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ORIGINAL RESEARCH PAPER

Histological, Bacteriological, and Parasitological Study and Animal Host Origin of Handmade Sausages Marketed in Eight Cities in Iran

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Abstract

The aim of this study was to examine the quality of handmade sausages using histological, bacteriological, and parasitological techniques. During 2023, 19 samples were randomly purchased from the market in eight cities in Iran, i.e., Tehran, Karaj, Tabriz, Hamedan, Borujerd, Mashhad, and Shiraz. Specimens were cut into three parts: i) fixed in 10% buffered formalin solution and sectioned and stained for histology according to the Iranian National Standard No. a6103, ii) minced and cultured on Plate Count Agar (PCA) medium and antibiogrammed, iii) used for genomic DNA extraction and conventional PCR to identify the animal host origin of the sausages and examine for coccidian parasites, *Toxoplasma gondii*, *Sarcocystis* spp., *Neospora caninum* DNA. Results showed that 4 samples were suspected of being parasitic cysts. PCR testing revealed that all four samples contained DNA of the coccidian protozoan parasite, and one sample contained DNA of *Neospora*. Additionally, based on PCR testing, 18 samples contained both chicken and beef meat, while one sample contained lamb meat. In the bacterial analysis, 3 samples contained a significant amount of *Bacillus subtilis*, and one sample contained *Bacillus cereus*. In the histological examination of the collected samples soybean, cartilage tissue, bone tissue, smooth muscle tissue, and dense connective tissue were observed. Therefore, it can be concluded that since there is no sanitary supervision or quality control in the production of handmade sausages, there is a possibility of using inferior meat products with lower nutritional value.

1. Introduction

It can be confidently said that sausages and cold cuts are among the oldest meat products, which were highly regarded by the Greeks. In fact, some sources trace the history of processed meat products back to two thousand and five hundred years ago. In the literature of Greece, there are references to sausages, cold cuts,

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and salami (a city in eastern Greece), and even Homer mentions sausages in his writings. In Iran, the history of making sausages and cold cuts, and the transition from handmade to factory production, dates back to 1928 in Bandar Anzali. In 1958 (1337 in the Iranian calendar), the first large sausage and cold cut factory was established in the southwest of Tehran. According to the Iranian National Standard, meat products must be free of prohibited tissues and organs from livestock and poultry (such as the digestive system of animals and poultry, including the lips of livestock, oral cavities, tongue, esophagus, gizzard of poultry, rumen and crop of poultry, rumens of livestock, milk glands, liver, intestines, rectum, glandular organs like the pancreas, salivary and adrenal glands, lymphoid tissues such as lymph nodes, thymus, spleen, Bursa of Fabricius, tonsils, and lymph follicles, respiratory system organs like the lungs, trachea, and larynx, reproductive system organs like kidneys, bladder, cloaca, mammary glands, meat from the head and face of the animal, heart, tail, skin, bone tissue, epithelial tissue, central nervous tissue, elastic cartilage, and transparent cartilage) (Asadi *et al.*, 2019).

Unauthorized tissues have low nutritional value and, from a hygiene standpoint, have a higher microbial load compared to muscle tissue. Additionally, some plant-based additives in meat products are considered allergens. Considering that the presence of unauthorized tissues in these types of meat products poses a serious threat to consumer health, and that the consumption of some of these tissues is forbidden in Islam and has religious prohibitions, the use of specific diagnostic methods to detect these tissues becomes necessary (Liscio and Hopley, 2016).

Given the high cost of certain food ingredients, producers may substitute them with materials that have lower costs. Fraud can also involve replacing a species with lower nutritional value in place of one with higher nutritional value. Some individuals may have allergies to certain diets, and using them can have irreversible consequences (Olsen and Borit, 2018).

The use of histological methods as a quality control test in the food industry has been employed since 1910. Histological methods are capable of directly identifying and differentiating the components of meat products, allowing for the direct detection of the composition and type of tissue in meat products. These methods can be used to detect tissue fraud. Therefore, histological methods can assist in the quality control of heat-treated meat products and complement the standards and regulations for meat and its products. The most common histological method used for detecting fraud in meat products is the standard tissue staining method (Hematoxylin-Eosin) (Hajimohammadi *et al.*, 2020).

Today, various methods are used for quality control of meat products, and each of these methods has its own specific capabilities. One of these methods is histological techniques. This method allows for the direct detection of a composition and tissue in meat products, and it can be used to identify tissue fraud in food products (Asadi *et al.*, 2023).

Histology can identify animal tissue structures (both authorized and unauthorized), such as muscle, connective tissue, bone, cartilage, and others, in meat products. Histology can also be used to identify non-meat proteins, such as plant-based proteins (wheat, soy, and flour), in meat products. This method has been used in meat products since 1910 (Sadeghi *et al.*, 2011). Muscle and connective tissues, which are the two main factors in evaluating the edibility quality of meat, can be identified by examining histological sections under a microscope. In this way, the higher the total percentage of muscle in a sample, the higher its edible quality will be. There are various techniques for staining cells or tissues, with Hematoxylin-Eosin staining being the most accepted and common method (Veuthey *et al.*, 2014).

In recent years, the production and presentation of meat products in front of customers and their display in stores have become widespread, and in most provinces of Iran, centers with this focus are actively operating. The production of these products in the presence of customers creates a sense of security. Currently, in Iran, if a store wants to sell its produced meat products under a brand, it needs approval from the Ministry of Industry, Mine, and Trade (SMT) as well as the Food and Drug Administration. However, if a store intends to produce meat products without a brand, it only requires a health permit and a license from the Guilds Organization. These meat products must be produced according to the Iranian National Standard No. 2303.

Along with the sense of assurance that the visibility of the production of these products provides to the customer, there may also be associated risks, such as the failure to use preservatives like nitrites (which may lead to botulism), the creation of new opportunities for fraud, and the pursuit of higher profits, among others. Given the widespread use of these products, their high price, and the lack of studies regarding the quality of these products, the present study aims to examine the components of these products using histological methods, measure the bacterial load, determine the animal species used to source the meat, and also conduct parasitological examinations of the collected samples.

2. Materials and Methods

2.1. Sample Collection

From June to February 2023, 19 sausage and cold cut samples were collected from production units in various cities, as outlined in Table 1. The samples were

immediately transferred to the laboratories of the Faculty of Veterinary Medicine, Bu-Ali Sina University, Hamedan, Iran, under the cold chain.

Table 1
Method of sampling handmade sausages from different cities in Iran in the present study

Province	City	Number of samples
Tehran	Tehran	4
Alborz	Karaj	3
East Azerbaijan	Tabriz	3
Hamedan	Hamedan	2
Loresatn	Borujerd	1
Mazandaran	Babol	2
Khorasan Razavi	Mashhad	2
Shiraz	Shiraz	2
Total		19

2.2. Histological Study

For histological examination (including the analysis of unauthorized tissues, percentage of skeletal muscle, connective tissue, and fat tissue), sampling was performed according to standard 6103 and a6103, and the samples were fixed in a 10% buffered formalin solution for at least 48 hours. After fixation, the samples underwent tissue processing and block preparation, and the tissue sections were cut using a microtome (Model DS4055, manufactured by Dideh Sabz, Iran) and stained using the Hematoxylin-Eosin staining method. To assess the percentage of skeletal muscle and connective tissue in the samples, a digital image analysis system was used, which included a light microscope (Medic M-107 BN, China) equipped with a camera (Dino-Late), imaging software (Dino capture V.2), and image analysis software (v.6 Image-Pro Plus). According to standard 6103, 36 sections were prepared for each sample. All tissue sections prepared for each sample were scanned using a slide scanner (PathScan Enabler IV, Plustek- USA), and the resulting images were analyzed with the mentioned software. The average image analysis for each sample was recorded. Since the aim of this study was to evaluate the quality of samples at the national level, and comparisons between provinces and cities were not intended (due to the limited number of production units and, consequently, the low number of samples taken from some cities), statistical comparisons were not made.

