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Research Paper / Araştırma Makalesi

Quality Determination of Traditional Fermented Sausages by Histological and Immunohistochemical Analyses

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ABSTRACT

In this study, it was aimed to determine the presence of animal tissues and plant materials in traditionally produced fermented sausages by using histological and immunohistochemical methods, and to determine the animal tissues that are not legally permitted. In the study, fermented sausage samples (n=18) produced by a traditional method in Mardin (Turkey) and sold in butcher shops between January and March in 2019 were used as a test material. Samples were taken from 5 different locations of each sausage for histological examinations, and routine histological tissue follow-up was performed. Paraffinized sections were stained with Hematoxylin eosin, Crossmon's trichrome and Immunohistochemical techniques. Striated muscle, collagen fibers, connective, adipose, nerve, glandular, cartilage, bone, lung, heart, spleen tissues, blood vessels, glandular epithelium and various plant materials were detected in the examined sections. Animal tissues that are not permitted but included in fermented sausages were determined in all samples. It was concluded that the identification of these tissues in fermented sausage contents individually is important and to examine fermented sausages by histological methods in terms of preventing fraud in addition to the analyses performed for the evaluation of microbiological and chemical quality parameters, and that foods produced locally and presented to consumption without being subjected for inspection may pose a health risk.

Keywords: Food adulteration, Histological examination, Traditional fermented sausage, Animal tissue, Plant material

Geleneksel Fermente Sucukların Kalitesinin Histolojik ve İmmünohistokimyasal Yöntemlerle Belirlenmesi

ÖZ

Bu çalışmada, geleneksel yöntemle üretilerek satışa sunulan fermente sucukların içeriğindeki hayvansal doku ve bitki materyallerinin histolojik ve immünohistokimyasal yöntemlerle belirlenerek, yasal olarak izin verilmeyen hayvansal dokuların varlığının araştırılması amaçlandı. Çalışmada 2019 yılı Ocak-Mart aylarında Mardin'de geleneksel yöntemle üretilerek kasap dükkânlarında satışa sunulan 18 fermente sucuk örneği test materyali olarak kullanıldı. Histolojik incelemeler için; sucukların her birinin 5 farklı bölgesinden örnekler alınarak rutin histolojik doku takibi uygulandı. Parafinlenmiş kesitler Hematoksilen eosine, Crossmon's trichrome ve Immunohistochemical teknikler ile boyandı. İncelenen kesitlerde çizgili kas, kollajen lifleri, bağ doku, yağ, sinir, bez, kıkırdak, kemik, akciğer, kalp, dalak dokuları, kan damarları, bez epiteli ve çeşitli bitki materyalleri tespit edildi. Sucuk örneklerinin tamamında sucuk içeriğine katılmasına izin verilmeyen hayvansal dokuların bulunduğu belirlendi. Sucukların mikrobiyolojik ve kimyasal kalite parametrelerinin değerlendirmeleri için yapılan analizler yanında, içeriğindeki dokuların bireysel olarak tanımlanarak hilelerin önlenmesi açısından, histolojik yöntemle de incelenmesinin önemli olduğu ve yerel olarak üretilerek denetime tabi olmadan tüketime sunulan gıdaların sağlık açısından risk oluşturabileceği kanaatine varıldı.

Anahtar Kelimeler: Gıda hileleri, Histolojik inceleme, Geleneksel fermente sucuk, Hayvansal doku, Bitki materyali

INTRODUCTION

Alongside socio-economic factors and religious beliefs, traditions also play a role in the consumption of meat and meat products [1]. Fermented sausage is a traditional meat product that is widely consumed in many countries of the world, especially in Mediterranean, Middle East and Southeast European countries [2, 3]. In Turkey, fermented sausage productions with traditional methods at homes and in small establishments are still continuing as well as industrially produced fermented sausages [4, 5].

