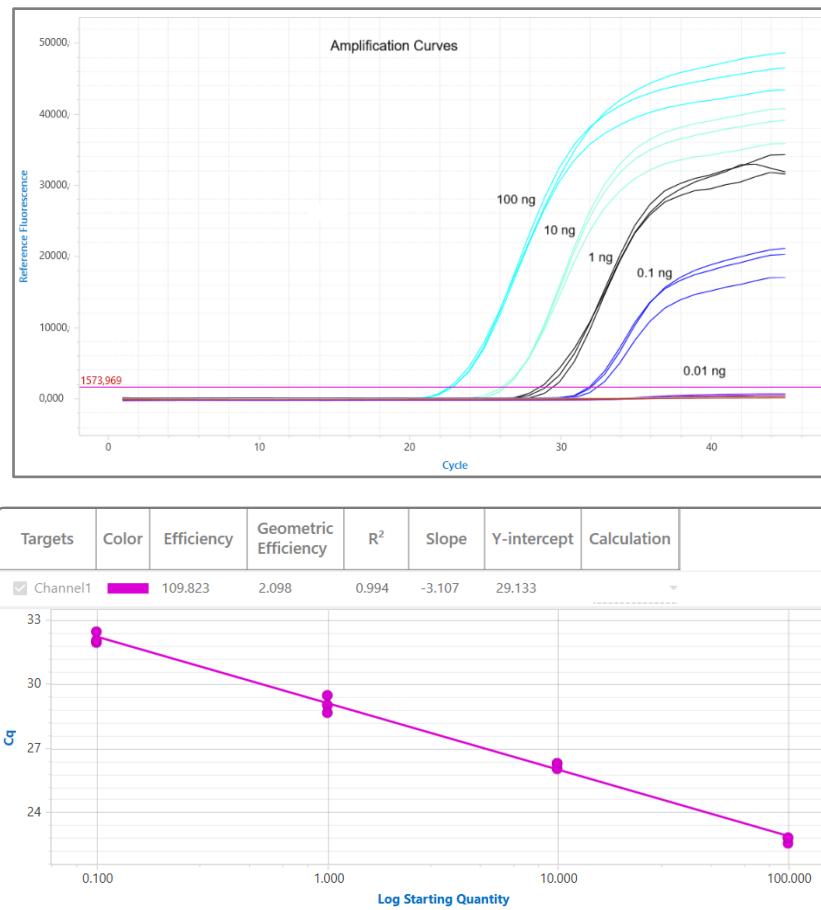


Figure S2. Amplification and standard curves obtained by real-time PCR with a TaqMan probe targeting the PLAG1 zinc finger protein gene using 10-fold serially diluted pig DNA from 100 ng/ μ L to 0.01 ng/ μ L (n=3 replicates).