2.3. Bacteriological Study

A portion of the samples was sent to the Bacteriology Laboratory of the Faculty for bacterial load assessment and antibiogram testing. For the bacteriological tests, 100 milliliters of buffered peptone water medium were first placed into a sterile wide-mouthed plastic sampling container with a sealed lid (sterilized by autoclaving). Then, under a suitable flame, a sausage (sample) was placed inside the container. The lid was sealed, and the container was thoroughly crushed and shaken well for 2-3 minutes. The resulting solution was then used for microbiological cultivation. For the total bacterial count in each milliliter of washing liquid, the surface plating method was used as described below: Serial dilutions of 0.1, 0.01, 0.001, 0.0001, 0.00001, and 0.000001 were prepared from the washing liquid. Then, 1 milliliter of each dilution (starting from 0.1) was separately transferred to plates containing Plate Count Agar, and the medium was spread using a sterile loop or spreader. Finally, the plates were incubated upside down at 37°C for 24-48 hours. After this period, the plates were examined. The number of colonies on each plate was counted, and the counted number was multiplied by the dilution factor. The average number of colonies from the different dilutions was recorded as the bacterial count per milliliter of washing liquid.
The identification of bacterial isolates was performed using macroscopic and microscopic tests, Gram staining, and biochemical tests, including catalase, sulfide production (SIM), motility, indole, citrate utilization, oxidation and fermentation of sugars (OF), Methyl-Red Voges-Proskauer (MR-VP), starch hydrolysis, casein hydrolysis, and gelatin liquefaction. The antibiotic sensitivity and resistance patterns of the isolates were determined using the disk diffusion method (Kirby-Bauer) based on the Clinical and Laboratory Standards Institute (CLSI) protocol. The results were read and recorded after 18 hours by measuring the diameter of the zone of inhibition and comparing it with available reference tables. For this purpose, antibiotic disks of chloramphenicol, cefixime, penicillin, cefazolin, ampicillin, and vancomycin were used. The procedure was as follows: a bacterial suspension with a turbidity equivalent to a 0.5 McFarland standard was prepared. Then, using a sterile swab, the suspension was inoculated onto a solid Mueller-Hinton agar medium. Sterile forceps were used to place the antibiotic discs on the surface of the medium. The plate was then incubated at 35°C for 18 to 24 hours. To read the results, the diameter of the zone of inhibition around the disc was measured in millimeters using a precise ruler, and the bacterial sensitivity to the antibiotic was reported. Additionally, part of the samples was considered for DNA extraction and determining the animal species used in the production of the products (Table 2). The remaining extracted DNA samples were stored at -80°C for further studies.

2.4. DNA Isolation and PCR Assay

For parasite specimen identification, genomic DNA was extracted from ca. 200 mg of each specimen using a commercial kit (MBST, Tehran, Iran) and examined for the presence of cyst-forming protozoan parasites DNA targeting different genes. Initially all of the

samples were tested with the common apicomplexan primer pair COC1 F: AAGTATAAGCTTTTATACGGCT / COC2 R: CACTGCCACGGTAGTCCAATAC amplifying 270-350 bp fragment of the chromosomal small-subunit (16S) rRNA of Toxoplasma gondii, Neospora caninum, eimeriid coccidia, isosporid coccidia, Sarcocystis, Cryptosporidium and Hammondia (Ho *et al.*, 1996). PCR-positive samples were further tested with species-specific primers targeting B1 gene of Toxoplasma gondii (B22: AACGGGCGAGTAGCACCTGAGGAGA/ B23 GGGTCTACGTCGATGGCATGACAAC, amplicon size ∼115bp) (Bretagne *et al.*, 1993), NC5 gene of Neospora caninum (Np21plus: CCCAGTGCGTCCAATCCTGTAAC / Np6plus: CTCGCCAGTCAACCTACGTCTTCT, amplicon size ∼337 bp) (Müller *et al.*, 1996), mitochondrial cytochrome c oxidase (cox1) gene of Sarcocystis species (SF1: ATGGCGTACAACAATCATAAAGAA / SR5: TAGGTATCATGTAACGCAATATCCAT, amplicon size ∼1100 bp) (Gjerde, 2013). All PCRs were performed with the Taq DNA Polymerase Master Mix RED® (Ampliqon, Odense, Denmark) in a SimpliAmp® thermal cycler (Applied Biosystems, Waltham, MA, USA). In each run, positive DNA control retrieved from a previous study (Khordadmehr *et al.*, 2023) and double-distilled water as negative control were included. The amplified products were detected by electrophoresis on 2 % agarose gels stained with DNA Safe Stain (SinaClon, Tehran, Iran) (Table 3).

3. Results

3.1. Microbiological Results

In four samples, a single colony was observed at a 0.1 dilution. Among these, three samples contained Bacillus subtilis, and one sample contained Bacillus cereus. Antibiotic sensitivity testing and the antibiotic resistance pattern of the isolates were determined using the disc diffusion method (Kirby–Bauer) as a standard procedure according to the (CLSI) protocol. After 18 hours, the zone of inhibition diameter was measured and compared with available tables for interpretation and recording. All 4 samples were sensitive to the listed antibiotics, with one sample resistant to cefixime and another sample resistant to cefazolin (Fig. 1).

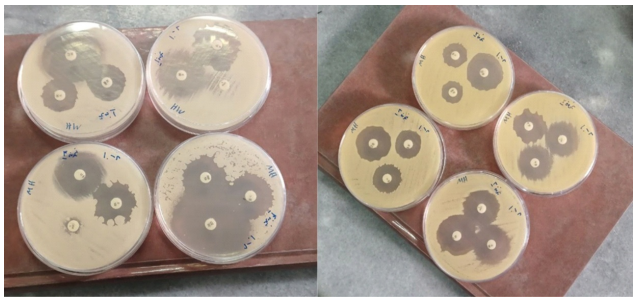

Fig. 1. Antibiotic sensitivity determination and the antibiotic resistance pattern of isolates obtained from handmade sausage samples

Table 2
Primers, target genes, and PCR conditions used in this study

Primers	Primer used (5′→3′)	Number of amplified base pairs (*bp*)
Cow	F: 5'-GCCATATACTCTCCTTGGTGACA-3′ R: 5'-GTAGGCTTGGGAATAGTACGA-3′	68
Sheep	F: 5'-ATGCTGTGGCTATTGC-3′ R: 5'-CCTAGGCATTTGCTTAATTTTA-3′	119
Chicken	F: 5'-CCTCCCAGCTCCATCAAACATCTCATCTTGATGAAA-3′ R: 5'-AAGATACAGATGAAGAAGAATGAGGCG-3′	133
Dog	F: 5'-GAGTTGATCCTTTTAGATTGTT-3′ R: 5'-AAGGGAATGATGAAAGACAT-3′	161
Donkey	F: 5'-CTATCCGACACACCCAGAAGTAAAG-3′ F: 5'-CTATCCGACACACCCAGAAGTAAAG-3′	439

Table 3
Specific primers designed for determining the type of microparasitic cyst species

Primers	Primer used (5'→3')	Number of amplified base pairs (*bp*)
B22	F: 5'- AACGGGCGAGTAGCACCTGAGGAGA -3'	115
B23	R: 5'- TGGGTCTACGTCGATGGCATGACAAC -3'	115
COC1	F: 5'- AAGTATAAGCTTTTATACGGCT -3'	270-350
COC2	R: 5'- CACTGCCACGGTAGTCCAATAC -3'	270-350
SF1	F: 5'- ATGGCGTACAACAATCATAAAGAA -3'	1100
SR5	R: 5'- TAGGTATCATGTAACGCAATATCCAT -3'	1100
Np21	F: 5'- CCCAGTGCGTCCAATCCTGTAAC -3'	337
Np6	R: 5'- CTCGCCAGTCAACCTACGTCTTCT -3'	337

Table 4
The unauthorized tissues observed in handmade sausages samples from provinces of Iran