According to the Turkish Food Codex Communiqué on Meat, Prepared Meat Mixtures and Meat Products, fermented sausages can be produced with minced bovine and / or ovine carcass meat and fat by mixing flavorings (salt, mustard, spice, spice extracts, aromatic herbs and aromatic plant extracts suitable for human consumption) and addition of offal is not permitted, and it should be labelled in accordance with the Turkish Food Codex Regulation on Food Labelling and Consumer Information. In the Turkish Food Codex Communiqué, it is also stated that sausage and similar products cannot be produced in retail establishments [6]. However, in the Turkish Fermented Sausage Standard (TS 1070) prepared by the Turkish Standards Institute, body cover fat, internal fat, tail fat, kidney fat and fat around kidney and bovine and ovine meat, which are separated from fat, bone, tendon, fascia, cartilage, lymph nodules, large nerves and blood vessels, can be used in the producing of fermented sausage [7].

Adulteration in meat products is frequently encountered as in other food products with high economic value [8]. Such applications in processed meats are not easily determined visually as in fresh meat. From past to present, in order to make more profit in the production of meat products, adulteration practices by using low-value and not allowed tissues instead of tissues with high-economic value have been encountered [9-11]. Animal tissues that are not allowed in the content of fermented sausage have been reported in several previous studies [11-16]. In studies conducted in our country and in other countries, animal tissues such as cartilage, glandular, lymph and bone tissues, tendon and fascia, spleen, tongue, rumen, salivary gland, lung and intestine were detected in the fermented sausages [12, 13, 17-19]. In addition to deceiving the consumer and unfair competition, adulteration in product also has the potential to cause health problems due to addition of tissues such as central nervous system, which may be a vector of infective agents or plant-based substances that may cause allergic effects [8, 11, 20]. Although herbal ingredients often used in most meat products, use of plant-derived ingredients in food without specifying at the label is not legally permitted because of their possible allergic effects in Turkey and the European Union countries [21, 22]. Solely chemical methods are not sufficient to evaluate all the properties of meat products [20]. Histological examination based on the determination and differentiation of ingredients in processed meat products by light or electron microscopy is a method that has been used since the early 1900s to detect tissue pieces and herbal contents in meat products and to determine the quality of meat products, and several researchers have stated that it is a reliable method that enables to determine even small amounts [11, 15-17, 20, 23-25].

In Mardin (Turkey), fermented sausage is produced with a traditional method in small butcher's shops and sold as Mardin Fermented Sausage during the winter season as in many other provinces in Turkey. These sausages are offered for sale without label. There are no previous studies on the determination of the individual tissues and ingredients in the Mardin Fermented Sausage, which is preferred by consumers because they thought it is natural and nutritious. In this study, it was aimed to determine the animal tissue and plant materials in the content of fermented sausages produced by traditional method and offered for sale by histological and immunohistochemical methods and to investigate the presence of tissues that are not legally allowed.

MATERIALS AND METHODS

Fermented sausage samples

In this study, 18 fermented sausage samples produced in different butcher's shops in Mardin (Turkey) and offered for sale in the same enterprise without labels were used as test materials. Samples were collected between January and March in 2019. Sausage samples weighing between 300 and 400 g were delivered to the laboratory in a container with ice batteries and examined.

Histological and immunohistochemical analysis

Standard morphologically based techniques were used to examine the histology of fermented sausage samples [15]. Pieces with a cut surface of 2-3 mm and a thickness of 5 mm were cut from 5 different areas of each fermented sausage sample for histological examinations. Specimens were placed in tissue follow-up cassettes and fixed in 10% formalin solution for 12-24 hours. Fixed tissue samples were dehydrated in graded ethanol (70, 80, 90%, and absolute) series, cleared in xylene. The tissues were then embedded in paraffin wax and 4-5 µm sections were taken with a rotary microtome (Thermo Scientific™HM 340E Electronic Rotary Microtome). For histological and immunohistochemical staining, 4 cuts from each sample thus a total of 72 serial sections were taken from paraffin blocks prepared from each of the 18 fermented sausage samples. Different special dyes were used to distinguish different tissue types in the sections. Hematoxylin-eosin (HE) staining was used for general histological examinations [26]. Crossman's trichrome is a connective tissue dye and has been used to distinguish between connective tissue fiber and muscle tissue in different organs [14, 20]. Each paraffin section was stained with Hematoxylin-Eosin (HE) stain (18 paraffin sections) and Crossmon's trichrome (18 paraffin sections). In addition, streptavidin-biotin peroxidase (SABP) complex method was performed (Invitrogen, USA) using two different antibodies including pan-

Cytokeratin Antibody (AE1/AE3) (18 paraffin sections) and Vimentin (18 paraffin sections).

