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Authentication of a food product based on DNA analysis of an added natural biological tracer: Testing the application to dry cured hams

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ABSTRACT

Food authenticity is crucial for all value chains, including many meat products. Various methods have been developed to authenticate meat products based on the intrinsic DNA information of the animals from which the products originated. In this study, we propose an alternative method of authentication based on DNA analysis of a biological tracer added to the product. To demonstrate its effectiveness, we conducted a pilot study focused on dry-cured hams that were authenticated using genetically characterised wheat flours, obtained from different accessions. The study consisted of three main steps: first, we analysed 23 wheat accessions using random amplified polymorphic DNA assays and seven wheat microsatellites to assess the accession homogeneity/homozygosity and establish accession specific microsatellite fingerprinting; next, we tested, as a proof-of-concept, the feasibility of using genetically characterised wheat flours as natural tracers when mixed with lard (used to cover the skin-free part of the legs) and ink (used to label the legs), which are routinely applied in ham production; finally, we tested the possibility of authenticating hams by retrieving DNA information from the applied matrices (lard and ink mixed with flour) on the legs that were cured and ripened for 16 months. The DNA fingerprinting was consistent throughout all evaluation stages enabling the authentication of the marked hams. One advantage of this system is that the tracer (and its DNA profile) is known only to the authentication system managers. This approach can be adapted to authenticate many other food products.

1. Introduction

Ensuring the authenticity of food is a major concern for all value chains, involving various stakeholders at different levels, such as farmers, processors, food industries, retailers, policy makers, regulators, and consumers (Charlebois, Schwab, Henn, & Huck, 2016; Huck, Pezzei, & Huck-Pezzei, 2016). Food authenticity can be compromised by fraudsters who are primarily motivated by economic advantages. Food fraud encompasses various deliberate and intentional illegal or unfair practices, including misbranding, substitution, adulteration, tax avoidance and smuggling, all aimed at creating economic benefits for fraudsters while causing harm to production chains and posing increased health risks to the consumers (Brooks et al., 2021; Kendall et al., 2019; Visciano & Schirone, 2021). The risk of fraud is particularly significant for luxury, high quality, or branded foods, including protected designation of origin (PDO) products (Di Pinto, Mottola, Marchetti, Savarino, & Tantillo, 2019; Manning, 2016).

The increasing number of food fraud has led to the development of many systems capable of defending and safeguard the entire production chain at every stage (Callao & Ruisánchez, 2018; Fontanesi, 2022; Hong et al., 2017). These systems utilize a combination of different approaches and are tailored to address the unique features and potential risks associated with each food product in its specific context.

Meat products are among the most frauded foods, with problems that could stem from the mislabeling of (i) the species of origin, (ii) the breed of origin for branded-breed products, (iii) the animal identification, (iv) the production systems, including traditional and PDO products and (v) quality attributes (Fontanesi, 2022). Many methodologies have been applied to address some of these concerns and to prevent and identify frauds (Vlachos, Arvanitoyannis, & Tserkezou, 2016). The first three

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mislabeling problems can be monitored by targeting intrinsic properties of the products which directly derives from the animals that were the sources of the meat. In these cases, DNA based methods have been widely implemented (Fontanesi, 2022). Methods designed for this purpose can detect species-specific DNA sequences (usually derived from mitochondrial DNA) which, in turn, discriminate different species, or other breed specific nuclear DNA markers that can distinguish between different breeds (Fontanesi, 2022; Fontanesi, Scotti, Gallo, Nanni Costa, & Dall'Olio, 2016; Tinarelli et al., 2021). Intrinsic characteristics of the animals from which the meat was obtained cannot be directly used to authenticate products that may come from various types of animals (at a within species level or population level) or for which there is no direct relationship or when establishing a direct link between individual animals and the final products would not be economically feasible. This is the case, for example, with PDO dry cured hams which can come from different heavy pig lines and crossbred pigs, making it too challenging or costly to utilize the intrinsic properties of the animals to trace the derived legs.

Italian PDO dry-cured hams are among the most representative typical food products of Italy. There are seven PDO dry cured hams listed in Italy (Crudo di Cuneo, Prosciutto di Carpegna, Prosciutto di Modena, Prosciutto di Parma, Prosciutto di San Daniele, Prosciutto Toscano and Prosciutto Veneto Berico-Euganeo) with a total production of more than 12 million hams and a retail economic value of about 3.1 billion euro per year (ISMEA-Qualivita, 2022). Ten additional PDO dry-cured hams are produced in several other European Union countries (Croatia, France, Portugal, Slovenia and Spain), along with several Protected Geographic Indication (PGI) dry-cured hams (European Commission, 2023a). It is also worth mentioning that the production of dry-cured hams in Europe is not only linked to the PDO or PGI systems but also includes many private labels and undifferentiated products, totaling an estimated annual production of more than 50 million hams (European Commission, 2023b). Typical country dry-cured hams are also produced in the United States and China (Toldrá, 2022).

In all systems, the transformation of pork meat into ham usually includes a curing period and a ripening period which increase the salt concentration and produce a partial dehydration of the animal tissues (Toldrá, 2022). The skin-free part of the legs is typically covered by lard mixed with pepper, salt and grain or rice flour in the greasing step to prevent excessive dehydration of this part of the legs. Labeling of the legs can be done with specific meat marking ink approved for food use or with other systems. Since ham comes from an entire part of the animal (i.e. the leg), only external or extrinsic components can be added during the processing steps, as described above. These natural components (of biological origin) may come from plants (pepper and flour) or from other animals (lard). Therefore, these other biological elements, different from the animal the legs come from, may contain DNA information that can be targeted for the authentication process of the entire ham.