City	Sample code	Soybean tissue	Cartilage tissue	Bone tissue	Parasitic microcyst	Smooth muscle	Dense connective tissue (percentage)	Skeletal muscle tissue (percentage)	Adipose tissue) (percentage)
Tehran	1			-		-	25.27	45.77	19.56
	2		-		-	-	15.14	36.11	20.14
	3	-	-	-	-	-	19.99	51.59	24.53
	4	-			-		19.21	61.06	17.03
Karaj	5		-			-	26.23	49.25	23.44
	6				-	-	16.01	61.23	18.18
	7	-		-	-	-	14.13	62.05	21.09
Tabriz	8	-		-	-		21.20	49.32	23.14
	9				-	-	12.95	53.89	14.07
	10			-	-	-	21.66	50.74	26.54
Hamedan	11	-	-	-		-	11.75	49.61	25.14
	12	-	-		-		9.58	62.24	17.65
Borujerd	13		-	-	-		19.21	58.32	19.11
Babol	14	-		-	-	-	15.68	52.47	11.57
	15		-		-	-	14.55	60.19	18.66
Mashhad	16		-		-	-	21.07	53.01	16.46
	17	-	-	-	-	-	18.57	55.95	20.88
Shiraz	18						21.44	57.59	14.91
	19		-		-	-	19.64	59.12	8.09
Average (percentage)		f57.89	42.10	52.63	21.05	26.31	18.06	54.18	18.95

3.2. Histology

In the histological study of the collected samples, a total of 57.89% (11 out of 19 samples) of the facial samples contained soybean plant tissue on average. Additionally, 49.10% (8 out of 19 samples) contained cartilage tissue, 52.63% (10 out of 19 samples) contained bone tissue, and 26.31% (5 out of 19 samples) contained smooth muscle tissue. In this study, microcystic sections of parasites were observed in 21.05% (4 out of 19 samples) of the tissue sections. On average, 18.06% of the compositions in these products were dense connective tissue, 54.18% were skeletal muscle tissue, and 18.95% were adipose tissue. The results, broken down by samples from each city, are shown in Table 4 (Fig. 2).Table 4 Results of the histological examination of the studied handmade sausage samples.

3.3. Species Identification

The results from the PCR test to determine the meat origin, using primers for chicken, cow, sheep, donkey, and dog, showed that out of the 19 samples, 4 samples contained the genome of both cow and chicken (simultaneously) (Fig. 3(A)). No dog or odd-toed ungulate genomes were detected in any of the samples (Fig. 3(B)). Finally, only one sample showed the sheep genome (Fig. 3 (C)).

3.4. Identification of Parasitic Cyst Species

In the investigation and species determination of the parasitic microcysts observed in the collected samples using the PCR method for *Toxoplasma gondii* (B22/B23), all samples were negative. Regarding the *Coccidia* primer (COC1/COC2), four samples (21.05%) were positive. When testing with the primer for *Sarcocystis species* (SF1/SR5), all samples were negative. For the *Neospora* primer (Np21/Np6), one sample (5.26%) was positive.

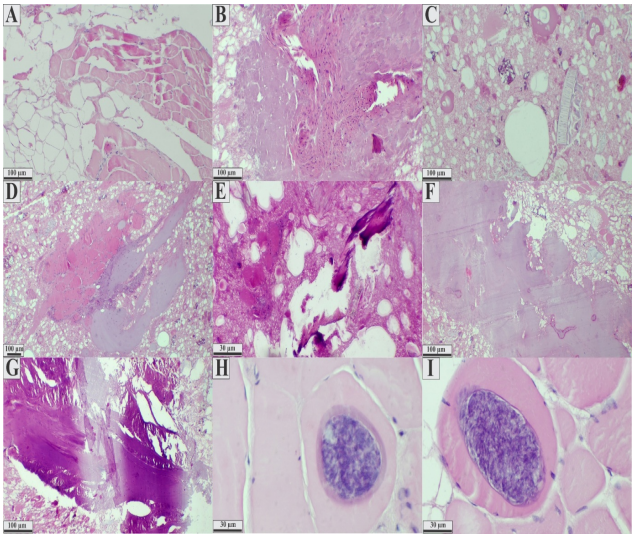

Fig. 2. Authorized and unauthorized tissues observed in the histological sections of the handmade sausages studied. A) Skeletal muscle tissue and fat; B) Smooth muscle tissue; C) Soy plant tissue; D) Cartilage tissue; E) Bone tissue; F and G) Dense connective tissue; H and I) Parasitic microcysts

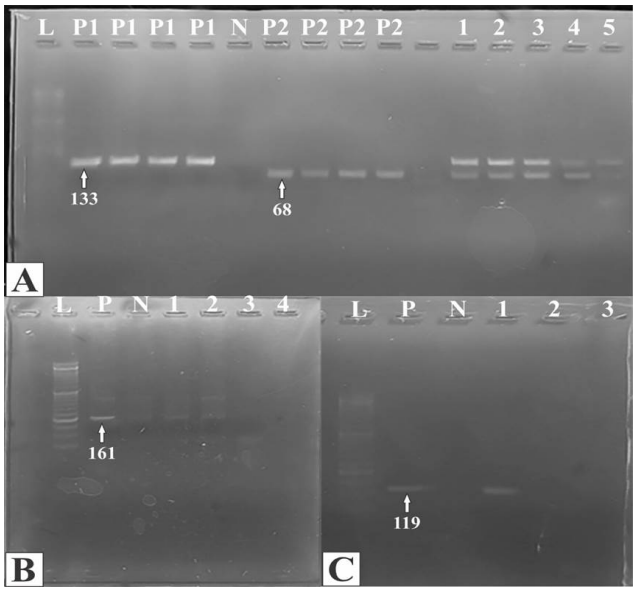

Fig. 3. PCR test images related to the origin of meat used in the production of handmade sausages. A) From left to right, the chicken primer (bp 133) and the cow primer (bp 68) are shown. In the corresponding band, both genomes are expressed in 4 samples, indicating the presence of both cow and chicken meat. B) In the examination with the dog primer (bp 161) and donkey primer (bp 439), no genome was observed in any of the samples. C) In the examination with the sheep primer (bp 119), only one sample was positive

4. Discussion

In the present study, which was conducted on handmade sausage samples collected nationwide, unauthorized tissues such as soybean, cartilage, bone, smooth muscle, and microcystic sections of parasites were observed. Additionally, a high proportion of dense connective tissue and adipose tissue was found in these samples. In the investigation of suspected parasitic cysts with PCR testing, four samples were positive for Coccidia primers, and one sample was positive for Neospora primers. In the bacterial examination of the samples, four samples at a 0.01 dilution contained a single colony, with three samples showing a significant presence of *Bacillus subtilis* and one sample containing *Bacillus cereus*. Among these, all four samples were sensitive to the antibiotic's chloramphenicol, cefixime, penicillin, cefazolin, ampicillin, and vancomycin, while one sample was resistant to cefixime and another was resistant to cefazolin. According to PCR testing, four samples contained both chicken and cow meat simultaneously, and one sample contained sheep meat.

Today, the demand for accurate and transparent information about food products from consumers is greater than ever, and meat products are no exception. The use of PCR technology in meat authenticity testing is an effective method (Cavin *et al.*, 2016). In a study aimed at identifying the animal species used in 91 beef samples through PCR, it was shown that 2.47% of the samples contained chicken meat, and 0.7% contained donkey meat (Mousavi *et al.*, 2015). In another study, the PCR test was used to investigate impurities in meat products in terms of the presence of soy. The results showed that 100% of sausage products, 20% of chicken cutlets, 33% of sausage products, 60% of hamburger products, and 15% of kebab products contained soy (Bazyari *et al.*, 2015). Alikord, *et al.* in 2017 used a composite polymerase chain reaction (PCR) to identify the meat consumed in raw and cooked meat products. The findings showed that, overall, six cases (17%) of fraud were detected, with four cases (11%) involving horse meat and two cases (6%) involving donkey meat. No instances of pork meat were found. The use of beef/sheep meat was confirmed in all samples, indicating that the use of other animal meats was solely to reduce economic costs (Alikord *et al.*, 2017). In a report by Eaqub Ali, *et al.* in 2015, PCR was used to determine the origin of raw materials in the production of meat products. The results indicated the presence of five non-halal species, including cat, pig, dog, monkey, and rat, in products that were sold as halal food (Eaqub Ali *et al.*, 2015).

