SPECIES AUTHENTICATION IN POULTRY MEAT PRODUCTS BY NEXT-GENERATION SEQUENCING

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Abstract

The growing demand for poultry meat and the complexity of the supply chain affect traceability. Therefore, the aim of this study was to apply DNA metabarcoding to verify the labelling compliance of multi-species poultry meat commercial products. Overall, the molecular identifications conducted in this study confirm that all products contain the species declared in the label and all the non-conformities regard the addition of one or more undeclared meat species which could reflect both unintentional and fraudulent behaviors. In particular, the presence of undeclared species, such as swine and bovine, were highlighted in 8/13 (60%) of the samples. Such pattern could be due to technological purposes or accidental contamination linked to inappropriate sanitation practices during processing. However, the presence of undeclared species can affect the ability to choose for consumers with of specific needs (e.g., ethic, religious) or health risk and should be not neglected. Results of this study show that metabarcoding is a promising tool to identify meat species in mixtures. Therefore, its application by food industry and competent institutions, could help to innovate the food management system with the creation of a favorable environment for the protection and respect of the consumer needs.

Keywords: meat product; metabarcoding; mixed poultry product; species substitution; NGS; meat product mislabelling

1. INTRODUCTION

Meat consumption has been shifting towards poultry, driven by two different forces. In lower income developing countries, poultry has lower price compared to other meats, while in high-income countries poultry meats are considered more convenient to prepare and perceived as a healthier food choice with a risk reduction of cardiovascular diseases (Falkovskaya & Gowen, 2020; Marangoni et al., 2015; OECD-FAO Agricultural Outlook 2021-2030). Poultry meat is the meat of domesticated birds, such as turkey and chicken, and poultry products foods such as sausages, patties, hamburgers are gaining growing interest among consumers due to their convenience in preparation (ready to cook), handling, and storage (Barbut, 2012; Kennedy et al., 2004). However, in these kinds of products, the ingredients are naturally less traceable due to international trade, market globalization, and long and complex food supply chains. In addition, with the booming of e-commerce, the opportunities for their fraud increased and mixed meat products are often considered among the most frequently adulterated foods (Di Pinto et al., 2015; Hassoun et al., 2020; Walker et al., 2013). In general, food frauds involving partial or full species substitutions are expected to increase economic gain with high-priced species being substituted by cheaper ones or even illegally trade matrices (Barbarossa et al., 2016). On the other hands, accidental species substitution could occurs and may be associated with unintentional cross-contamination in processing plants sharing common machinery or equipment to produce different meat products or improper human handling (Keyvan et al., 2017). Whether intentional or not, the incorrect description of meat products is an issue of primary importance not only for economic value, but also for the potential public health risks. Indeed species substitution could have a direct impact on health of consumers when meat not compliance with hygiene requirements or even coming from illicit trade is used (Vidal Junior et al., 2020). Moreover, species substitution affects the possibility for the consumers to choose products based on ethical issues as sustainable production, animal health and wellness, health problems or religious laws (Bertolini et al., 2015; Chuah et al., 2016). The implementation of control systems is fundamental,

and several methods, including molecular, chromatography, spectroscopy, and/or spectrometry, as well as imaging approaches, have been used for the authentication of meat products (Ballin, 2010; Ellis et al., 2015; Fengou et al., 2021; Ropodi et al., 2017). However, considering the high stability and highly specificity of DNA present in almost all tissue types, molecular approaches are considered the most appropriate allowing the differentiation even in cases of closely related species. Many DNA-based methods such as DNA sequencing, species specific PCR, randomly amplified polymorphic DNA (RAPD), restriction-fragment-length polymorphism (RFLP), real-time PCR, Droplet digital PCR (ddPCR) loop-mediated isothermal amplification (LAMP), and touchdown PCR (TD-PCR) have effectively and largely been developed, tested and used for identification and differentiation of animal species in meat products both in single ingredient commodities and in complex matrices (Cai et al., 2017; Di Pinto et al., 2015, 2019; Haider et al., 2012; Kumar et al., 2017; Lin et al., 2019; Nischala et al., 2022). However, these analytical techniques require knowledge about which species to search for, and therefore are not appropriate for detecting all the species used in mixed meat products. Currently, DNA metabarcoding, the combination of DNA barcoding with Next Generation Sequencing platforms (NGS), could plays an important role in food authentication without the requirement for previous knowledge of the supply chain, production process, ingredients or about the species to search for. Thank to these new generation of sequencers, all the DNA molecules extracted from the matrices can be simultaneously amplified and sequenced allowing species identification also in complex foods containing multiple ingredients. Although application of metabarcoding to trace ingredients is still in its infancy, several studies have tested this approach for species identification in different food products including dairy (Ribani et al., 2018), seafood (Giusti et al., 2017; Piredda et al., 2022), commercial plant (Bruno et al., 2019), herbal medicinal (Anthoons et al., 2021), candies (Muñoz-Colmenero et al., 2017) honey (Prosser & Hebert, 2017; Wirta et al., 2021), probiotics (Patro et al., 2016) and pet food (Palumbo et al., 2020; Preckel et al., 2021). Few studies applied metabarcoding to investigate the species composition in artificially prepared mixtures or commercial

meat and meat products (i.e., sausages, balls, canned luncheon meat, minced meats, kebab) made of several animal species including beef, camel, horse, sheep, deer, swine and/or poultry (Cottenet et al., 2020; Dobrovolny et al., 2019; Pan et al., 2020; Preckel et al., 2021; Xing et al., 2019). Despite the paucity, these studies have shown that metabarcoding is a promising tool to species authentication also able to reveal the presence of unexpected taxa in addition to those declared.

In this study, the DNA metabarcoding approach will be applied to multi-species poultry meat products to verify the declared list of ingredients in term of animal species. Results of this work, by highlighting the potential application of DNA metabarcoding for food authentication and traceability, could innovate the food management system throughout the supply chain.

2. MATERIALS AND METHODS

2.1 Sampling

A total of 10 prepacked and 3 prepacked for direct sale poultry meat products samples including sausages, cutlets hamburgers and meat patties, reporting in the ingredient list as manufactured using pure chicken (6), pure turkey (2), chicken and turkey meat (4) and chicken, turkey, and swine meat (1) (Table1) were purchased from different markets and supermarkets in the Apulia region (Italy). Specifically, the three prepacked for direct sale samples were from in-house butchery markets.

Samples were stored at -20 °C until processed. Positive control was generated using an artificial DNA pool constructed from 50 ng of VERYfinder Poultry Pure DNA Extract – HEAT TREATED MEAT (Generon, Italy), 50 ng of VERYfinder Turkey Pure DNA Extract – HEAT TREATED MEAT (Generon, Italy), 50 ng of VERYfinder Bovine Pure DNA Extract – HEAT TREATED MEAT (Generon, Italy), and 50 ng of VERYfinder Swine Pure DNA Extract – HEAT TREATED MEAT (Generon, Italy).

2.2. DNA extraction, purification and sequencing

Total genomic DNA was extracted from all samples starting from aliquots of 25 mg of each sample using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) following producer instructions. To verify the purity of the extraction reagents, blank negative control (no added tissue) was included. DNA concentration and purity were established by evaluating the ratio A260 nm/A280 nm using a BioPhotometer D30 filter (Eppendorf, Milan, Italy). Then, the DNA was amplified using the primer pairs previously tested by Pan et al. (2020), consisting in mini-COI-F: 5′-GGTCAACAAATCATAAAGATATTGG-3′ (Folmer et al., 1994) and mini-COI-R:5′-ACTATAAAGAAGATTATTACAAAGGC-3′ (Pan et al., 2020), amplifying a fragment of about 136bp of the cytochrome oxidase I (COI) mitochondrial gene. The sequencing was carried out on the Illumina NextSeq platform (2×150 bp) by LGC Genomics GmbH (Berlin, Germany). PCR negative controls (no template) were included during the amplification step of library preparation. Raw sequences were deposited in the Sequence Read Archive (SRA) under the BioProject (under submission).

Illumina paired-end raw reads were pre-processed to generate Amplicon Sequence Variants (ASVs) using DADA2 R package (Callahan et al., 2016). Briefly, primers were removed, forward and reverse trimmed based on the Quality score and, the reads filtered, were then used to train the error model using machine learning approach. Then forward and reverse were dereplicated to generate unique sequences and denoised (collapsed) in amplicon sequence variants (ASVs) applying the trained error model. Finally forward and reverse reads were merged and checked for chimera sequences. Representative sequence for each ASV were taxonomic assigned blasting the representative sequences against GenBank in remote mode using the standalone blast + suite (Altschul et al., 1990; Camacho et al., 2009) and assignments with a similarity of <90%, representing potential low-quality reads, were discarded. Sequences assigned in the range 100-98% of similarity were assigned at species level (Barbuto et al., 2010) and merged. Molecular results were then compared with the list on ingredients reported on the labels.

3. RESULTS

The Illumina sequencing of the 13 meat samples generated a total of 5,695,822 raw reads and filtering reduced the dataset to 5,473,877 reads. Positive control sample confirmed the efficiency of the primers and generated 396,128 raw reads reduced to 376,484 (Supplementary Table 1). Taxonomic assignments revealed the presences of four species *Bos taurus*, *Gallus gallus, Meleagris gallopavo* and *Sus scrofa*.

The comparison between the results of the molecular identification and ingredient lists confirmed that all the samples contained the species reported on the label. In eight cases 8/13 (61.5%) the presence of unexpected species was highlighted. All the six samples labeled as pure chicken were found to contain additional species: turkey in 4/6 cases (67%), turkey, swine and bovine in 2/6 (33%). As regards the 2 samples of turkey hamburger, the analysis confirmed the presence of turkey, but in one case (50%) also the presence of chicken. Within the 4 chicken and turkey products, one sample (25%) revealed also the presence of bovine. Finally, in the chicken, turkey and swine sausages molecular data confirmed the species reported on the label (Figure 1 and Table 1). In the sample Sample 04 (chicken hamburger) the presence of the avian feather mites *Proctophyllodes sylviae* (Acari: Astigmata), an important symbiont of birds, was even detected.

4. DISCUSSION

The globalization of meat supply and the consequent increase in the complexity of supply chains has significantly increased the risks of food fraud. The European Union (EU) is one of the world's largest poultry meat producers. However, EU imports high value poultry products, including breast meat and poultry preparations, mainly from Brazil, Thailand, and Ukraine, while the EU exports poultry products of lower value (European Union, 2022). Such complexity reduces the ability of both regulators and industry to effectively oversee food supply chains, creating further confusion and

weakness that can facilitate inadequate practices. Furthermore, considering the reduced worldwide regulatory monitoring that appears to have occurred during the pandemic, the COVID-19 pandemic played a role in the observed increase in food fraud incidents from January to June 2020 compared to the same period in 2019 (Brooks et al., 2021).

Overall, the molecular identifications conducted in this study, focusing on poultry meat products, confirm that all products contain the species declared in the label and all the non-conformities regard the addition of one or more undeclared meat species which could reflect both unintentional and fraudulent behaviors. The primers pair used in this study were proved to be able to identify 51 edible animal species (swine, bovine, poultry, ovine, caprine, and some fish and shrimp), also including *Homo sapiens* (Pan et al., 2020); however, only four edible species (chicken, turkey, swine, and bovine) have been detected in the analyzed products. In particular, in this study, we revealed a high rate of non-conformity (61.5%) as found by Xing et al. (2019), who reported a non-conformity rate of 59% in processed meat and poultry products. Despite the reduced number of samples, this study represents one of the few studies available for poultry products and it corroborates the hypothesis that processed foods with no morphological structure are vulnerable to species substitution, either intentionally or unintentionally, practiced at any stage of the supply chain (Lianou et al., 2021). However, we have to consider that chicken, turkey, swine, and bovine, represent the top consumed meat types worldwide and are expected to be routinely present in the butchery, so their presence in the products could be related to an accidental introduction due to the fact that different raw materials are processed within the same processing plants (Di Pinto et al., 2019; Marchetti et al., 2021). Indeed, cross-contamination, can easily occur when improperly cleaned equipment is used to process meat of multiple species. This hypothesis could be the reason why, all the samples purchased from in-house butchery markets contain four types of meat independently to those declared in label and suggesting more risks of incident in prepacked for direct sale products than in prepacked ones. On the other hand, the addition driven by economic benefit cannot be excluded since undeclared meat could be

intentionally and illegally incorporated into the products for technological purposes. In particular, the presence of bovine or porcine DNA could be due to the fraudulent addition to poultry of water containing proteins of porcine or bovine origin, aimed at aid carcasses water retention (Fuseini et al., 2017; Lianou et al., 2021). Yet, in the prepacked for direct sale sausage sample, pork casings could have been used to contain the products. In addition, more mechanically recovered meat (MRM), often produced from pork and chicken carcass, is added as cheap protein source to meat products such as sausages, hamburgers, or cured meats (Surowiec et al., 2011). Similarly, the undeclared bovine presence could have been due to intentional addition of non-fat dry milk powder to increase taste and to improve binding qualities (Di Pinto et al., 2015, 2019). Whatever was the reason or source, the non-conformity due to the presence of undeclared species has always consequences for the consumers, and this is especially important in the case of poultry meat products which are often chosen and purchased on the bases of specific needs. Indeed, poultry products are allowed in presence of strict religious restrictions on the consumption of pork and bovine, as is the case of Muslim food laws (Halaal) and in Jewish food laws (Kashrut) that forbid swine meats, or Hindus that abstain from eating beef meat (Ng et al., 2022). For this reason, the simultaneous presence of bovine and swine (three samples) and bovine (two samples) found in our samples in addition to poultry meats, should be seriously considered. Indeed, Europe's societies are undergoing change and, even if Italy cannot be considered as multiethnic or multicultural country right away, we can predict an increase of consumers with different cultural values for which the undeclared presence of bovine and swine will have more impact and weight in comparison with the traditional Italian consumers.

In addition to ethical aspects, species substitution includes violation of the EU traceability requirements of the transparent food labelling systems set forth respectively in Reg. EC 178/02 and Reg. EU 1169/2011, as well as the code of conduct on the management of food allergens established by the recent Commission Regulation (EU) 2021/382 (Mottola et al., 2022). A full traceability of ingredients is fundamental for people with allergies to milk or with allergic reactions to gelatin

(Caponetto et al., 2013; Zin et al., 2021). Moreover, although the allergy to meat itself has historically been considered quite rare, cases of allergy to meat from mammals and birds, beef, pork, lamb, and poultry have become more common starting around 20 years ago (Marques et al., 2021; Wilson & Platts-Mills, 2018, 2019).

Despite the European Union Commission defines food fraud as "*any suspected intentional action by businesses or individuals for the purpose of deceiving purchasers and gaining undue advantage therefrom, in violation of the rules referred to in Article 1(2) of Regulation (EU) 2017/625*", to date from a regulatory point of view, it lacks a specific body of legislation and a clear and shared definition of "food fraud", as well as details concerning the approaches of discriminating between accidental and intentional. In the routine analysis of samples in public laboratories, mass concentrations below 1% (w/w) are generally reported as possible process contaminants and do not constitute a violation of declaration since substitution at such low levels should not have an economic advantage (Al-Kahtani et al., 2017). However, this requires specific discipline and great inspection attention. Indeed, any possible accidental or low-level presence linked to the unintentional presence and accidental traces of a type of food product with another species during processing and handling must in any case be indicated on the label as "May contain traces". On the contrary, to prevent or react on food fraud-incidents, companies need to plan mitigation practices and prevention strategies for species replacement practices, making use of the Food Fraud Vulnerability Assessment, an effective measure for specific risk management for the food industries, food authorities and consumers (Barrere et al., 2020; Marchetti et al., 2021). A comprehensive food fraud and adulteration prevention program could be a decisive and fundamental development factor in the innovation of the processed poultry sector for more accurate and truthful labeling (Di Pinto et al., 2019). The main tools for controlling vulnerabilities include the traceability plan vulnerability and severity analyses and assessment of risk, evaluation of the preventive measures in place, identification of critical points for controlling origin of the fraud, establishing a system for monitoring and critical limits, corrective actions and

verification and validation of the system. Specifically, the development of standardized tests (Pan et al., 2020) play a crucial role in meat product authentication and in the mitigation of food fraud related to species substitution.

Our outcomes show that metabarcoding is a promising tool to identify meat species in mixtures which often contain multiple animals including species not routinely used, which were not suspected to be present or for which real-time PCR methods are not available (Piredda et al., 2022). In addition, the high sensitivity of metabarcoding approach could also help the estimation of hygienic conditions of meat supply chain. Indeed, Pan et al. (2020) detected the presence of fly and cockroach in their artificial meat mixture samples, probably due to a contamination of laboratory working environment, showing that the application of metabarcoding on food products could trace not only the mislabeling but also the history of environmental and hygienic-sanitary conditions. In this sense, very positive outcomes emerge from our poultry samples in which none 'unexpected' eukaryotic taxa are detected except for one sample in which we detected trace of avian feather mites belonging to *Proctophyllodidae.* However, such presence cannot be related to a scarce hygienic condition since the general avian slaughter process operations does not remove avian skin, and thus could justify the *Proctophyllodidae* presence. Furthermore, it does not represent a sanitary risk given that, to date, this avian mite has not been identified as harmful for human health. Similarly, the presence of *Proctophyllodidae* seems to be not an animal welfare issue given that they are bird ectosymbionts that play an important biological function in cleaning bird feathers (Dona et al., 2019). Interestingly, also traces of human DNA are absent in our samples suggesting that the strong obligation of masks and gloves probably due to pandemic times, could have avoid the occasional contamination with the operator's saliva from talking while work with a consequent improvement of safety.

Despite the great potential of metabarcoding approach, the semiquantitative nature of metabarcoding approach is well known and the limitations for DNA quantification have been reported in several field including in meat products (Cottenet et al., 2020; Preckel et al., 2021). Different reasons

contribute to this bias as tissue type, genome size, copy number for nuclear regions, number of mitochondrial in cells/tissues/organs (Ren et al., 2017). Moreover, since metabarcoding is a PCR-based method, variations in primer binding capacity, could overestimate low abundant taxa with higher primers affinity or underestimate high abundant taxa with lower primer affinity, especially in complex food matrices with competitive affinity of animal species. On the other hand, in most of the cases, the labels of sampled products didn't include the proportion of animals used in the mixtures so, a qualitative approach for the comparison remains the most practicable way for the metabarcoding assessments in meat products.

5 CONCLUSIONS

Poultry meat product species substitution result from the combination of opportunities, motivations, and inadequate control measures. The poultry meat is the fastest growing segment in the world meat market (Roiter et al., 2021) and it is necessary to conduct baseline studies of the current state, identify strengths and weaknesses of the supply chain specific, build vulnerability assessment and critical control point system. The development and application of strategies and tools for traceability are required especially for processed poultry meat products because these products are often the target of consumers with of specific needs (e.g., ethic, religious) or health risk (e.g., cardiovascular diseases, obesity, diabetes) that should be respected. The innovative approach of DNA metabarcoding could be a suitable method helping the authentication of animal species in mixed meat products. Its application in routine assays may verifying compliance with food labeling for the protection of consumers and contribute to the achievement of the European Green Deal objectives for the food systems.

Figure 1. Comparison between species reported in label and metabarcoding identification. Number and type of species reported in Label (yellow on the left) and Metabarcoding identification (blue on the right) with unexpected species highlighted in bold. Mislabeled samples are indicated with asterisk (*)

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