In this work, we report a pilot application of an innovative DNA-based authentication system, that describes, as case study, the implementation in a dry-cured ham value chain. This authentication system relies on DNA analysis of an added natural (biological) tracer constituted by flour, obtained from genetically characterised wheat accessions. This tracer is known only to the owner and manager of the authentication system. The natural tracer (flour), being a common edible component of the processing steps of the dry-cured ham production, does not alter or modify the integrity of the final product. The natural tracer can be selected or changed based on its DNA features, which authenticate the legs and then the final dry-cured hams. Different levels of authentication can be achieved for various purposes, such as authenticating different batches, production plants, years of production, private labels and more.

2. Material and methods

2.1. Pigs and legs

Four pigs were slaughtered in a commercial abattoir after reaching 160 kg live weight and nine months of age, in accordance with the production rules of the PDO Prosciutto di Parma dry-cured ham. Therefore, a total of eight legs were included in the traceability trial based on DNA analyses of the flour wheat preparations (containing lard and inks) used to brand the legs, as described below. The animals were raised on a pig farm, adhering to EU and national regulations. The animals were not treated in any way for the purpose of this study and were not slaughtered for the purpose of the study, so no ethical issues were raised.

2.2. Natural tracers: wheat accessions and flours

In this study, wheat flour was used as natural tracer as a proof of concept. Wheat flour was chosen because, at the time of the study, there was much more DNA information available for this species compared to the rice, which could have been used as an alternative, considering the potential problems that wheat flour could create to celiac people (see the discussion below). The flour used in this study was produced from common wheat (*Triticum aestivum*) and durum wheat (*Triticum durum*). These species were selected because their autogamous reproduction system allows for the production of highly genetically homogeneous batches of seeds. This genetic homogeneity is necessary to produce flour that is genetically uniform when derived from the same wheat accession. Across accessions, there is usually a high level of genetic variability. This variability across accessions can be used to obtain genetically distinguishable flours.

A total of 23 wheat accessions were used in this study: 11 from common wheat, indicated as Tae-1 to Tae-11, and 12 from durum wheat, indicated as Tdu-1 to Tdu-12. These accessions were provided by the Società Italiana Sementi (Italian Seed Society, SIS) and were derived from old varieties or newly selected lines that were not further reproduced, and that for these reasons were not marketed. Seeds obtained from all these accessions were used to grow plants to test the genetic purity of the accessions and then to produce flours. To confirm genetic purity within each accession, seeds were germinated *in vitro* and leaves from a total of 50 plants were used for DNA extraction and Random Amplified Polymorphic DNA (RAPD) analysis, as reported below. Subsequently, approximately 100 g of seeds from each of these 23 wheat accessions were ground separately to produce 23 raw flours. These flours were then characterised at the DNA level using microsatellite markers as detailed below.

2.3. Preparation of lard and food ink matrices with flours

After having characterised these wheat lines with microsatellites, 19 out of 23 were retained (see below). Then, a total of 19 different lard matrix series were prepared, each containing flour in 1%, 5%, 10% and 15% w/w ratios, derived from one of the 19 retained wheat accessions. These matrices were prepared by mixing for 10 min the two components (lard of commercial origin and flour obtained as described above) until amalgams were formed. These matrices were then stored for 18 months in a curing plant where hams are regularly processed to replicate temperature and humidity conditions of the ham curing and ripening processes. Each month, a few g of these amalgams was taken for DNA extraction and microsatellite analysis as described below.

In addition, a lard matrix based on 1% of Tae-2 variety derived flour (prepared in the same manner as described above) was applied to the skin-free part of four legs to simulate the routine greasing phase of the curing and ripening processes of the hams. About 200 mg of this matrix was taken twice, every eight months (till 16 months of age) and then used for DNA extraction and microsatellite analysis as reported below.

Two food grade inks allowed for meat marking (Allura Red, E133; and Brillant Blue FCF, E155) were used to prepare ink matrix series with 1% and 5% w/w ratios of flour derived each from two varieties (Tae-1 and Tdu-1). These liquid matrices were stored for 18 months and used to extract DNA and for microsatellite analysis every three months, as described below. In parallel, these liquid matrices were also used to imbibe two ink pads and then to wet two timbres (one for each wheat variety), which were used to stamp the skin of a total of eight hams (for each ink matrix, two flour concentrations and two wheat varieties) at the beginning of the curing and ripening phase. Stamped skin regions were scratched every four months (up to 16 months of age) for DNA extraction and microsatellite analysis as reported below.

2.4. DNA extraction

DNA extraction was carried out using different protocols based on the type of matrix being investigated. DNA extraction from wheat flour and leaves was carried out using a modified CTAB [2% (w/v) cetyltrimethylammoniumbromide; 1.4 M NaCl; 100 mM Tris-HCl; 20 mM EDTA; pH 8] based method (Murray & Thompson, 1980), as described in Mulcahy et al. (1993). For DNA extraction from lard and flour matrices, ink and flour matrices and inked skin scratched specimens the NucleoSpin Food Mini kit (Macherey Nagel, GmbH & Co. KG, Düren, Germany) was used following the manufacturer's instructions. The quality and quantity of the extracted DNA were measured using a Nanophotometer P-330 instrument (Implen GmbH, München, Germany) and 1% agarose gel electrophoresis in 1 × TBE buffer, with DNA staining using 1 × Gel Red Nucleic Acid Gel Stain (Biotium Inc., Hayward, CA, USA). DNA was then diluted with ultrapure sterile water at a final concentration of 25 ng/μL for subsequent analyses.

2.5. PCR and RAPD analyses

Genetic purity and integrity within each wheat accession (defined based on the seeds of each selection having the same DNA profile) was verified through Random Amplified Polymorphic DNA (RAPD) analysis of DNA extraction from plant leaves. PCR was carried out using ten dodecamer primers purchased from Operon Technologies (Alameda, CA, USA), selected among the C and AD series according to their amplification efficiency: OPAD01, OPAD02, OPAD03, OPAD04, OPAD05, OPAD06, OPAD16, OPC12, OPC13, and OPC16. PCR was obtained in a final volume of 25 μL containing 50 ng of template DNA, 10 mM Tris-HCl (pH 9.0 at 25 °C), 50 mM KCl, 0.1% Triton X-100, 0.05 mM dNTP (dATP, dCTP, dTTP and dGTP), and 0.03 U/μL of *Taq* DNA Polymerase (Promega, Madison, WI, USA), one Operon primer and 1.5 mM $MgCl_2$, both latter components at optimized concentration to obtain consistent amplification. Amplification reactions were obtained in a SimpliAmp thermal cycler (Thermo Fisher Scientific, Carlsbad, CA, USA) with the following profile: 2 cycles of 30 s at 94 °C, 30 s at 36 °C, 120 s at 72 °C; 20 cycles of 20 s at 94 °C, 15 s at 36 °C, 15 s at 45 °C, 90 s at 72 °C; 19 cycles of 20 s at 94 °C (increased 1 s/cycle), 15 s at 36 °C 15 s at 45 °C, 120 s at 72 °C (increased 3 s/cycle), followed by 5 min at 72 °C. Amplified products were separated through 2% agarose gel electrophoresis in 1 × TBE buffer and then staining with 1 × Gel Red Nucleic Acid Gel Stain (Biotium Inc.).

2.6. PCR and microsatellite analyses

Nine microsatellite markers (simple sequence repeats of the Xgwm series; Table 1), retrieved from the literature (Maccaferri, Sanguineti, Donini, & Tuberosa, 2003; Röder et al., 1998) were used to genotype the wheat derived specimens (wheat flours, lard and flour matrices, lard and flour preparations applied and then recovered from the legs, ink and flour matrices and inked skin scratched specimens). Microsatellites were selected based on the following characteristics: at least five alleles described in the literature, to obtain high discriminating power; localization on different chromosomes; amplified fragments of all alleles <350 bp. PCR was carried out using a SimpliAmp thermal cycler (Thermo Fisher Scientific) in a total volume of 10 μL, containing about 50 ng of template, 0.1 μM of each primer, 200 μM of each dNTP, 1.5 mM of $MgCl_2$ and 0.5 U of *Taq* DNA polymerase (Thermo Fisher Scientific). The PCR profile was as follows: an initial denaturation step at 95 °C for 8 min, followed by 40 cycles of amplification (95 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min) and a final extension step at 72 °C for 60 min. Amplified products (1 μL of the amplified volume) were electrophoresed on a capillary sequencer (Genetic Analyzer ABI Prism 310 or ABI3100; Applied Biosystems, Waltham, MA, USA) with standard ladders (Liz-500 or Rox-500). Microsatellite profiles were analysed using GeneScan software (Applied Biosystems).

2.7. Statistical analyses

The diversity index (*DI*) of each microsatellite locus was calculated with the following formula: $DI = 1 - \sum p_j^2$, where p_j is the frequency of the *j*th allele across all 23 accessions (Weir, 1996). The matching probability or the probability of identical genotypes (Weir, 1996), was defined as $PI = \sum_i p_i^4 + \sum_i \sum_{j>i} 2(p_i p_j)^2$, where p_i and p_j are the frequencies of the *i*th and *j*th alleles in the analysed wheat accessions, and multiplied over all selected microsatellites (Paetkau & Strobeck, 1994). Allele frequency data used in the calculation were derived from the analysed accessions. The proportion of microsatellite loci with shared alleles was

Table 1
Microsatellites used in this study to genotype the 23 wheat accessions.

Locus	Forward primer (5′-3′)	Reverse primer (5′-3′)	Repeat[a]	Alleles Opata/ Synth. (bp)[b]	Allele size range (bp)[c]	No. Of alleles[d]	DI[e]	PI[f]
Xgwm43	CACCGACGGTTTCCCTAGAGT	GGTGAGTGCAAATGTCATGTG	P (CA)	184/176	–	–	–	–
Xgwm46	GCACGTGAATGGATTGGAC	TGACCCAATAGTGGTGGTCA	Im (GA)	186/179	144–177	9	0.8166	0.0369
Xgwm88	CACTACAACTATGCGCTCGC	TCCATTGGCTTCTCTCTCAA	Im (GT-GA)	162/-	115–149	8	0.7902	0.0535
Xgwm95	GATCAAACACACACCCCTCC	AATGCAAAGTGAAAAACCCG	P (AC)	128/116	114–118	3	0.6323	0.1513
Xgwm99	AAGATGGACGTATGCATCACA	GCCATATTTGATGACGCATA	P (CA)	117/120	104–130	7	0.7826	0.0666
Xgwm136	GACAGCACCTTGCCCTTTG	CATCGGCAACATGCTCATC	P (CT)	278/321	–	–	–	–
Xgwm368	CCATTTCACCTAATGCCTGC	AATAAAACCATGAGCTCACTTGC	P (AT)	259/271	244–271	8	0.6484	0.0428
Xgwm448	AAACCATATTGGGAGGAAAGG	CACATGGCATCACATTTGTG	P (GA)	203/243	208–238	8	0.8081	0.0547
Xgwm610	CTGCCTTCTCCATGGTTTGT	AATGGCCAAAGGTTATGAAGG	Im (GA)	172/162	154–170	5	0.7070	0.1087

[a] Type of simple sequence repeat: P, perfect repeat; Im: imperfect repeat. The repeat motif is reported within the brackets.
[b] Alleles of the Opata durum and of the wheat synthetic accessions, considered as references, as defined in Röder et al. (1988).
[c] Allele size range observed from the analysed accessions. Two microsatellites were excluded from the analyses of the accessions (see the text).
[d] Number of alleles identified in the analysed accessions.
[e] Diversity Index calculated for the analysed accessions.
[f] Probability of Identity calculated for the analysed accessions.

chosen as a measure of genetic similarity between pairs of accessions, using the Simple Matching coefficient (*SM*) as determined by the following formula, according to Sneath and Sokal (1973): $SM_{ij} = m/n$, where m is the number of loci with shared alleles, n is the total number of analysed microsatellites, i and j are the two compared accessions.

3. Results

3.1. Experimental flow and steps of the tested authentication systems

The flow of the experimental design and the summary of the obtained results are reported in Fig. 1. The first part of the study involved the genetic characterisation and DNA fingerprinting of the wheat lines from which the natural tracers (wheat flours) were obtained (Fig. 1a). The second part tested, as a proof of concept, the possibility of using genetically characterised wheat flour as a natural tracer when mixed with two components/matrices routinely used in PDO ham production (Fig. 1b). The third part tested the possibility to authenticate PDO hams by retrieving DNA information from the applied natural tracer to the legs (Fig. 1c) that were processed following the standard procedures of the Parma ham PDO system (European Union, 2023).

3.2. Genetic characterisation of the natural tracer: DNA analyses of the wheat accessions

We first evaluated whether the wheat accessions, from which wheat flours would be obtained and used as natural tracers, had homogeneous genetic profiles. This was a prerequisite that could potentially made possible to use their DNA features for the authentication system designed in this study. The aim of this initial screening was to determine if the selected wheat populations were genetically heterogeneous and not homozygous at all loci: this problem could sometimes happen considering that during the development of the accessions, some residual heterozygosity could remain. Only accessions that were homozygous at all loci could be chosen, as simple and stable DNA fingerprints would provide the needed profiles useful for matching the natural tracer (flour derived from the wheat accessions) throughout all authentication steps.

All analysed plants from the same line had the same RAPD profile, indicating that all investigated accessions and then all subsequent seed batches originated from a genetically homogeneous line (Fig. S1). Therefore, this initial step did not exclude any of the 23 wheat lines from being considered eligible for the use of their wheat as natural tracers.

Next, we genetically characterised the 23 derived wheat flour batches using microsatellite markers (i) to further assess if these wheat lines were genetically homogeneous, (ii) to obtain an accession specific and reliable DNA fingerprinting useful to discriminate the different lines (whose ID could be also recovered after DNA analysis of the natural tracers applied to the targeted food product), and (iii) to evaluate if the 23 accessions could be distinguished, based on the DNA fingerprints obtained from the analysed microsatellite panel.

Out of nine microsatellite markers used, seven (Xgwm46, Xgwm88, Xgwm95, Xgwm99, Xgwm368, Xgwm448 and Xgwm610) produced clear and consistent allelic profiles. Two microsatellites (Xgwm136 and Xgwm43) were excluded due to low amplification efficiency and/or unreliable allele detection. The genotyping data for all 23 accessions, obtained from the remaining seven microsatellites, can be found in Table S1.

The number of alleles, Diversity Index (DI) and Probability of

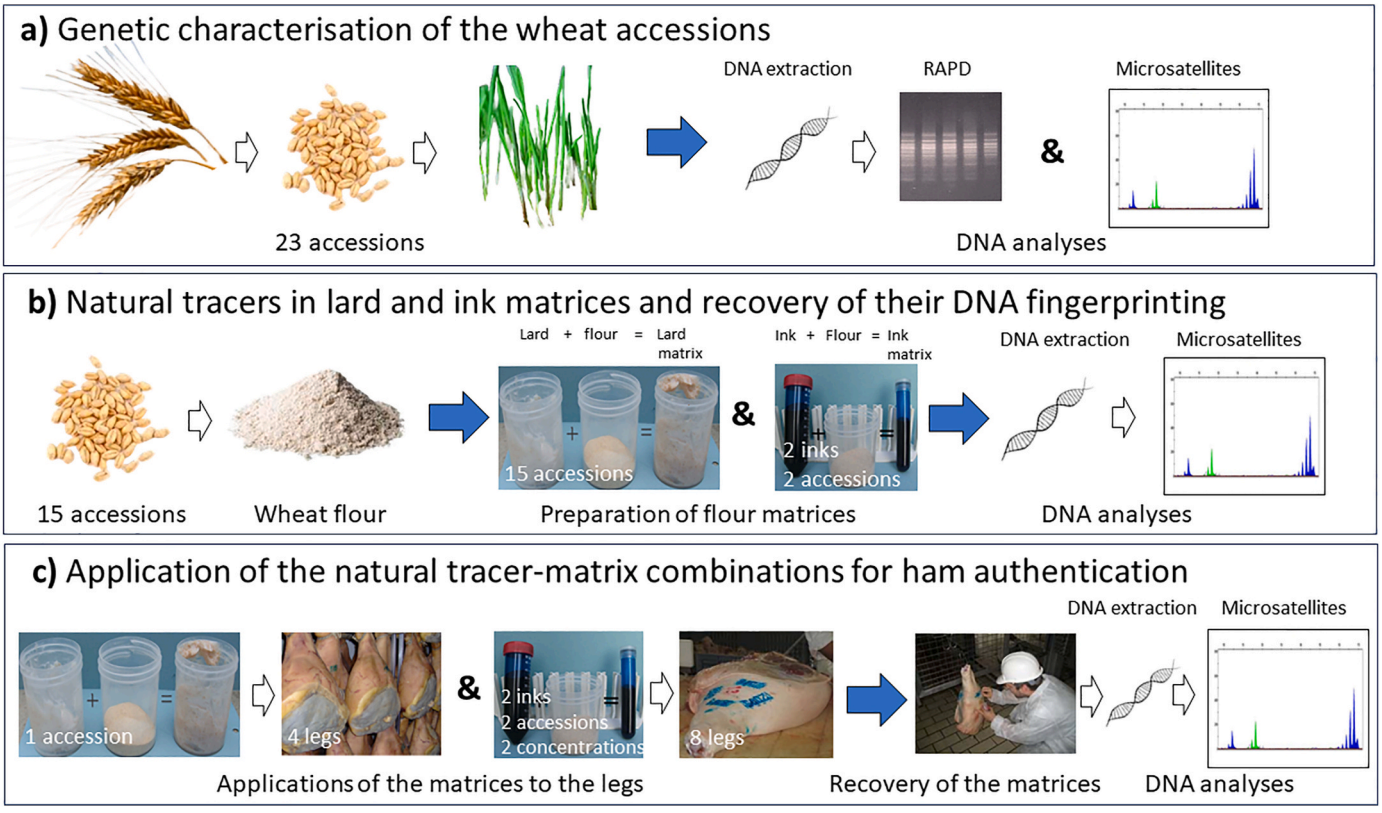

Fig. 1. Schematic representation of the experimental design and workflow. **a)** The 23 wheat accessions are genetically characterised starting from plant derived DNA using RAPD analyses, as a first step to evaluate genetic homogeneity of the accessions, and then microsatellite analyses, to further evaluate genetic homogeneity and identify the loci useful for the subsequent steps. **b)** Flour was then prepared from 15 accessions that passed the first step. Lard and ink matrices where prepared by mixing flour at different w/w concentrations and the corresponding wheat microsatellite profiles were then recovered after a certain period. **c)** Different lard and ink matrices were applied to several legs and then the microsatellite profile of the corresponding wheat flour accessions was retrieved from a certain time from the specimens obtained from the curing legs.

Identity (PI) of these microsatellites are reported in Table 1. The number of alleles ranged from 3 (Xgwm95) to 9 (Xgwm46). The Diversity Index ranged from 0.6323 to 0.8166 for Xgwm95 and Xgwm46, respectively. The Probability of Identity ranged from 0.0369 (Xgwm46) to 0.1513 (Xgwm95). Therefore, among the investigated microsatellite panel, Xgwm46 was the most informative locus and Xgwm95 was the least informative locus for the analysed accessions. Based on these seven microsatellites, the combined PI for detecting two accessions and then flours with identical genotypes at all loci was 5.1E-09. Eight out of 23 analysed accessions (two common wheat accessions: Tae-7 and Tae-11; and six durum wheat accessions: Tdu-1, Tdu-4, Tdu-8, Tdu-10, Tdu-11 and Tdu-12) had heterozygous genotypes for one microsatellite: Xgwm95 or Xgwm368 or Xgwm448 (Table S1).

Table 2 reports the Simple Matching (*SM*) coefficients for all pair combinations of accessions, based on the seven analysed microsatellites. Accessions Tdu-3, Tdu-5 and Tdu-6 shared identical genotypes for all microsatellites analysed. Similarly, accessions Tdu-10, Tdu-11 and Tdu-12 exhibited identical microsatellite profiles at all seven microsatellite loci. As a result, three accessions from each of these two groups could not be differentiated through DNA fingerprinting, indicating that they may have originated from the same selection and breeding programs. Furthermore, Tdu-10, Tdu-11 and Tdu-12 which shared the same microsatellite profiles, also displayed identical heterozygous genotypes at microsatellite Xgwm368, suggesting a very close genetic relationship.

3.3. Natural tracers in lard and ink matrices and recovery of their wheat DNA fingerprinting

The flour derived from the remaining 15 accessions was then mixed at different w/w ratios (from 1 to 15%) with lard to obtain flour-lard matrices and test the possibility of recovering wheat DNA fingerprinting over a period of 1.5 years, under the same environmental conditions to which hams are typically maintained. Wheat DNA analysis was successful for all matrices with varying concentrations of flour. All microsatellites were successfully amplified and the alleles previously identified were also detected for the corresponding accessions. As expected, allele signals were, stronger when the flour concentration was higher (10 and 15% w/w) compared to the other two concentrations tested (1 and 5% w/w). Obtained PCR products from the templates originated from the higher flour w/w concentrations had to be diluted (1:5–10) to fit within the detection range of the capillary sequencer and obtain reliable microsatellite genotype.

Similar results were seen in the microsatellite amplification of DNA extracted from matrices prepared with 1 and 5% w/w concentrations of Tae-1 and Tdu-2 flours in Allura Red and Brillant Blue FCF inks. However, no consistent effects of flour concentration were observed in this case.

Then, the trial continued with the application of the lard matrix, obtained by mixing Tae-2 flour at 1% w/w with the porcine lard, to four legs. Tae-2 variety was chosen because its genetic profile was clearly distinguishable from that of all other lines and because we had available enough flour derived from this accession to produce the needed mixed wheat/lard matrix. These legs, in addition to other four legs, were also labeled with eight ink matrices, each constituted by one of the two tested inks (Allura Red and Brillant Blue FCF), one of the two tested flour concentrations (1 and 5% w/w), with flour derived from one of the two tested wheat accessions (Tae-1 and Tdu-2). These two accessions were chosen because their microsatellite profiles could be distinguished and because we had available enough flour that could make it possible to prepare the necessary ink matrices. Samples of each type of matrix was obtained every four (ink) or eight (lard) months from the eight legs during the curing and processing period. DNA amplification was again successful for all specimens and for all tested microsatellites, except for Xgwm368, for which alleles where not always detectable for all 1% w/w ink matrices sampled at 12 and 16 months (50% success rate). This could be probably due to the low amount of flour and then wheat DNA that

Table 2
Simple Matching (SM) coefficients between pairs of accessions based on the seven analysed microsatellites. In bold, SM coefficients that showed identical DNA fingerprinting between the pair of accessions.

	Tae-1	Tae-2	Tae-3	Tae-4	Tae-5	Tae-6	Tae-7	Tae-8	Tae-9	Tae-10	Tae-11	Tdu-1	Tdu-2	Tdu-3	Tdu-4	Tdu-5	Tdu-6	Tdu-7	Tdu-8	Tdu-9	Tdu-10	Tdu-11	Tdu-12
Tae-1																							
Tae-2	2/7																						
Tae-3	0/7	3/7																					
Tae-4	1/7	1/7	2/7																				
Tae-5	3/7	3/7	2/7	2/7																			
Tae-6	2/7	2/7	3/7	2/7	2/7																		
Tae-7	3/7	3/7	2/7	2/7	3/7	5/7																	
Tae-8	4/7	4/7	1/7	4/7	2/7	1/7	2/7																
Tae-9	3/7	3/7	1/7	1/7	2/7	4/7	4/7	2/7															
Tae-10	3/7	3/7	1/7	5/7	3/7	2/7	2/7	4/7	2/7														
Tae-11	6/7	4/7	1/7	5/7	1/7	3/7	3/7	2/7	2/7	5/7													
Tdu-1	0/7	0/7	1/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7												
Tdu-2	0/7	0/7	1/7	0/7	0/7	0/7	1/7	0/7	0/7	0/7	0/7	3/7											
Tdu-3	1/7	1/7	2/7	1/7	2/7	2/7	3/7	1/7	2/7	1/7	1/7	1/7	2/7										
Tdu-4	0/7	0/7	1/7	0/7	0/7	0/7	0/7	1/7	2/7	0/7	0/7	1/7	2/7	0/7									
Tdu-5	1/7	1/7	2/7	1/7	2/7	2/7	3/7	1/7	2/7	1/7	1/7	1/7	2/7	**7/7**	0/7								
Tdu-6	1/7	1/7	2/7	1/7	2/7	2/7	3/7	1/7	2/7	1/7	1/7	1/7	2/7	**7/7**	0/7	**7/7**							
Tdu-7	1/7	1/7	1/7	1/7	1/7	2/7	2/7	1/7	2/7	1/7	1/7	1/7	1/7	5/7	0/7	5/7	5/7						
Tdu-8	0/7	0/7	1/7	0/7	0/7	0/7	1/7	0/7	0/7	0/7	0/7	3/7	5/7	2/7	1/7	2/7	2/7	1/7					
Tdu-9	1/7	1/7	0/7	0/7	0/7	1/7	1/7	0/7	1/7	0/7	1/7	2/7	2/7	4/7	0/7	4/7	4/7	4/7	2/7				
Tdu-10	2/7	2/7	1/7	0/7	2/7	0/7	2/7	2/7	1/7	0/7	0/7	2/7	4/7	1/7	1/7	1/7	1/7	0/7	5/7	1/7			
Tdu-11	2/7	2/7	1/7	0/7	2/7	0/7	2/7	2/7	1/7	0/7	0/7	2/7	4/7	1/7	1/7	1/7	1/7	0/7	5/7	1/7	**7/7**		
Tdu-12	2/7	2/7	1/7	0/7	2/7	0/7	2/7	2/7	1/7	0/7	0/7	2/7	4/7	1/7	1/7	1/7	1/7	0/7	5/7	1/7	**7/7**	**7/7**	

could be sampled and then extracted and the lower PCR efficiency of this microsatellite.

4. Discussion

This study demonstrated, as a proof of concept, that it is possible to authenticate a food product (in this case, dry-cured ham) by adding a genetically characterised food tracer (in this trial, flour) that, in turn, is the target of the DNA analyses. The addition of the tracer to a food product can be based on the specific peculiarities of the food production system, that can be identified case-by-case. In the case of the ham, we identified two possible types of applications/food tracers, lard and ink (Toldrá, 2008), which were mixed with flour produced from the seeds of a wheat accession. Their use is possible because lard and ink are already used in the processing and labeling steps of the legs. Therefore, they can be applied without significantly changing the common and standard procedures that are already part of the ham production system.

This work combined information derived from plant genetics and meat science to apply a natural (biological) food tracer that could be easily multiplied/reproduced for bulk applications. The 23 accessions were also genetically characterised using seven microsatellites (Maccaferri et al., 2003; Röder et al., 1998). Eight accessions still had some residual genetic non-uniformity. Therefore, even if the heterozygous profile could be useful to characterise the accessions, these wheat lines were not further considered as tracers, due to the potential instability of their genotyping information over their multiplication steps. Two groups of accessions of durum wheat (Tdu-3, Tdu-5 and Tdu-6; and Tdu-10, Tdu-11 and Tdu-12) could not be genetically distinguished using the seven tested microsatellites, probably due to their similar phylogenetic origin. Other microsatellites should be analysed to further evaluate if they could be considered genetically different natural tracers and then used as alternative natural tracers in this authentication system.

Microsatellite fingerprinting was conducted on the wheat accessions throughout the entire production process. This process includes seed multiplication to produce the necessary amount of flour for marking large batches of hams, as well as all intermediate verification steps using two types of matrices: lard and ink mixed with varying concentrations of flour. The microsatellite fingerprinting of the wheat accessions made it possible to unequivocally recover, in turn, the information of the corresponding wheat variety that was used to mark the hams.

The food matrix used as tracer in this proof-of-concept study (wheat flour) can be easily substituted by rice (*Oryza sativa*) flour to avoid the potential problems of gluten intolerance to the consumers (Rai, Kaur, & Chopra, 2018; Woomer & Adedeji, 2021). Rice is naturally gluten-free; therefore, it usually substitutes wheat flour in the lard matrix used to cover the skin-free muscle part of the hams, to prevent it from excessive drying. *Oryza sativa* is another autogamous plant, which has similar genetic characteristics to wheat. Genomic information in rice is now well advanced with the availability of many markers, including informative microsatellites, useful to set a similar study to what here reported for wheat lines (McCouch et al., 1997; Temnykh et al., 2000; Vieira, Faustino, Lourenço, & Oliveira, 2022; Wang & Han, 2022). Other plant based natural tracers could eventually be considered to cover a broader range of different food products, in addition to dry-cured hams.

The approach we propose for a meat product is conceptually similar to the method used to authenticate Swiss Emmental cheese and a few other Swiss PDO cheeses (Casey, Isolini, Amrein, Wechsler, & Berthoud, 2008; Lüdin, Von Ah, Rollier, Roetschi, & Eugster, 2016). In these cases, the natural tracers (biological markers) were several selected lactobacilli strains with unique insertion sequence elements. These lactic acid bacteria were cultivated and added during the cheese manufacturing process. As they are compatible with the PDO specifications, they can contribute to the maturation of the cheese. The insertion sequences of these bacteria were then targeted by PCR using DNA extracted from the cheese to recover the specific insertion sequence elements of the added bacteria strains and then authenticate the cheese, based on positive/-negative PCR results (Casey et al., 2008; Lüdin et al., 2016). This process needed to optimize the production of lactic acid bacteria starter cultures, define the optimal quantity of freeze-dried bacteria to be added to the milk that was then transformed into cheese (in order to avoid anomalous fermentations from one hand, but also to be able to recover the bacteria DNA over the maturation time of the cheese, on the other hand) and evaluate the sensitivity of the PCR based assay. In the context of authentication, one potential risk of using cultivated bacteria is that the strains could be eventually easily reproduced by the fraudsters, who could get them directly from some cheeses and start a multiplication activity. There are however some actions that can prevent or minimize this risk: for example, the use of mixture of different bacteria strains (which might have different insertion sequence elements) and a rotational principle from time to time to change the combinations of strains (Lüdin et al., 2016).

The authentication system that we tested for dry-cured hams can be further strengthened by mixing two or more flours derived from different accessions. These flours should have different DNA fingerprinting. This approach can be similar to the method proposed for cheese with different bacteria strains (Lüdin et al., 2016), as mentioned previously. This will further increase the number of possible "combined natural tracers", whose DNA fingerprinting can be derived from the combined profiles of the respective mixed flour accessions. The microsatellite profiles we obtained for the 23 wheat accessions will be useful to properly combine the flours that will give additional genetic information and further reduce the Probability of Identity. To simplify the interpretation of the fingerprinting, it is also possible to select only few microsatellites and the combination of the accessions with the simplest (or more robust and easily interpretable) genotype profile not overlapping with stutter bands.

In routine analyses, it is also possible to reduce the number of microsatellites to be genotyped (Gomez-Raya et al., 2008), as what would be needed is to first check the presence of the DNA of the added natural tracer, which could preliminarily be matched according to the microsatellite profile, compatible with that of the accession used to obtain the flour. In this first analysis, the microsatellite with the highest DI or the lowest PI should be considered. Anyway, each microsatellite analysed in this pilot study has a quite low PI, which reduces the possibility that by chance a commercial flour or a flour derived from another line or accession would have the same microsatellite profile. Other microsatellites can be subsequently added in the analysis if a lower PI is subsequently needed (DeNise et al., 2004).

A natural tracer based on a complex organism, i.e. a plant, which can be easily cultivated and multiplied only starting from seeds, eliminates the risk that fraudsters would be able to re-cultivate the same accessions, without the use of the right seeds. Therefore, the authentication system based on wheat or rice flour (or flour of another autogamous plant) has a lower risk to be bypassed than a system based on bacteria (like the case based on lactobacilli strains, as mentioned above for the cheese; Lüdin et al., 2016), which could be potentially isolated from the food product and re-cultivated (if not destroyed/killed by the food processing steps). The complexity of the genome of a plant is also another element that can further contribute to strengthen the authentication system based on plant originated material, as applied to the dry-cured ham in this study. A complex genome means that it would not be possible to artificially synthetize it. Many other DNA markers could be useful to further characterise this natural tracer that can be also derived from genetically close accessions (Singh et al., 2019). Some other markers could be more robust to get the DNA profile from processed products, which can also have a long curing and maturation time, reducing the problem of low amplification efficiency of some markers in some matrices (Fanelli et al., 2023; Giancaspro et al., 2016).

Other synthetic DNA molecules (also referred as DNA tags, molecular tags) have been proposed as potential anti-counterfeiting markers useful for applications on food and non-food products (Altamimi et al., 2019;

Bloch et al., 2014; Jung, Hogan, Sun, Liang, & Hayward, 2019; Kuzdraliński et al., 2023; Liu, Xing, Sun, Ge, & Chen, 2023). These DNA tagging approaches usually rely on the encapsulation of the DNA molecules to prevent DNA degradation and facilitate the capture of the elements to be analysed. For example, among the applications in food science, silica particles with encapsulated synthetic or natural DNA, iron oxide nanoparticles absorbing DNA sealed with dense silica coating or a synthetic microbial spore system have been proposed to label milk and dairy products (Bloch et al., 2014), extra-virgin olive oil (Puddu, Paunescu, Stark, & Grass, 2014), the surface of a fruit (Liu et al., 2023) or a wide range of food products (Qian et al., 2020). Most of these systems are quite promising being cost effective but they are all based on the addition of some artificial/synthetic components to the food products which could not be always compatible with food specifications or that could not be acceptable by the consumers. Our approach based on a natural biological tracer, that is flour in the described pilot study, can overcome these problems and limits derived by the PDO specifications. The components of the food product are not modified or altered based on the common and already accepted composition of the ingredients.

One limitation of the specific application in the illustrated case study is that the natural tracer can remain until the ham is commercialized as a whole product, which still includes the lard and the skin. When the ham is sliced, the natural tracer is not maintained on the product, as the lard and skin of the hams are removed. To overcome this limitation, other applications of the natural tracer might not be directly done on the product itself. Following the application suggested for other systems (Eroglu, Emekci, & Athanassiou, 2017), the natural tracer could potentially be placed on the packaging of sliced ham. Further studies are necessary to assess the logistics for its use in this scenario and the associated application systems.

Other genomic technologies can be also used to characterise the DNA of the natural tracer and then detect DNA markers useful to identify without any doubts the origin of the products. Next generation sequencing based on targeted or untargeted approaches or single nucleotide polymorphism genotyping platforms can be addressed to analyse not only the DNA of the animals from which the products were directly obtained (Bovo, Utzeri, Ribani, Cabbri, & Fontanesi, 2020; Ribani et al., 2018a,b; Schiavo et al., 2020) but also the DNA of plant origin that is contained in the added tracer. Other studies are however needed to establish the depth of sequencing and the bioinformatic pipelines useful to obtain DNA marker information, select and filter informative markers and the relevant genotyping results from the sequencing or genotyping steps. In the future, whole genome sequencing could be used to genetically characterise all plant accessions developed by plant breeders. This information could be used to pre-select the varieties from which the biological tracer (such as flour) will be produced.

5. Conclusions

We have demonstrated, as proof-of-concept, that an authentication system of a food product can be based on the addition of a genetically characterised natural tracer that does not alter or modify the production procedures or specifications of the product in question. The owners or managers of the seeds used to obtain the natural tracers (the flours) control this authentication system, as the wheat accession used is not disclosed or shared with others. Implementing this system on a large scale based on flour requires careful planning in advance, as the seeds must be multiplied to obtain the necessary amount of the natural tracer. Logistical problems should be taken into consideration, including the land area required to cultivate enough seeds to produce the necessary amount of flour. The authentication system tested in this study can be adapted and utilized to authenticate and differentiate various batches of hams. This includes hams from a particular production plant, those produced in different months or years, or those labeled with specific private labels. The system is very difficult, if not almost impossible, to be bypassed by fraudsters. This is due to the possibility to rotate different accessions, which are only known by the owners/managers of the system, and create mixed flours. This introduces a high level of flexibility and adaptability to ensure a complete and precise authentication system that can prevent many frauds.

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CRediT authorship contribution statement

Luca Fontanesi: Writing – review & editing, Writing – original draft, Validation, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Anisa Ribani:** Methodology, Formal analysis. **Francesca Bertolini:** Validation, Methodology. **Stefano Ravaglia:** Methodology, Investigation, Formal analysis. **Marco Pancaldi:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2024.110547.

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