Studies show that incorrect labeling of meat products occurs in more than 20% of cases (Ballin *et al.*, 2009). The mixing of chicken meat and by-products in meat products labeled as red meat has been reported in recent years (Nešić *et al.*, 2017).

In a study by Hosseni *et al.* in 2014, ten different brands of red meat sausages, made from samples collected in Tehran province, were analyzed using PCR to determine the type of meat used. The specific PCR results revealed that, contrary to the labels on the products, all samples contained poultry by-products (Hosseni *et al.*, 2014). In another study, Lakzadeh and *et al.* in 2016 used Real-Time PCR to identify and measure the amount of chicken tissue in meat products labeled as red meat. The results of the study indicated the presence of 84% and 26% chicken tissue, respectively, in sausage and burger samples (Lakzadeh *et al.*, 2016). Sadeghpour, *et al.*, in 2022 investigated the authenticity of 47 samples of raw meat and red meat products from Tabriz, specifically focusing on the mixing of chicken meat or other poultry parts, using PCR technique. The results showed that poultry by-products were mixed in both processed products with standard certification, such as fully and semi-processed products, as well as in raw meats. Overall, 87.23% of the collected samples were found to be fraudulent (Sadeghpour *et al.*, 2022). Al-Qassab, *et al.*, in 2019 conducted a study on 114 samples of red meat sausages with 40%, 55%, and 70% meat content from 10 different companies in Tehran. After extracting DNA from the sausages, they were amplified using multiplex PCR. The results showed that in 60 sausages (52.6%), only

chicken DNA was detectable. In 48 sausage samples (42.1%), both cow and chicken DNA were present, and in 6 samples (5.3%), only cow DNA was detectable. The use of chicken meat in sausages labeled as red meat may be due to the lower price of chicken compared to red meat, which allows producers to make a higher profit (Al-Qassab *et al.*, 2019). The results of the present study, which focused on handmade sausage samples, showed that 4 samples contained both chicken and cow meat, and 1 sample contained sheep meat.

Julini, *et al.* in 1979 reported the use of unauthorized animal tissues such as nerves, bones, skin, kidneys, and mammary glands in sausages (Julini *et al.*, 1979). Prayson, *et al.* in 2008 conducted histological studies on eight different brands of hamburgers in the United States. They found that all eight brands contained unauthorized tissues including connective tissue, blood vessels, fat, and peripheral nerves. Additionally, skeletal muscle was observed in seven samples, plant tissue in four brands, cartilage tissue in three brands, and bone tissue in two hamburger brands (Prayson *et al.*, 2008). In another study, the presence of unauthorized tissues was investigated in 20 samples, including kebab, sausage, handmade hamburger, and koobideh kebab. The histological test results showed the presence of tissues such as connective tissue in all samples, gizzard in two samples, fat in seven samples, soy in eleven samples, cartilage in five samples, and ovary in one sample (Latorre *et al.*, 2015). In the present study, soy plant tissue, cartilage, bone, smooth muscle, and dense connective tissue were observed, which is consistent with previous studies.

In addition, Jahed Khaniki, *et al.* in 2004 found that the contamination rate of samples with Sarcocystis parasites was 25.6%, and the presence of tissue masses was 5%, with a significant amount of plant tissue also observed (Jahed Khaniki and Rokni, 2004). Furthermore, Jahed Khaniki and Kia in 2006 studied the prevalence of Sarcocystis cysts in Iranian hamburger meat products using histological methods, and the results indicated that the contamination of samples with Sarcocystis parasites was approximately 60% (Jahed Khaniki and Kia, 2006).

In another study, raw hamburgers sold in Tehran were examined for Sarcocystis cysts. The results showed that no parasite cysts were observed in any of the industrially produced samples; however, one handmade sample was found to contain parasite cysts (Hosseini *et al.*, 2008). The results of the present study regarding the observation of parasitic cysts were consistent with previous reports, but they did not align with the species of parasite most commonly reported in studies on Sarcocystis cysts.

Molecular detection of N. caninim herein reported is consistent with previous studies reporting widespread and considerable prevalence of the parasite in muscular tissue of mammalian and avian hosts (refs). In other researches on meat products N. caninum DNA and cysts were reported by PCR (refs) and histology (refs). However, it should be remembers that microscopic cysts of the parasite could be located in one portion of the examined tissue collected from molecular analysis while absent in another portion taken for histological examinations (Karimi *et al.*, 2023).

Unfortunately, the issue of fraud in meat products, which disrupts public health, contradicts religious and ethical principles, and undermines public trust in a system of fair trade, is a very important topic in the food industry. Therefore, it is essential to make adherence to standards and quality control in meat products mandatory for producers, with serious supervision over their practices. Additionally, identifying the type of meat used in meat products is important for religious, health, and economic reasons. In this regard, the present study is the first to examine handmade sausages from across Iran using histological testing and PCR techniques to detect fraud in meat products.

5. Conclusion

Research indicates that the use of unauthorized tissues in meat products is possible. Based on the histological findings in the present study, it can be concluded that some handmade sausages across the country contain unauthorized tissues, Neospora microcysts, and coccidiosis. The primary meat used is beef, although chicken and lamb meat were also observed. Considering that handmade sausages are not subject to health supervision or quality control, there is a possibility of using substandard meat products. Therefore, it is important to emphasize that more rigorous control and supervision should be implemented by regulatory and health organizations.

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ORIGINAL RESEARCH PAPER

Molecular Detection of *Neospora Canium* in Bovine Colostrum: A Possible Risk Factor for Neonatal Infection

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Abstract

Neospora caninum is an obligate intracellular parasite that is both vertically and horizontally transmitted in cattle leading to significant economic losses in the cattle industry. The aim of this study was to investigate the prevalence of colostrum contamination in Neospora positive cows as a potential route for horizontal transmission. To this end, whole blood and colostrum from 44 fresh cows were analyzed using polymerase chain reaction (PCR) and nested PCR methods to detect the presence of the parasite DNA. In addition, serum samples were tested with a whole cell-based enzyme-linked immunosorbent assay (ELISA) to identify antibodies against the parasite. The results showed that 19 out of 44 colostrum samples (43.18%) contained the parasite genome. In addition, 16 of 18 (88.9%) Neospora -positive cows and 13 of 14 (92.9%) seropositive cows were found to have secreted infected colostrum. The relative risk (RR) of colostrum infection was significantly higher in blood PCR-positive cows (RR=7.7, P=0.0002) and seropositive cows (RR=4.6, P<0.0001) compared to uninfected cows. Based on these results, it is suggested that colostrum may be considered as a potential source of horizontal transmission in newborn calves and that methods for removing the infection such as pasteurisation may be required to reduce the risk of transmission.

1. Introduction

Neospora caninum is an obligate intracellular apicomplexan protozoan, poses a significant threat to the cattle industry due to fetal losses (Dubey and Schares, 2011). The infection can be transmitted horizontally (exogenous transmission) or vertically (endogenous transmission) (Wouda *et al.*, 1998). The probability of abortion in infected cows is 3 to 7 times higher than in non-infected cows (Dubey and Schares, 2011). Infected fetuses may be aborted or, if delivered naturally, may be congenitally infected (Wouda *et al.*, 1998).

Colostrum is the primary source of passive acquired immunity, predominantly containing IgG1, as well as immune cells such as macrophages and neutrophils, which contribute to the secretion of complement factors, cytokines, and antimicrobial peptides (Stelwa-

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gen *et al.*, 2009). Studies on the contamination of colostrum with *Neospora* have been limited; however, recent research has focused on the mechanisms of the innate immune response, including Toll-like receptor molecules, which play a crucial role in the induction of pro-inflammatory and adaptive immune responses (Marin *et al.*, 2007; Rivera *et al.*, 2021).

Oocytes, tachyzoite or tissue cysts ingestion is reported as the main route for horizontal transmission. Neospora DNA was first detected in colostrum of infected cattle indicating the colostrum to be a route of the parasite transmission (Lindsay and Dubey, 1990). Based on a previous laboratory study showing a sensitivity for *N. caninum* tachyzoites in HCL-pepsin (Lindsay and Dubey, 1990), consumption of contaminated colostrum was considered to be uninfective. However, other studies reported oral transmission of *N. caninum* through colostrum. Experimental consumption of colostrum, milk or milk replacer, even one week after birth, can cause infection in calves (Moskwa and Cabaj, 2007).

The frequency of *Neospora caninum* tachyzoite contamination in bovine colostrum and milk may vary depending on the stage of lactation or dairy cow breed. Consequently, a considerable number of tachyzoites in colostrum or milk could result in infection of the calf (Moskwa and Cabaj, 2007).

Depending on the stage of lactation and the number of tachyzoites in the colostrum or milk, the contamination of colostrum and milk with tachyzoites may change The permeability of intestine in the first few hours after the birth is crucial for the efficient transfer of colostrum immunity to newborn calves. The high permeability of intestinal cells in newborn calves makes them highly susceptible to macromolecules and pathogens. Therefore, contaminated colostrum can serve as a potential source of pathogens for calves before their gut closure (Moskwa and Cabaj, 2007).

The horizontal role of colostrum in transmission of pathogens is also accounted as a topic of interest. The blood circulation of the parasite and the response of the immune system can probably affect the presence of the parasite in the breast tissue and its secretion in the colostrum. The primary objective of this study was to determine the prevalence of colostrum contamination in infected cows and to evaluate the correlation between the presence of the parasite in colostrum, the serological immune response, and the detection of parasite DNA in blood.

2. Materials and Methods

2.1. Animals and Sampling

Based on an effect size of 0.6 (Mohammed and Aaiz, 2025; Ayati *et al.*, 2023), α<0.05 level and a 1-ß=0.95, the sample size was determined. Sampling was performed in an industrial Holstein dairy cattle herd located in Qazvin Province, Iran, with cows evenly distributed across parities one through four. Blood and colostrum samples were collected from 44 full-term cows. Blood was collected using 10 ml EDTA K3 Venoject and plain 10 ml Venoject from the jugular vein, and colostrum in 70 ml sterile bottles (at least 10 ml from each quarter). The buffy coat and serum were separated by centrifugation at 1500×g for 10 minutes. All samples were stored at -20°C before analysis.

2.2. Indirect ELISA Test

Two million (2×10^6) parasite tachyzoites were cultured at room temperature for three days. 300 L of blocker buffer (100 ml PBS + 5 g powdered milk) were added to each well of the plate and incubated at 37°C for one hour. The parasites were washed, and 100 L of the serum samples were added diluting at a ratio of 1:100. HRP-conjugated sheep anti-bovine IgG heavy chain was added to the wells and incubated at 37°C for one hour. After three washes and a thirty-minute incubation, 100 L of substrate was added. Finally, 50 L of 2.5 M sulfuric acid was added as the stop solution. The optical density (OD) was measured at a wavelength of 450 nm. The S/P ratio was calculated as follows: S/P= (sample-negative control) / (positive control-negative control). An S/P value less than 0.5 (the cut-off point for negative ELISA) was considered as negative (Schares *et al.*, 2004).

2.3. The Polymerase Chain Reaction (PCR) Conforming

DNA extraction from colostrum and blood buffy coat was performed using the Tissue Genomic DNA Extraction Mini Kit and the Blood Genomic DNA Extraction Mini Kit, respectively, both from FAVORGEN Company (Meiri-Bendek *et al.*, 2002). The extracted DNA was stored at -20°C until used.

PCR: NC5 gene from *N. caninum* was detected in buffy coat samples using Np6 forward (5′-CTCGCCAGTCAACCTACGTCTTCCT>-3′) and Np21 reverse (5′ -CCCAGTGCGTCCAATCCTGTAA CC >-3′) primers (Muller *et al.*, 1996). The PCR was programmed as 10 minutes at 95C for primary denaturation and 35 cycles of 95C for 1 minute, 65°C 1 min and 72°C for 1 minute and a final extension at 72°C for 10 min.

Nested-PCR: 1 μl of the PCR products was subjected to nested-PCR for confirming the presence of *N.caninum* NC5 gene using 5′-GTGTTGCTCTGCTGACGTGT-3′ forward and 5′-TACCAACTCCCTCGGTTCAC-3′ reverse primers (Ayati *et al.*, 2023). The nested PCR was programmed as 10 minutes for primary denaturation at 95°C and 35 cycles of 1 minute at 95°C for denaturation, 45 sec-

onds at 54°C for annealing and 1 minute at 72°C for extension. Finally, the reaction was completed with a final extension at 72°C for 10 minutes.

Statistical Analysis: Chi-square test was used to describe the relationship between variables and logistic regression to the model and predict the factors determining the presence of parasites in colostrum. Analyzes were performed at $\alpha<0.05$ level by IBM SPSS Statistics for Windows, version 19 (IBM Corp., Armonk, N.Y., USA).

3. Results

Neospora caninum DNA was detected in 19 out of 44 colostrum samples (43.18%). *Neospora caninum* DNA was also detected in colostrum of 16 out of 18 (88.9%) blood positive and 13 out of 14 (92.9%) seropositive cows (Table 1).

Table 1
Correlation between blood PCR and serum ELISA-Ab with colostrum PCR results

		Colostrum PCR		*P*-value	Relative Risk (CI 95%)
		Positive	Negative		
Blood PCR	Positive	16 88.9%	2 11.1%	0.0002	7.7 (2.6-22.6)
	Negative	3 11.5%	23 88.5%		
ELISA -Ab	Positive	13 92.9%	1 7.1%	<0.000	4.6 (2.2-9.6)
	Negative	6 20.0%	24 80.0%		

Table 2
Reliability of blood PCR and serum ELISA-Ab for detection of *N. canium* infection

		ELISA-Ab		*P*-value	*kappa*
		Positive	Negative		
Blood PCR	Positive	13 72.2%	5 27.8%	<0.001	0.708
	Negative	1 3.8%	25 96.2%		

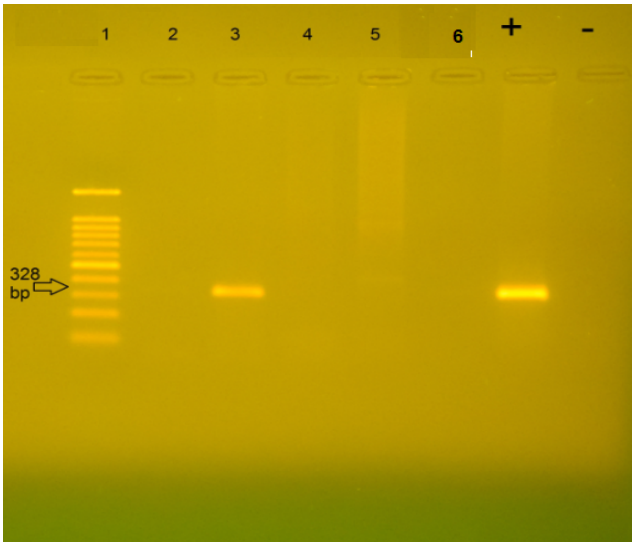

Fig. 1. PCR results with primers NP6 and NP21, bp 328 (1: standard DNA, 2 to 6: tested samples, +: positive control, -: negative control)

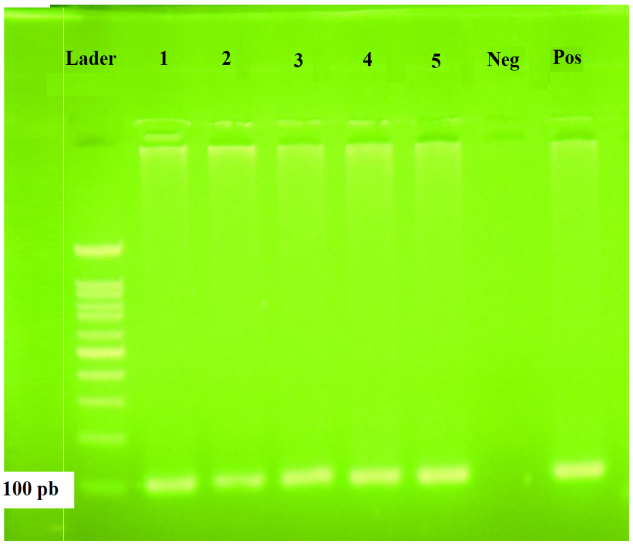

Fig. 2. Nested-PCR results with bp100 primer (Standard DNA (Lader), 1 to 5: positive samples, positive: positive control, negative: negative control)

4. Discussion

Neospora caninum-DNA was detected in 18/44 (40.90%) and 19/44 (43.18%) blood and colostrum samples, respectively. As presented in Table 1, Neospora DNA was detected in the colostrum of 88.9% (16/18) of cows positive by blood PCR and 92.9% (13/14) of cows seropositive by ELISA. These findings indicate that a substantial proportion of cows testing positive for the parasite-either through molecular detection in blood or serological assays-produce colostrum containing parasite DNA.

This point becomes more significant when despite the antibody response, Neospora parasite can still be detected in the bloodstream of infected cows (Ayati *et al.*, 2023; Okeoma *et al.*, 2004). Therefore, because of the high rate of blood circulation mammary glands can be considered as a target for the parasite in newborn cows.

The genome of *Neospora caninum* was first identified in the colostrum of infected cattle in 2007 (Moskwa *et al.*, 2007). Previous studies reported the mammary glands as one of the target tissues of the parasite. They noted that the parasite was present in the mammary glands during the acute phase of the disease and it was cleared when the disease became chronic (Lopez-Perez *et al.*, 2006). The presence of the parasite in the colostrum in the early lactation is due to the natural hyperaemia of the mammary glands in this lactation stage (Gotze *et al.*, 2010).

Genomic detection methods such as PCR are capable of identifying DNA from both viable and non-viable pathogens, whereas bioassay techniques are required to detect only live pathogens. Therefore, assays targeting specific pathogens must be meticulously designed and rigorously validated to enable accurate interpretation of results and definitive conclusions (Valasek and Repa, 2005). With this understanding, if genomic detection by PCR is assumed as the criterion for identifying live parasites, then the data suggest that oral infection with *N. caninum* through colostrum could serve as a potential mechanism for horizontal transmission, resulting in the infection of newborn calves shortly after birth (Uggla *et al.*, 1998). It is shown that one-week-old calves can be experimentally infected by ingesting *N. caninum*-infected colostrum or milk replacer (Davison *et al.*, 2001). The susceptibility of *N.caninum* tachyzoites to HCL-pepsin is reported. However, certain factors, such as encapsulation of the parasite by biological materials like placenta, may shield the tachyzoites from the acidic gastric environment, facilitating their safe passage into the intestine and subsequent infection of the host animal (Dijkstra *et al.*, 2001). Moreover, concentration of the parasite in colostrum and milk can significantly vary among the host cows and during stages of lactation and it can be influenced by immunological conditions. Thus, calves may potentially contract the infection through the colostrum under specific circumstances, particularly when high numbers of tachyzoites are present in the colostrum or milk during the lactation period.

The high intestinal permeability and incomplete function of the HCL-pepsin system in the abomasum of new born calves increase the absorption of macromolecules from the colostrum within the first 24 hours of life. This phenomenon guarantees the effective absorption of maternal immunity (Lopez and Heinrichs, 2022). Nevertheless, there is still a potential risk of infection for newborn calves due to environmental exposure or the ingestion of colostrum contaminated with pathogens.

The immune function of periparturient cows may be suppressed in various ways due to metabolic, hormonal and immunological disturbances. Consequently, clinical mastitis and other diseases are more common during this period (Vlasova and Saif, 2021). The pathogens are likely not only present in the secretions of the mammary glands but also in the circulatory system. Umbilical endothelial cells are immunoreactive when they encounter parasites, stimulating the transcription of genes encoding for inflammatory and immunomodulatory molecular genes, which in turn lead to innate and acquired immune responses (Taubert *et al.*, 2006). A previous study indicated the presence of *N. caninum* DNA in the milk of seropositive cows, which can be followed by lactogenic transmission. Other researchers have observed the absence of *Neospora* DNA in colostrum samples, peripheral blood mononuclear cells (PBMC) of heifers and calves, as well as in the umbilical cord (5), which is attributed to the infection occurring prior to delivery. In this case, the bradyzoite form of the parasite may enter the tissues due to the weak immune responses. Furthermore, the expression of genes and the production of anti-inflammatory cytokines such as IL-10 in the umbilical cord may also contribute to the observed weakness of the immune system (Taubert *et al.*, 2006).

Moskwa *et al.* (2007) reported that due to the presence of high levels of specific anti-Neospora IgG antibodies in the colostrum, it is unlikely that the infection will be transmitted through colostrum. Consequently, the presence of contamination in colostrum cannot be the definitive reason for the transmission of the parasite due to the presence of antibodies. The mechanism of this neutralisation is not yet clear, but colostrum can be considered as a source of contamination and transmission (Moskwa *et al.*, 2007).

Immunomodulatory agents suppress the responses of the immune system and lead to colonization of the parasite in the tissue, such as the mammary glands. TH2 lymphocytes stimulate antibody production by the humoral immune system, which leads to the production of specific antibodies, including IgG. These antibodies have the capacity to neutralize the parasite.

However, although they neutralize the parasite in the tissue and colostrum of the cow, they still have the potential to transmit the infection to calves (Taubert *et al.*, 2006).

The relative risk of colostrum contamination was 7.7 (95% CI: 2.6-22.6, p-value <0.0002) and 4.6 (95% CI: 2.2-9.6, p-value<0.000) for dams that tested positive for *N. caninum* by PCR and ELISA, respectively (Table 1). These results indicate that the risk of colosterum contamination was significantly higher in mothers that tested positive than in those that tested negative (7.7 and 4.6 times, respectively). Therefore, both the PCR and ELISA tests are useful markers of the risk of colosterum contamination in cattle.

The Kappa coefficient is a statistical measure used to evaluate the agreement between two diagnostic tests and is a standard method for assessing the consistency and reliability of classified data. In this study, a Kappa value of 0.708 (P-value<0.001) between PCR and ELISA indicates good agreement between the results of the two tests detecting *N. caninum* infection. This value means that their diagnostic agreement is beyond chance and is reliable. Generally, a Kappa between 0.61 and 0.80 indicates acceptable and reliable agreement (Cohen, 1960). Therefore, these two tests provide similar and dependable results in diagnosing the disease in question. (Table 2).

The reliability of blood PCR and serum ELISA-Ab tests for detecting *N. caninum* infection is highlighted in Table 2. Although both tests demonstrate a good level of agreement, there are instances where their results differ. The blood PCR test appears to be more accurate in identifying positive cases, but the ELISA-Ab test may be more susceptible to producing false positive results. These findings suggest that using both tests for combination can lead to a higher level of diagnostic accuracy in *N. caninum* infection.

5. Conclusion

If the presence of the parasite genome is interpreted as the presence of viable parasites, colostrum may be considered a significant potential source of horizontal transmission in newborn calves. Therefore, it is recommended to implement biosecurity measures, such as pasteurization, to reduce this potential risk. Furthermore, studying the distribution of the parasite in mammary gland tissue and its correlation with parasitemia and the immune response status may contribute to a better understanding of the disease pathogenesis. It can be suggested to design an observational study to investigate the potential risk of transmission through colostrum and milk to calves, as well as to assess the viability of the parasite in these samples.

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ORIGINAL RESEARCH PAPER

Evaluating the Prevalence of *Isospora* in Parrots in Golestan Province

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Golestan province
Parasitic infection

Abstract

Introduction: Parasitic infections in pet birds are common issues in veterinary medicine. *Isospora* is an important pathogenic agent in birds, particularly in various species of parrots, causing diarrhea and feather loss. This study investigates the prevalence of *Isospora* infection in parrots in Golestan Province.
Materials and Methods: In this study, fecal samples were collected from parrots that were brought to veterinary clinics in Golestan Province by their owners between October 2022 and May 2023, as well as from parrots kept in pet shops. The samples were examined using the sucrose flotation method.
Results: Among the 51 samples examined, 6 birds showed clinical signs of lethargy, 12 exhibited feather loss, and 9 had watery diarrhea. The results of both the flotation and direct microscopic examinations indicated that none of the birds were infected with *Isospora*.
Conclusion: The findings suggest a low prevalence of *Isospora* in parrots of Golestan Province. Based on the negative test results, it can be concluded that *Isospora* is not widely prevalent in this region, and the clinical symptoms observed in parrots are likely due to other parasitic, bacterial, or viral infections.

1. Introduction

Isosporiasis is a parasitic disease caused by protozoa of the *Isospora* genus, which has been reported in various species of migratory, domestic, and caged birds (Lindsay *et al.*, 1997). Several studies have shown that many bird species have co-evolved with specific species of *Isospora*. The severity of this disease can range from subclinical infection to severe and fatal cases. The transmission of this parasite among captive birds, especially species at risk of extinction or those kept for breeding and reintroduction programs, is a significant concern (Goodgame, 1996).

One of the critical issues in bird care facilities is assessing the impact of housing different bird species in shared enclosures on the risk of severe isosporiasis caused by host-incompatible *Isospora* strains. Further research is needed to better understand interspecies transmission of this parasite and potential host shifts (Maria de Fatima *et al.*, 2025).

Isosporiasis is a type of coccidiosis caused by *Isospora*, although other protozoa can also be responsible for the disease. Epidemiological studies have shown that *Isospora* is significantly prevalent among wild bird populations. This parasite has a complex life cycle, which includes an intestinal phase and possibly a systemic phase, the latter of which may be associated with atoxoplasmosis. Both intestinal and systemic isospori-

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asis can lead to severe mortality in bird populations, including rare and endangered species (Chen *et al.*, 2025). While anticoccidial treatments have been effective against intestinal isosporiasis, no definitive treatment has been reported for its systemic form. Infected birds may exhibit symptoms such as feather loss, lethargy, and diarrhea, which, if not diagnosed and treated promptly, can lead to mortality (Tokiwa *et al.*, 2025).

Despite the known impact of *Isospora* on ornamental birds, its prevalence in parrots, particularly in Golestan Province, remains unclear. Investigating this parasite in local parrot populations will help bridge this knowledge gap and provide insights into its epidemiology. Comprehensive research in this field can contribute to a better understanding of the disease's epidemiology, improve control strategies, and reduce the risks associated with this parasite in valuable bird populations.

2. Materials and Methods

2.1. Study Type and Population

This study was a descriptive, cross-sectional investigation conducted on parrots in Golestan Province to determine the prevalence of *Isospora* infection. The study included parrots brought to veterinary clinics by their owners between October 2022 and May 2023, as well as parrots kept in pet shops within the province.

2.2. Sampling and Sample Preparation

Fecal samples were collected from various parrot species during the specified period. For sample preparation, 2 grams of feces were collected from each bird. Initially, a direct smear was prepared from each sample and examined microscopically. The samples were then mixed with 10 mL of distilled water in a beaker and stirred continuously for 5 minutes to obtain a homogeneous solution.

Once homogenized, the solution was filtered and transferred into a test tube. The tubes were centrifuged at 1,500 rpm for 5 minutes. After centrifugation, the supernatant was discarded, leaving only the sediment at the bottom of the tube. A pre-prepared saturated sucrose solution was then added to the tube until the liquid level reached halfway. The mixture was carefully stirred again to ensure uniformity. After adding more sucrose solution to completely fill the tubes, they were left undisturbed for 20 minutes. Finally, a microscopic slide was prepared from the surface of the sample for further examination.

3. Results

Out of the 51 samples examined, 6 birds (11.7%) exhibited clinical signs of lethargy, 12 birds (23.5%) showed feather loss, and 9 birds (17.6%) had watery diarrhea. The results from both flotation and direct examination methods revealed that none of the birds were infected with *Isospora* (Fig. 1).

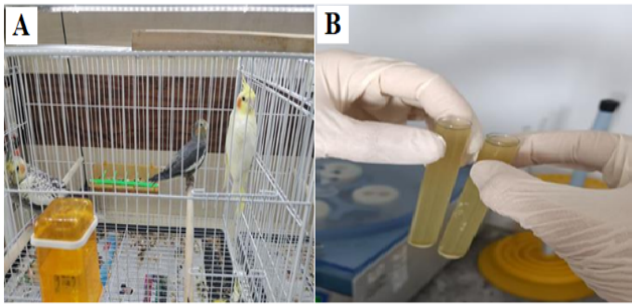

Fig. 1. Collected Samples from Parrots in Golestan Province. A) Studied parrots B) Sucrose flotation method

Among the studied birds, the species most frequently reported were cockatiels (*Nymphicus hollandicus*) with 26 cases, lovebirds (*Agapornis* roseicollis.) with 9 cases, and budgerigars (*Melopsittacus undulatus*) with 7 cases (Table 1).

Table 1
Prevalence of *Isospora* and Clinical Symptoms in Studied Parrots by Bird Species

Bird Species	Frequency (%)	Isospora		Clinical Symptoms			
		Positive	Negative	Diarrhea	Feather loss	Lethargy	Asymptomatic
Nymphicus hollandicus	26 (51)	0	26	4	5	2	15
Agapornisroseicollis	9 (17.6)	0	9	3	2	2	2
Melopsittacusundulatus	7 (13.7)	0	7	1	2	1	3
Psittaculakrameri	6 (11.8)	0	6	1	1	1	3
Aratingasolstitialis	2 (3.9)	0	2	0	1	0	1
Pyrrhuramolinae	1 (2)	0	1	0	1	0	0
Total	51 (100)	0	51	9	12	6	24

4. Discussion

Parasitic infections represent a significant health concern for both domestic and wild birds, potentially having adverse effects on their overall well-being. Protozoa, such as *Eimeria* and *Isospora*, are among the leading causes of gastrointestinal infections in birds. While *Isospora* is more commonly found in passerines, *Eimeria* is prevalent in industrial poultry farms and pigeons (Hemmati *et al.*, 2023). These parasites can cause a range of clinical signs, including bloody diarrhea, depression, anorexia, and, in severe cases, mortality (Sanchez-Cordon *et al.*, 2007).

In this study, we investigated the presence and prevalence of *Isospora* infections in parrots in Golestan Province. Our findings revealed a very low prevalence of this infection among captive parrots in the province. These results contrast with previous studies that reported a higher prevalence of *Isospora* in other domestic and wild birds.

Relatively few studies have investigated parasitic infections in domestic birds in Iran. One such study by Tavasoli M *et al.* (2004) examined gastrointestinal parasites in domestic birds in Urmia, finding that approximately 47% of the canaries studied showed signs of infection (Tavasoli and Dastjerd, 2004). Another study by Islampanah *et al.* (2015) in Tehran and Alborz provinces focused on the prevalence of various *Eimeria* species in poultry, discovering multiple *Eimeria* species in both broiler and laying flocks (Islampanah *et al.*, 2015).

International studies have also examined similar topics. For example, Freitas *et al.* (2003) investigated coccidiosis in canaries in Brazil and found that 167 out of 327 canaries had coccidial infections (De Freitas *et al.*, 2003). Saki *et al.* (2012) studied 64 canaries in Turkey of which 18 were infected with *Isospora* (Saki and Ozer, 2012). A study conducted in New Zealand found that around 40% of migratory birds were infected with various parasites, although no clinical symptoms were observed. In Iran, Hemmati *et al.* (2022) examined *Isospora* and *Toxoplasma* infections in deceased canaries and reported that 7 out of 50 canaries examined had oocysts in their feces (Hemmati *et al.*, 2023).

There are several factors that can influence the results of studies on parasitic infections in birds, including the species studied, geographic location, bird management practices, and the environment in which birds are kept. For instance, in this study, we focused only on parrots brought to veterinary clinics or housed in pet shops, while many other studies have examined wild birds or passerines.

One possible reason for the lack of observed *Isospora* infections in this study could be the captivity of the birds. Captive domestic birds typically have limited contact with wild birds and are generally exposed to fewer environmental parasites. In contrast, wild birds are often exposed to a broader range of parasites, and the prevalence of parasitic diseases among them is higher (Box, 1975). Additionally, the level of awareness among bird owners and pet shop employees regarding avian parasitic infections may have influenced the findings.

Furthermore, this study relied solely on microscopic methods and standard tests for detecting *Isospora*. It is recommended that future studies incorporate molecular techniques, such as PCR (Polymerase Chain Reaction), to more accurately identify *Isospora* infections. These methods offer higher sensitivity and accuracy and are capable of detecting cases that might otherwise go undiagnosed.

Considering that 52.9% of the birds in this study exhibited at least one clinical sign, and *Isospora* was not identified, we suggest that future studies investigate other common parasitic, bacterial, and viral infections in parrots to identify the underlying causes of these clinical symptoms. These diseases can significantly impact bird health and welfare, and their investigation could contribute to improved health management in bird populations.

In conclusion, this study indicates that *Isospora* has a low prevalence in parrots in Golestan Province. This finding may contribute to improved health monitoring and the prevention of parasitic diseases in domestic and ornamental birds. However, it is important to note that the negative results do not definitively rule out *Isospora* infection in parrots. Factors such as the timing of tests, sample collection and transportation methods, and the sensitivity of the testing methods used can all affect the accuracy of the results. Therefore, we recommend further studies to assess the prevalence of *Isospora* in different regions, which will provide a better understanding of its distribution and epidemiology.

5. Conclusion

The results of this study, which indicate the absence of *Isospora* infection in parrots in Golestan Province, suggest a low prevalence of this parasite in the region. However, to enhance the accuracy of the findings and provide a more comprehensive understanding of *Isospora* prevalence in parrots, it is recommended that long-term studies with larger sample sizes be conducted. Such studies will offer more reliable data and improve our ability to assess the true distribution of this parasite in parrots within the province.

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ORIGINAL RESEARCH PAPER

Advanced Strategies for Mycotoxin Detoxification in Animal Feed: Challenges, Risks, and Innovations

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Abstract

Mycotoxins are toxic secondary metabolites produced by molds, posing a serious threat to livestock health, feed quality, and food security. These harmful compounds originate from environmental stressors and improper feed storage, leading to immune suppression, metabolic disturbances, and reduced productivity in animals. Given their stability and resistance to degradation, mycotoxins persist in feed materials, necessitating effective detoxification strategies to mitigate their risks. Their widespread occurrence across diverse climatic conditions underscores the necessity of global surveillance and intervention measures. Additionally, understanding species-specific sensitivity to different mycotoxins is critical for optimizing protective strategies in livestock. This review examines biological degradation, enzymatic neutralization, and advanced adsorbents, evaluating their efficacy, safety, and cost-effectiveness. Additionally, regulatory frameworks and innovative detection technologies are explored to ensure compliance with global feed safety standards. By synthesizing recent scientific advancements, this study provides a comprehensive perspective on mycotoxin mitigation, offering insights into sustainable livestock management and improved nutritional outcomes. These strategies contribute to enhancing feed security and reducing mycotoxin-related risks, supporting efficient animal production systems worldwide.

1. Introduction

Ensuring food safety remains a fundamental global concern, shaping national and international health policies aimed at hazard prevention. Both microbiological and chemical contaminants impact food security, with mycotoxins-fungal secondary metabolites-recognized by the World Health Organization (WHO) as major contributors to foodborne illnesses (Adelere *et al.*, 2025).

Animal nutrition plays a critical role in maintaining health, optimizing physiological functions, and supporting resilience against environmental stressors. An ideal diet must integrate energy, protein, essential minerals, beneficial microorganisms, and bioactive feed components, all tailored to enhance immunity, digestive efficiency, and reproductive performance (Tegzes, 2025). Livestock feed formulations are strategically designed not only to meet nutritional demands at minimal costs but also to sustain production efficiency, welfare, and overall health (Saghir and Bancroft, 2024).

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2. Chemical Contaminants and Mycotoxins

Chemical contaminants are undesirable substances present in food or animal feed, originating from production processes, environmental exposure, or post-harvest handling. Among these, naturally occurring toxins such as alkaloids and mycotoxins pose significant risks. Mycotoxins are toxins produced by specific molds that grow under warm and humid conditions, affecting a wide range of agricultural commodities, including grains, nuts, dried fruits, coffee beans, and spices (Chain *et al.*, 2024).

Despite the identification of over 300 mycotoxins, aflatoxins, deoxynivalenol (DON), zearalenone, fumonisins, ochratoxins, and T-2 toxin remain the most concerning contaminants in animal feed. These toxins exert neurotoxic, hepatotoxic, carcinogenic, and immunosuppressive effects, leading to nutrient malabsorption, altered gut microbiota, impaired reproduction, and increased disease susceptibility (Santos Pereira *et al.*, 2019, Tegzes, 2025). Chronic exposure further reduces livestock productivity and compromises global food security, contributing to economic losses and sustainability challenges (El-Sayed *et al.*, 2022).

3. Mycotoxin Presence in Animal Feed and Risk Prevention Strategies

The phrase "If feed exists, mycotoxins will exist" reflects the inevitability of fungal contamination in agricultural systems (Santos Pereira *et al.*, 2019, Tegzes, 2025). Molds responsible for mycotoxin production proliferate during feed storage and processing, and even properly dried feed can become susceptible to contamination when environmental conditions favor fungal growth. Proper drying and controlled storage remain among the most effective strategies for preventing mycotoxin formation (Organization, 2023).

4. Mycotoxin Regulation and Control Measures

Given the severity of contamination risks, international regulatory agencies, including the FDA, European Union, and agricultural policy frameworks, enforce strict limits on mycotoxin concentrations in food and feed (Santos Pereira *et al.*, 2019, Yang *et al.*, 2020). However, effective prevention strategies must begin at the source, implementing a comprehensive approach combining:

- Good Agricultural Practices (GAP) to minimize fungal colonization during crop production.
- Good Manufacturing Practices (GMP) & Good Hygiene Practices (GHP) to ensure safe processing and storage and reduce post-harvest contamination.
- Hazard Analysis and Critical Control Points (HACCP) for early identification and mitigation of contamination risks (Fumagalli *et al.*, 2021).
- If mycotoxin contamination occurs, detoxification methods such as:
- Physical strategies (sorting, washing, extraction, heat treatment, irradiation, adsorption),
- Chemical approaches (alkaline treatment, ozone treatment),
- Biological solutions (microbial degradation and enzymatic detoxification) can significantly reduce contamination levels (Liu *et al.*, 2022).

Given regional variations in legal regulations, integrated preventive frameworks, including early warning systems and contamination monitoring, have been established in countries with advanced agricultural sectors, such as the European Union (Table 1)(Fumagalli *et al.*, 2021).

Table 1
Mycotoxin detoxification, regulation, and monitoring approaches

Category	Methods & Strategies
Detoxification	- Physical: Sorting, drying, heat treatment, extraction with solvents - Chemical: Binding agents, alkaline treatment, ozone exposure - Biological: Microbial degradation, enzymatic neutralization
Regulation	- FDA & EFSA mycotoxin limits for livestock feed - National agricultural safety guidelines - Risk assessment and contamination threshold enforcement
Monitoring	- Rapid test kits for mycotoxin detection - Laboratory analysis for toxin quantification - Feed surveillance systems and early warning networks

Table 2
Effects of Mycotoxins on different animals

Animal	Major mycotoxins affecting health	Key health impacts
Sheep	Aflatoxins, Ochratoxins, Zearalenone	Liver toxicity, immunosuppression, reproductive issues, reduced wool quality
Cattle	Aflatoxins, Fumonisins, DON, T-2 toxin	Reduced milk yield, feed inefficiency, liver damage, metabolic disorders
Horses	Fumonisins, