Tissue and species identification in minced meat and meat products from Italian commercial markets by DNA microarray and histological approach

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Summary
Adequate testing and adulterant detection of food products are required to assure its safety and avoid fraudulent activities. Adulteration/substitution of costlier meat with a cheaper or inferior meat is one of the most common fraudulence in meat industry. Aim of this study was the development of a screening protocol combining DNA microarray approach and histological examination to identify animal species, exclude the presence of unwanted tissues and cells and check the reality of the meat label. 101 samples of bovine minced meat (Group 1) and ready to cook meat products (Group 2) were collected from supermarkets in Torino, Italy. DNA microarray revealed that 25.7% of samples were positive for species not declared on the label, swine being the most common. Histology showed the presence of cartilage, bone and glandular tissue. A higher presence of bacteria and inflammatory cells was detected in Group 1. Bacterial cells associated to inflammatory cells were detected with a higher score in Group 2. Sarcocystis were present in 83.3% samples of Group 1 and 49.1% of Group 2. This study confirmed that the mislabelling of meat products is not uncommon. The combination of DNA microarrays and histology can increase the monitoring capacity in bovine meat industry.

Introduction

Meat and meat products represent an important source of protein for the human diet (Font-i-Furnols and Guerrero 2014). Recently, due to changes in animal production, product processing, consumer needs and people awareness of meat safety, the authenticity of meat and meat products has been at the forefront of attention of consumers, manufacturers, and regulators (Mousavi *et al.* 2015,

Danezis *et al.* 2016). In particular, according to the EU Regulations, these products must respect the requirements of food hygiene regulations and must be labeled with detailed information about their ingredients (European Regulations[1]).

The authenticity of meat and meat products should be assessed by checking the ingredients declared in the label and excluding the presence of unauthorized tissues and cells (Ballin 2010).

Moreover, the monitoring of animal species present in these products is fundamental to prevent adulteration and to protect consumers in terms of economic, health and religious aspects (Ballin 2010, Kane and Hellberg 2015, Mousavi *et al.* 2015).

Since different types of meat have a different quality and price (e.g. usually red raw meat such as beef and sheep are more expensive than the other meat), the fraudulent addition or replacement of valuable species by less valuable ones may occur. For example, several authors reported the practice of mixing beef with cheaper meats such as chicken, horse and pork (Ballin 2010; Parchami Nejad *et al.* 2014) or with plant proteins such as soybean or grain derivatives (Flores-Munguia *et al.* 2000).

Moreover, mislabelling or improper labelling may not cite allergens that can be matter of concern for food-allergic people (Pascoal *et al.* 2004, de la Cruz *et al.* 2017), as well as the risk of cross-contamination with pork meat that raises religious problems. Alongside the possible presence of not declared species, some authors also reported undesirable organs from slaughtered animals, including viscera, hyaline cartilage, udder, skin, spleen, fat, bone and central nervous tissue, replacing the meat (Botka-Petrak *et al.* 2011, Latorre *et al.* 2015, Tafvizi and Hashemzadegan 2016). Apart from the adulteration issues, some animal tissues such as central nervous tissue can be vectors of infectious agents transmissible to humans (Herde *et al.* 2005).

Currently, molecular techniques are the methods of choice for species identification in meat products. DNA analysis has some advantages, such as the high thermal stability of DNA and the specificity of the genetic code (Ballin 2010, Yosef *et al.* 2016). Several authors developed methods for species identification in meat products based on end point PCR (Calvo *et al.* 2002), real time PCR (Martín *et al.* 2009), and sequencing analysis (Iijima *et al.* 2006). Meanwhile DNA microarray approach is one of the fastest-growing technologies, based on the classical PCR followed by a LCD array hybridisation. Previous studies already reported efficient and reliable meat species identification by DNA microarray technique (Iwobi *et al.* 2011, Yosef *et al.* 2014, Beltramo *et al.* 2017).

Formerly, many researchers reported histological methods as a simple, economic and efficient approach for the identification of unauthorized tissues, herbal content, different microbial and parasites in meat products (Prayson *et al.* 2008, Botka-Petrak *et al.* 2011, Sadeghinezhad *et al.* 2015, Hafeez *et al.* 2016).

Aim of this study was to check the correct labelling of meat and ready to cook bovine meat products, combining the DNA microarray approach to identify

the animal species with the histological examination, to check the composition and safety of meat.

Materials and methods

Sample collection

In this study, 101 samples of bovine minced meat (without other ingredients reported in label) (Group 1, n = 48) and ready to cook bovine meat products (Group 2, n = 53) were collected from different local supermarkets (referred as A, B, C, D, E and F) in Torino, Italy. In both experimental groups the samples were collected from retail distributors A, B, C, and D, while the market E & F were designed for exclusive sampling for Group 1 and Group 2, respectively. Samples characteristics were recorded, including product type, ingredients, and date of sampling. Each sample was divided into two parts: the first part was stored in sterile tubes at - 20 °C to prevent DNA degradation for species identification by DNA Microarray analysis; the second part was fixed in 10% buffered formalin and processed for tissue identification by histological methods.

DNA microarrays

Meat samples were thawed and put in a sterile Petri dish, then they were manually mixed by using sterile scalpel and nipper. DNA was extracted using a Nucleo Spin Food kit (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions. DNA was extracted from 1 g of tissue (5 aliquots of 200 mg each, in order to have good sample representation). DNA was stored at - 20 °C until further use.

PCR assays were performed using a 2X "All-in-One" Master Mix (Roche, Basel, Switzerland) in a final volume of 25 µL: each reaction contained 1X Master Mix, 1.5 µL of the Meat PCR biotinilated primers supplied with the Meat LCD Array kit (Chipron, Berlin, Germany) and 5 µL of sample's DNA.

Amplifications were performed in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) according to the following protocol: 10 min at 95 °C to activate the Hot-Start Taq polymerase; then 40 cycles with a denaturation step at 94 °C for 30 seconds, an annealing step at 57 °C for 45 seconds, an elongation step at 72 °C for 45 seconds, and a final step at 72 °C for 2 minutes.

DNA for each sample was analyzed using the Meat 1.6 LCD Array kit. Each array presents 8 chips, with 14 species-specific capture probes fixed to each chip, spotted as duplicates. The detectable species with this kit are: cattle (*Bos taurus*), buffalo (*Bubalus bubalis*), pork (*Sus scrofa*), sheep (*Ovis aries*), goat (*Capra hircus*), horse (*Equus caballus*), donkey (*Equus*

asinus), rabbit (*Oryctolagus cuniculus*), hare (*Lepus europaeus*), chicken (*Gallus gallus*), turkey (*Meleagris gallopavo*), goose (*Ansa albifrons*), mallard duck (*Anas platyrhyncos*) and muscovy duck (*Cairina moschata*). The PCR products were identified on the LCD Array following the manufacturer's instructions (Chipron). Biotinilated amplicons were linked to a streptavidin-peroxidase conjugate after hybridization at 35 °C to the probes on the array. A peroxidase substrate was added to highlight spots with amplicon-probe hybridization. A PF3650u LCD-array scanner (Pacific Image Electronics, Torrance, CA, USA) was used to visualize the dark precipitate. The default detection cut-off threshold was the 1,700-pixel value (pv).

Histological screening test

For histological screening, five different aliquots were randomly obtained from each sample. Each aliquot was fixed in 10% neutral buffered formalin and paraffin embedded. Paraffin blocks were cut into 4 µm-sections and stained with haematoxylin and eosin (H&E) by means of a Leica ST5010 Auto-strainer XL Machine (Leica Biosystem, Wetzlar, Germany). In total, 505 sections from 101 meat products were prepared and observed under a light microscope (U-MDOB3, Olympus optical CO. LTD, Tokio, Japan) to detect the presence or absence of different tissues, and to identify the presence of microorganisms, parasites, inflammatory cells and other ingredients.

Statistical analysis

The number of observations for each parameter was recorded from each section and the mean score of five slides was obtained for each sample, and the two groups (Group 1 and 2) were compared. Scores obtained were reported as the mean ± standard deviation. The normality of distributions was evaluated by Kolmogorov and Smirnov test. Mann-Whitney test was applied to compare the two groups. Regarding the presence of animal tissues and

Table I. *Results of DNA Microarray analysis for Group 1 (minced meat, N = 13) and Group 2 (meat products, N = 13)*

Sample ID	Group	Product label	Market ID	Substituted species
4	1	Choicest minced of adult bovine	C	Beef, pork, chicken and turkey
5	1	Ground from adult bovine ragu	C	Beef, pork, chicken and turkey
6	1	Ground chosen from adult bovine	B	Beef, pork and turkey
7	1	Ground chosen from adult bovine	B	Beef and pork
11	1	Adult bovine hamburger (without other ingredients)	D	Beef and pork
16	1	Adult bovine hamburger (without other ingredients)	B	Beef and pork
17	1	Adult bovine hamburger (without other ingredients)	B	Beef and pork
22	1	Thigh minced of adult bovine	E	Beef, chicken and turkey
23	1	Choicest minced of adult bovine	C	Beef, pork, chicken and turkey
24	1	Minced of adult bovine	C	Beef, pork, chicken and turkey
30	1	Minced of adult bovine	C	Beef and pork
32	1	Choicest minced of adult bovine	C	Beef and pork
44	1	Adult bovine hamburger (without other ingredients)	B	Beef, pork and chicken
3p	2	Hamburger	A	Beef and pork
5p	2	Adult bovine hamburger	B	Beef and pork
15p	2	Pizzaiola hamburger	A	Beef and pork
16p	2	Hamburger	A	Beef and pork
17p	2	Hamburger	A	Beef and pork
23p	2	The American 100% black angus meat	F	Beef and pork
24p	2	The American 100% black angus meat	F	Beef and pork
29p	2	Cheeseburger	C	Beef and pork
33p	2	Cheeseburger	C	Beef and pork
39p	2	Calf hamburger with olive	B	Beef and turkey
41p	2	Calf hamburger with olive	B	Beef, turkey and chicken
44p	2	Minced for sauce	C	Beef and pork
53p	2	The Piedmont hamburger	F	Beef and chicken

ARTICLE AHEAD OF PRINT

of parasites, each sample was considered positive if the investigated items were present at least in 1 slide (Gibson-Corley *et al.* 2013). Fisher's exact test was applied to determine the possible association of the presence of bacteria and inflammatory cells, parasites and different tissues in the two groups, as well as the species contamination. Statistical analysis was performed using Graph Pad Prism (Graph Pad Software, La Jolla, Ca, USA).

P-values smaller than 0.05 were considered statistically significant.

Results

DNA Microarray results

DNA Microarray analysis showed that 26 out of 101 samples (25.7%) were incorrectly labelled, containing different animal species besides the declared ones (Table I).

In Group 1, the minced meat samples showed contamination in 13 out of 48 samples (27.1%). In 6 cases the contamination was due to the presence of a single species (swine), whereas in 7 cases the contamination was due to multiple species. Totally, contaminations were found in 4 out of the 5 supermarkets investigated (Table I).

In Group 2, the samples of meat products analysed were from 5 different manufacturers. Incorrect labelling was found in 13 samples out of 53 (24.5%), showing the presence of different species aside from bovine. In 10 cases the contamination was due to swine, whereas in the other cases the contaminant species were chicken and turkey. The non-compliant samples were found in all the 5 supermarkets taken in account (Table I).

No significant association was revealed between the species contamination results and the examined group.

Histology screening results

Light microscopy showed skeletal muscle tissue characterized by muscular fibers surrounded by nuclei as well as peripheral nerves, vascular tissue
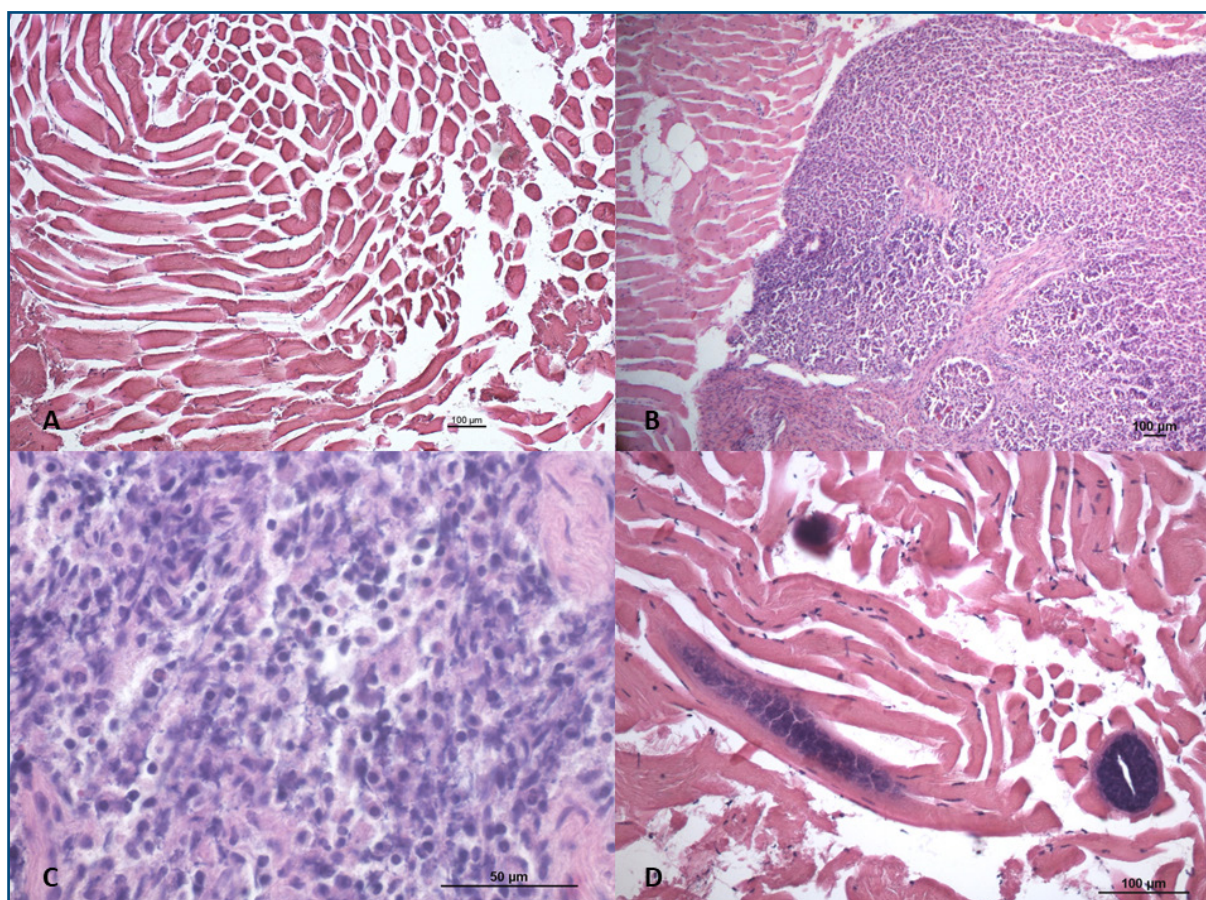

Figure 1. A. *Histological image of adult bovine minced meat.* Cross and longitudinal-sections of muscular fibres, with peripheral nuclei (H&E). **B.** *Glandular tissue found in an adult bovine hamburger along with striated muscle fibres (H&E).* **C.** *Adult bovine hamburger, bacterial contamination associated with inflammatory cells infiltrating the skeletal muscle (H&E).* **D.** *Bovine minced meat, longitudinal and cross sections of* Sarcocystis *spp. localized in the muscle fibres (H&E).*

and adipose tissue in samples both of minced meat and meat products (Figure 1A). Fifteen (31.3%) samples out of 48 in minced meat, and 13 (24.6%) out of 53 in meat products, showed the presence of hyaline cartilage fragments in at least 1 out five aliquots (Table II), characterized by the presence of chondrocytes in a collagenous extracellular matrix. Forty (83.3%) samples in Group 1 and 38 (71.7%) in Group 2 revealed pieces of bone tissues in at least one out the 5 aliquots analysed, with the osteocytes embedded in mineralized bone matrix, whereas 5 (10.4%) samples in Group 1 and 4 (7.6%) in Group 2 presented glandular tissue (Figure 1B) in at least 1 out of the 5 aliquots analysed, recognisable by the

characteristic epithelial structure. The number and distribution of the positive slides in the two groups concerning cartilage, bones and glandular tissue is reported in the Figure 2 (A, B, C).

No statistically significant associations were found between the presence of the analysed tissues and the group. Central nervous tissue and skin tissue were never found in the examined samples.

In addition to the identification of other tissues than meat, histology revealed the presence of inflammatory cells and bacteria (Figure 1C, Table II) in 46 (95.8%) samples out of 48 in Group 1 and in 50 (94.3%) out of 53 in Group 2. The bacterial were represented by cocci-shaped bacteria, and

Table II. *Tissues other than meat as well as bacteria and inflammatory cells found in minced meat and meat products revealed by histological examination.*

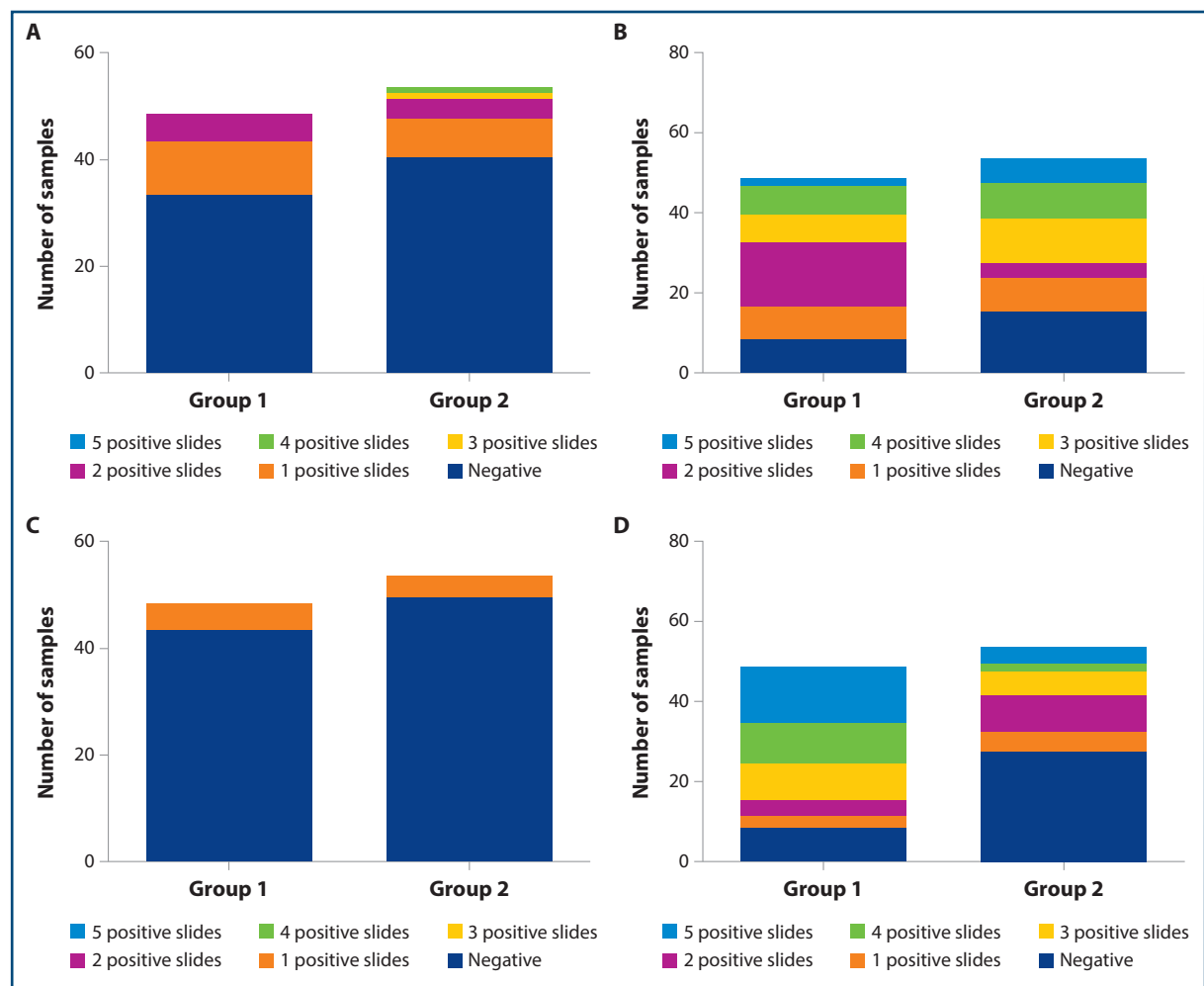| Groups	Cartilage	Bones	Glands	Bacteria	Inflammatory cells
Minced meat	15 (31.3%)	40 (83.3%)	5 (10.4%)	46 (95.8%)	25 (52.1%)
Meat products	13 (24.6%)	38 (71.7%)	4 (7.6%)	50 (94.3%)	40 (75.5%)

Figure 2. *Number and distribution of the positive slides concerning cartilage (**A**), bones (**B**), glandular tissue (**C**), and parasites (**D**) in the two examined groups.* Slides were considered positive when at least one finding of investigated items (cartilage, bones, glandular tissue or parasites) was observed.
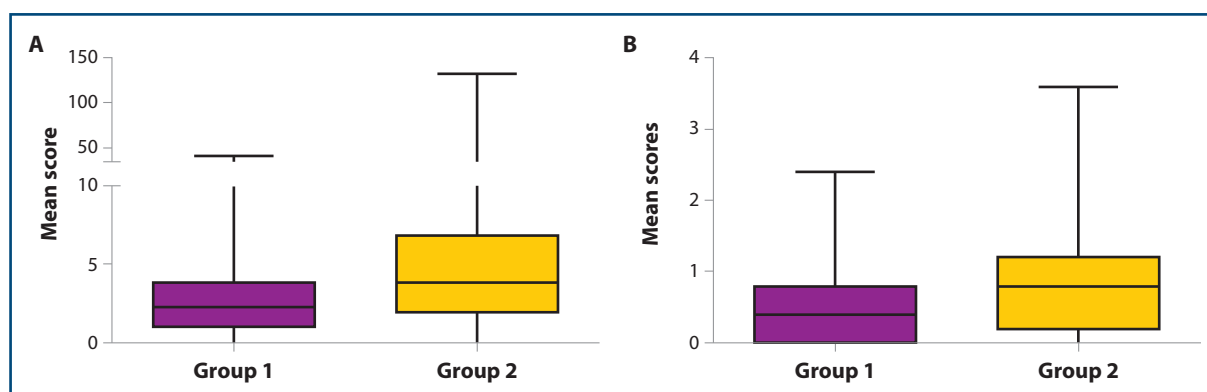
Figure 3. A. *Bacterial cell detected in the two groups (mean scores), p < 0.01.* **B.** *Inflammatory cells detected in the two groups (mean scores), p < 0.01.*

rod-shaped bacteria. Twenty-five (52.1%) samples in Group 1 and 40 (75.5%) in Group 2 showed the presence of different types of inflammatory cells. In Group 1, expected to be without ingredients other than meat, 6 (12.5%) samples out of 48 showed plant cells.

A significantly higher presence of bacteria (p < 0.01) and inflammatory cells (p < 0.01) was detected (Figure 3) in meat products compared to minced meat samples, when comparing the mean score calculated for the 5 replicates of each sample.

Bacterial associated to inflammatory cells were detected with a significantly higher mean score in Group 2 (p < 0.05). Moreover, 1 (2.1%) sample of minced meat and 7 (13.2%) samples from meat products showed the simultaneous presence of bacteria and inflammatory cells in at least 1 out 5 slides, without revealing a significant association between the findings and the group.

Examination of histological sections revealed the presence of at least one parasite in one slide per sample (Figure 1D), which were identified as *Sarcocystis*, in 40 (83.3%) out of 48 samples of Group 1 and 26 (49.1%) out of 53 samples of Group 2, revealing a statistically significant association (p < 0.01) of the finding with the groups, with the highest incidence in the Group 1.

The number and distribution of the positive slides in the two groups is reported in Figure 2D. Inflammation was not detected along with parasites in the positive samples.

Discussion

Due to the rising awareness of the public health and lifestyle improvement, consumers pay more attention to quality and safety of meat. On the other hand, high importance of a clear and trustworthy identity of the species in meat products has become important due to economic, safety and religious issues.

Analytical methods are applicable based on different types of fraud, but it seems that there is not a perfect analytical tool able to provide an answer for all the existing problems. Each single technique has its own characteristics and individual limitations, particularly in minced and homogenised meat, where animal tissues may occasionally be mixed with various ingredients. Recently, to strengthen analytical methods, some multivariate techniques have been suggested to be more effective to determine meat authenticity as reviewed by Vlachos and colleagues (Vlachos *et al.* 2016).

In this survey the histological analysis allowed to detect specific tissues, sometimes unwanted, as well as to identify various microorganisms, inflammation and other ingredients in different meat products. Furthermore, the prevalence of *Sarcocystis* spp. was easily assessed by histology, suggesting considerable concerns in this regard. Our study reveals a high substitution rate and insufficient or improper labelling in minced meat and meat products sold on the different chain supermarket in Italy without a significant association with the groups. Overall trends indicate that cheaper species can be mixed with more expensive species for economic purposes as well as unintentional cross contamination in the processing procedures may occur.

The rate of mislabeling in the present study was 25.7% which is slightly higher than a recent study conducted in U.S. which reports the 21% of mislabeling rate for ground meats (Kane and Hellberg 2015). Another study carried out in Istanbul reported a 53.4% of samples of meat and meat products incorrectly labelled (Özpinar *et al.* 2013).

The reasons for the presence of DNA of undeclared species could be either the deliberate introduction of meat from other species in order to commit a fraud or the consequence of a cross-contamination in the production chain. DNA microarray method used in the present study is essentially qualitative but the manufacturer declares that the signal

intensity, given by the DNA probe hybridization, is somehow proportional to the amount of DNA in the sample. The majority of non-compliant samples showed a weak signal, probably explainable with unintentional cross-contamination, but 7 samples had a very strong signal: all of them were minced meat and five of them were prepared directly at selling points. This finding shows that a particular attention should be paid by controllers to minced meat prepared in small production sites belonging to shops and supermarkets: in these sites there could be a higher risk of contamination either intentional, to get rid of leftover meat, or unintentional, due to very poor cleaning procedures, giving rise to heavy contamination loads.

The histological technique based on light microscopy has been extremely useful to determine the content of specific animal tissues like skeletal muscle, blood vessels, peripheral nerve, connective and fat, or components not mentioned in the label such as bone, cartilage, glands and vegetables in different meat products (Prayson *et al.* 2008, Ghisleni *et al.* 2010, Sentandreu and Sentandreu 2014, Sadeghinezhad *et al.* 2015, Hafeez *et al.* 2016, Malakauskiene *et al.* 2016). In the present study histology revealed high frequency of unauthorized tissues in various commercial meat products sold in the Italian supermarkets. A similar survey was carried out by Ghisleni and colleagues (Ghisleni *et al.* 2010) who investigated tortellini meat-filling coming from four Italian commercial brands by light microscopy in combination with image analysis, confirming that histology and image analysis are reliable tools in order to identify various animal tissues in a processed meat product. In another study, microscopic examination was used in order to estimate the meat content in American Hotdogs; outcomes revealed that the amount of skeletal muscle in most of brands was less than 10% of the cross-sectional surface area while bone and cartilage were present in all samples (Prayson *et al.* 2008). Although, due to the meat processing procedures, the presence of bone and cartilage fragments is not completely unexpected; according to Prayson and colleagues (Prayson *et al.* 2008) there is a general correlation between cost of the products and proportion of meat and bone. Bacteria were a frequent finding in this investigation, possibly related to environmental contamination, as also shown by Hafeez and colleagues (Hafeez *et al.* 2016) on the surface of meat sandwiches contaminated with different shaped bacteria. The coexistence of bacteria and inflammatory cells could be the proof of an ante-mortem infection.

Since histology is a reliable and accurate method for BSE risk assessment (Ghisleni *et al.* 2010, Iulini *et al.* 2012), in our investigation particular attention was devoted to detect the presence of central nervous tissue, though all slides were negative. Similarly, Ghisleni and colleagues (Ghisleni *et al.* 2010) and Prayson and colleagues (Prayson *et al.* 2008) did not report any positive samples for BSE risk material from tortellini meat filling. A recent study carried out in Piedmont (Italy) (Meistro *et al.* 2015), examining six histological sections for each sample, revealed the presence of *Sarcocystis* spp. in 16 (64%) out of 25 samples of bovine minced meat, which indicates a lower frequency compared with the first group of our study (83.3%). Conversely, the frequency was higher in the second group (49%).

Previous studies carried out in Italy showed a prevalence of Sarcocystosis in cattle above 80% (Domenis *et al.* 2011) and 91% (Chiesa *et al.* 2013). In some European countries, there is a trend to raw or undercooked bovine minced meat consumption. In Italy and particularly in Piedmont region, this type of consumption is common (Meistro *et al.* 2015). Therefore, it is highly recommended to cook fresh beef products before consumption or, alternatively, to freeze them for at least 3-5 days, in order to destroy *Sarcocystis* cysts (Roberts *et al.* 2005).

Conclusions

Within the last decades, a wide range of analytical methods have been employed to reveal the composition of meat and meat products as well as other aspects related to meat authenticity. Seeking out the development of molecular methods for food authentication, still it seems that the use of traditional methods along with innovative techniques may provide useful advantages to achieve a more comprehensive outcome of the food quality control. In conclusion, the combination of DNA microarrays and histology will increase the monitoring capacity of bovine meat food process.

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