Avidin Biotin Peroxidase Complex (ABC) technique was performed according to the standard procedure prescribed in the commercial kit (Zymed, Histostain Plus Kit, California, USA) to demonstrate vimentin and cytokine activity in tissues. Primary antibodies used in the current study are provided in Table 1.

Table 1. Characteristics of the antibodies used in this study.

Monoclonal/ polyclonal	Company	Catalog number	Target antigen	Target Species	Dilution and Incubation
Monoclonal	Santa-Cruz (Dallas, Texas, USA)	sc-81714	pan-Cytokeratin Antibody (AE1/AE3)	-	1/50- 1 hour
Monoclonal	LSBio (Seattle, Washington, ABD)	LS-C40181	Vimentin	Pig, Human, Monkey, Mouse, Rat, Bovine, Cat, Dog, Hamster, Horse, Rabbit, Chicken	1/100- 1 hour

Phosphate buffer solution (PBS) (pH 7.4) was applied to the tissues as negative control, and primary antibodies were applied the control tissues recommended by the manufacturers of primary antibodies as positive control. The 5 µm thick sections were taken on adhesive slides with the help of a microtome from the prepared paraffin blocks. Sections were kept in 3% H_2O_2 solution for 10 minutes to block non-specific antigenic sites and washed for 3-5 minutes in PBS. The periphery of the tissue was wiped, the tissues were placed horizontally in a humid environment, and primary antibody was dropped on it, and incubated for one hour then washed for 3-5 minutes in PBS. After wiping the periphery of the cross section, the secondary antibody (with biotin) was dropped and incubated for 30 minutes at room temperature. Again, it was washed for 3-5 minutes in PBS and the periphery of cross section was wiped. Then Peroxidase-streptavidin was dropped and incubated for 30 minutes at room temperature. The slides were washed for 3-5 minutes in PBS and wiped. While positive cells were fixed with DAB chromogen, ground staining was done with Gill Hematoxylin for 1-3 minutes. It was later closed with entellan.

All slides were photographed with a digital camera (Olympus DP71) and digital programmers (DP Controller and the DP Manager) fitted to a microscope (BX-51, Olympus) (using x10, x20, x40).

RESULTS

The histological analysis results of the examined sections are presented in Table 2. Legally restricted animal tissues were detected in all sausages examined (18, 100%) along with meat and fats, which are the allowed main contents. Skeletal muscle, connective tissue, gland tissue, adipose tissue, bone and cartilage tissue were remarkable in all sections (Figures 1A-O and 2A-O).

In histological examination, herbal materials were observed in all of the examined sections from each of the sausage sample. Cortex of ranunculus root parenchyma, poppy seed and root of plants were present along with the frequently used spices such as paprika and allspice (Figure 3).

In the triple staining method (Crossman trichrome), one of the applied staining methods, elastic fibers were stained in pinkish red, nerve fibers sheaths in blue, connective tissue and cartilage intermediate substrates in tones of blue, and muscle and epithelial tissues were stained in reddish tones whereas cell nuclei were stained purplish black (Figure 1A, C, D-I, L-O). In Hematoxylin-Eosin staining, cell cytoplasm of different tissue types was stained in pink and red tones; cell nuclei were stained in purple (Figure 1B, J, K).

It was shown whether the tissue types were of epithelial or mesenchymal origin with the antibodies used in immunohistochemical staining and cytokeratin vimentin. As a result of staining, the positive reaction was stained with a dark brown color (Figure 2).

DISCUSSION

In this study, skeletal muscle, adipose and connective tissue allowed to be present in sausage samples and animal tissues that are not legally allowed to be added to the sausage dough were detected in all of the fermented sausage samples. Although skeletal muscle and connective tissue are the main components found in sausages, the quality of sausage is closely related to the amount of these components [9]. Although a histometric measurement was not performed in this study, in the microscopic examination of the preparations, the level of the presence of connective tissue in 9 (50%) of the samples was the same or more than the muscle tissue. The level of connective tissue determined in this study is quite compatible with the results of İnce and Özfiliz [19].

Although the skeletal muscle contains certain amounts of connective tissue [17], the nutritional value of fermented sausages containing large amounts of connective tissue is decreased due to the fact that the lower bioavailability of the connective tissue protein than the muscle protein [27, 28]. In the Turkish Food Codex, the collagen connective tissue protein is allowed to be maximum 20% of the total meat protein in the fermented sausage [6].

Table 2. Histological examination results of fermented sausage samples

Sample No	Skeletal Muscle*	Connective Tissue	Adipose Tissue	Collogen Fibres	Nerve Tissue	Glandular Tissue	Cartilage and Bone Tissue	Lung Tissue	Heart Tissue	Spleen Tissue	Blood Vessels	Glandular Epitelium	Plant Materials
1	+	+	+	+		+	+				+		+
2	+	+	+	+	+	+	+	+				+	+
3	+	+	+	+		+	+		+		+	+	+
4	+	+	+	+	+	+	+			+	+		+
5	+	+	+	+		+	+						+
6	+	+	+	+	+	+	+	+				+	+
7	+	+	+	+		+	+				+		+
8	+	+	+	+		+	+		+	+		+	+
9	+	+	+	+	+	+	+	+			+		+
10	+	+	+	+	+	+	+					+	+
11	+	+	+	+		+	+						+
12	+	+	+	+		+	+				+		+
13	+	+	+	+	+	+	+					+	+
14	+	+	+	+		+	+				+		+
15	+	+	+	+	+	+	+					+	+
16	+	+	+	+		+	+						+
17	+	+	+	+	+	+	+				+	+	+
18	+	+	+	+		+	+			+	+	+	+
Total n (%)	18 (100)	18 (100)	18 (100)	18 (100)	8 (44.4)	18 (100)	18 (100)	3 (16.7)	2 (11.1)	3 (16.7)	9 (50)	9 (50)	18 (100)

*(+) indicates the presence

In the present study, nerve, glandular, cartilage, bone, lung, heart, spleen tissues, blood vessels and glandular epithelium were found in the investigated samples (Table 2, Figures 1 and 2). Similarly, in studies conducted with histological examination, it has been reported that disallowed tissues are detected in meat products. In several previous studies in Turkey, tendon and ligament, nerves, cartilage and salivary gland were found by Atasever et al. [17] in the samples of fermented sausages offered for consumption in Konya province; skin and digestive system epithelial tissues, cellular structures of internal organs, cartilage and bone were reported by Ayaz et al. [12] in the fermented sausages sent to the Food Control Laboratory in Ankara; Sezer et al. [4] observed epithelial tissue, sero-mucous glandular epithelium, smooth muscle tissue, cartilage and bone tissue in fermented sausages produced by traditional methods in butcher's shops and heat-treated sausages purchased from markets in Kars. Kılıç Altun et al. [18] found cartilage tissue, lymph tissue, and connective tissue in the fermented sausage samples offered for sale in Erzurum. İnce and Özfiliz [19] also have reported that they detected nervous tissue, smooth muscle, tendon, ligament, bone and cartilage tissues in fermented and heat-treated sausages obtained from different companies.

In studies conducted in other countries; it has been reported by Ghisleni et al. [9] that nervous tissue, blood vessels, adipose tissue, upper digestive system mucosa, cartilage and glandular tissues are present besides skeletal muscle in the meat filling of tortelline, which is a popular pasta unique to Italy. Marcincak et al. [29] have reported bone tissue in a traditional meat product purchased from markets in Slovakia and the Czech Republic, and Latorre et al. [11] have reported presence of cartilage and lymph nodes in sausages in Iran. Glandular and nervous tissues were also detected in cured sausage samples in a study conducted by Malakauskiene et al. [13] in Lithuania. In a study of Mokhtar et al. [14] conducted in Egypt, adulteration was determined in 88% of minced meat and 100% of sausages, similar to this study bone, cartilage, smooth muscle fibers, nerve cells, tendon were present in sausages.

Similar to the results that we obtained from this study, previous studies have shown the presence of not allowed tissues in meat products. In this study, the presence of bone tissue may suggest that remaining part of meat trimmings and meat scraps are added to the fermented sausage dough. Similarly, use of the offal such as heart, lung and spleen with low commercial value as well as glandular tissue with no commercial value suggests the possible tendency gaining unfair profit by reducing the production cost of fermented sausage It is thought that the diversity between the findings of the studies may be due to the differences in production practices, the staining methods of the histological sections and the number of examined samples.

Ingredients of plant origin as flavoring agents have been used in the production of fermented sausage as well as animal tissues [6]. In this study, plant materials such as cortex of ranunculus root parenchyma, paprika, poppy seed, allspice, root of plants were detected in the examined sections. Although soya was detected in all processed meat products examined by Latorre et al. [11], it was not detected in this study. In many countries including Turkey, soya is in the list of substances that cause allergies, and it is a legal requirement to be indicated on the product's label [21, 22]. Poppy seed was determined similar to the study conducted by Mokhtar et al. [14] in addition to paprika and allspice, which are frequently used by fermented sausage producers in Mardin (Turkey). Addition of tissues such as central nervous system, a possible vector of infective agents, [8, 11, 20] may cause health problems and use of plant-derived ingredients in sausages without specifying at the label is not legally permitted because of their possible allergic effects [21, 22].

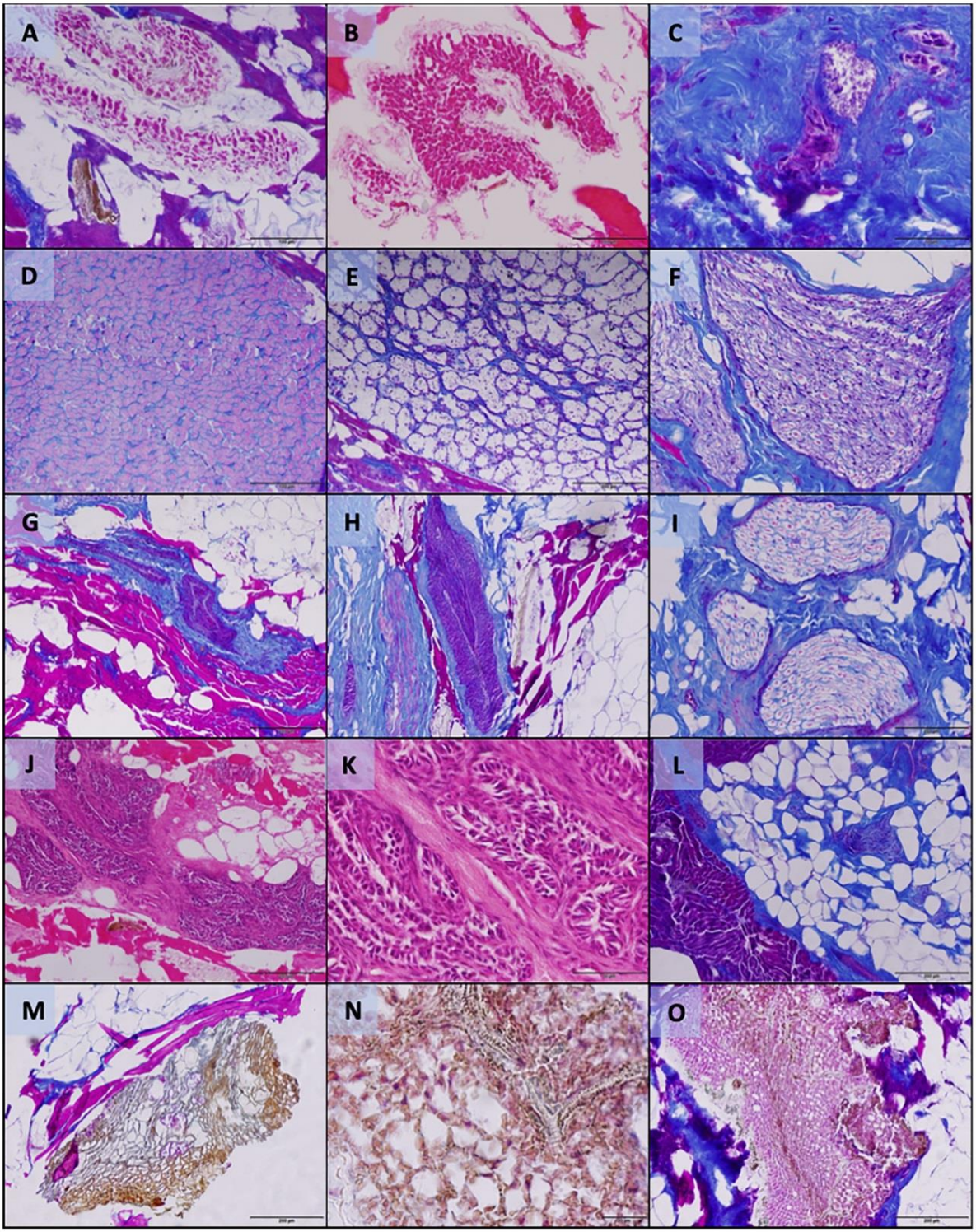

Figure 1. Representative examples of different types of tissues identified by microscopic analysis in fermented sausage food preparations. A, B) Histological section shows hearth tissue (C) Spleen tissue in cross section with connective tissue. (D) Cross section of a connective tissue and skeletal muscle(E) Glandular tissue and blood vessels in examined fermented sausage (F) Cross section of a peripheral nerve fascicle (G, H) Spleen trabecule with skeletal muscle and adipose tissue in examined fermented sausage. J-L) Histological section shows glandular tissue with adipose tissue in fermented sausage. M-O) Histological section shows lung tissue with bronches. Stain of plate: Hematoxylin and Eosin (B, J, K). Crossman trichrome stain (A,C, D-I, L-O).Bars of plate: 20 µm (N), 50 µm (C, K), 100 µm (A, B, D, F), 200 (E, G, H, I, J, L, M, O)

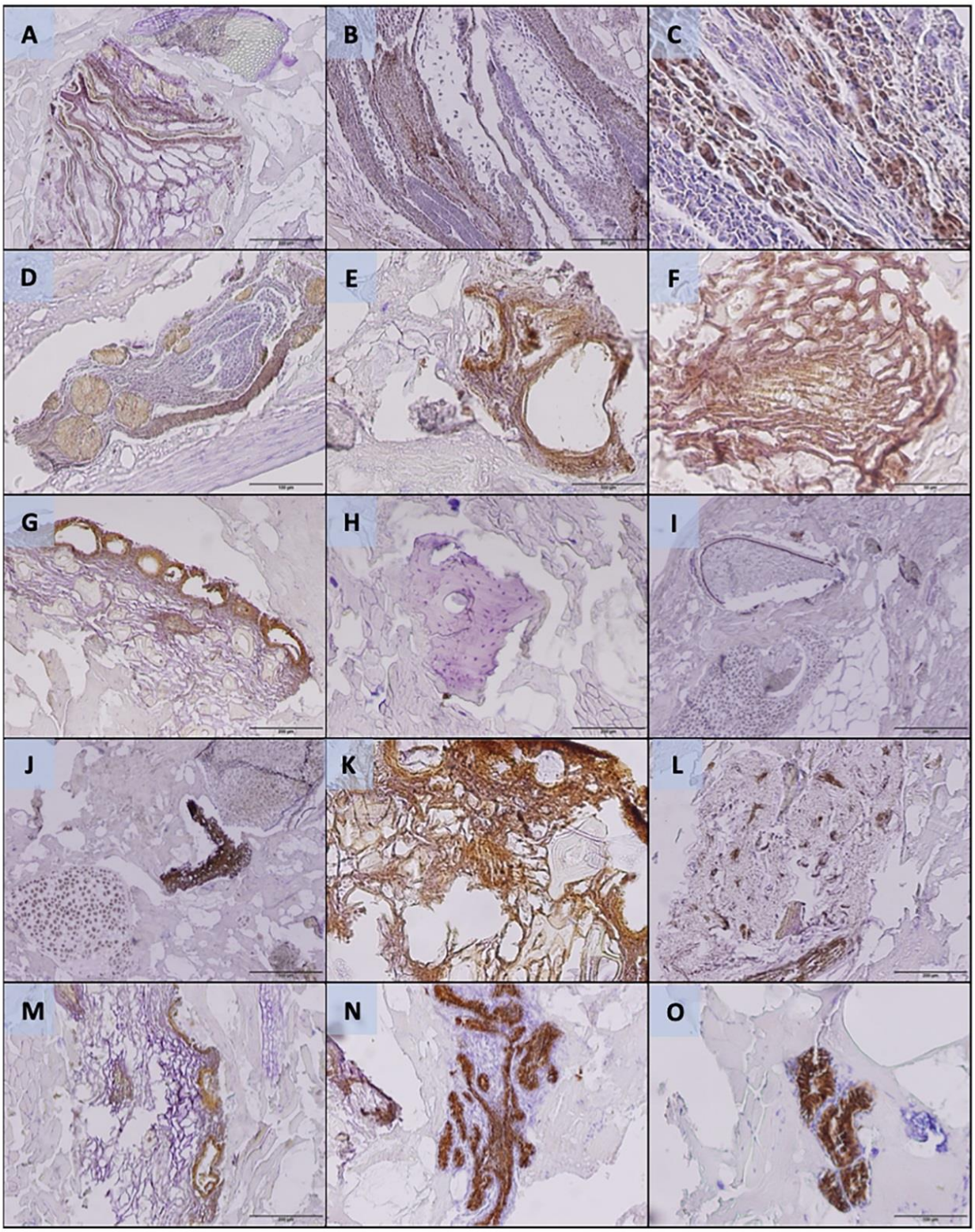

Figure 2. Immunohistochemistry positivity of different animal tissue. Cytokeratin AE1/AE3 positive immunoexpression in all glandular luminal and glandular epithelial cells of (C-G, K, M-O) with blood vessels endotel. Vimentin positive immunoexpression in cartilage and bone tissue (B, H-J). Spleen spleen immunostained for vimentin. sinusoid wall shows strongly immunoreactive rod-like structures running parallel to the long axis and sinusoidal endothelium is immunoreactive (L). Stain of plate: Immunoperoxidase-3,39-diaminobenzidine (DAB chromogen and counterstained with hematoxylin). Bars of plate: 20 µm (C), 50 µm (F, K), 100 µm (D, O), 200 (A, B, G, H, L, M), 500 µm (E, I, J, N).

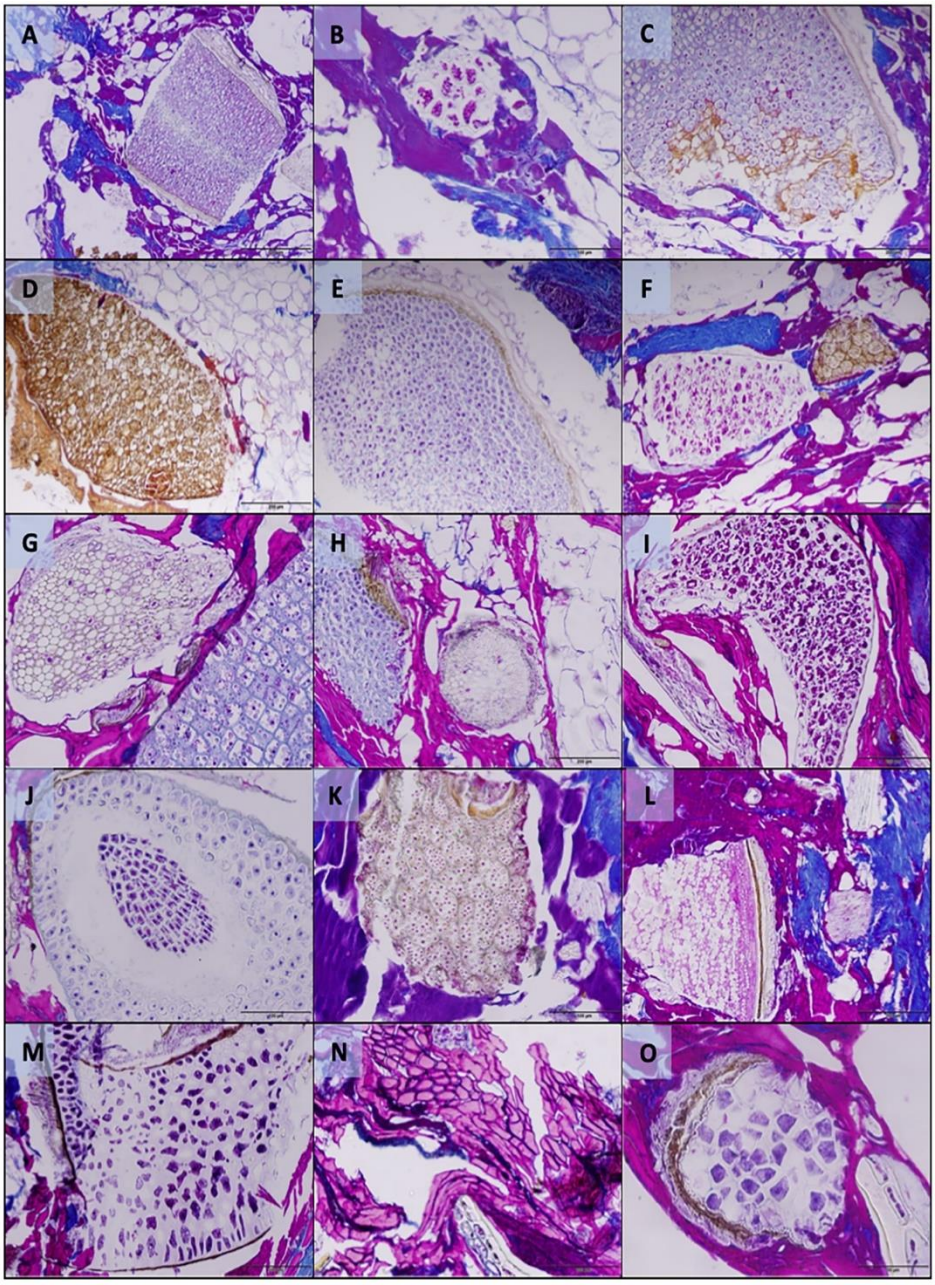

Figure 3. A-0) Histological presentation of plant tissues in fermented sausages. C, D, F) Cortex of ranunculus root parenchyma with starch grains. Note the yellow color additives. B, F, I) Chromoplasts inside plant cells in paraffin block section taken from a sausage sample prepared by adding red chili pepper to the product mortar. A, C, E, G, H, J) Cross section of poppy seed. K) Leucoplasts in a paraffin block section taken from a sausage sample prepared by adding allspice to the product mortar. L, M, N, O) Root of plants. Stain of plate: Crossmon's trichrome. Bars of plate: 50 µm (A, E, O), 100 µm (B, J, K), 200 (C, D, F, G, H, I, L, M, N)

CONCLUSION

In this study, animal tissues that are legally not allowed to be added to the sausage dough were detected in all of analyzed fermented sausage samples. Such fraud practices may pose a risk for consumer health, as well as may result in reduction in nutritional value and overall quality of the product, economic deceit of the consumer and unfair competition among producers. Therefore, determination of the individual animal tissue types and plant materials in meat products with histological examination together with other quality control analyses such as microbiologic and chemical methods in order to detect fraudulent productions is considered important for ensuring food safety and quality.

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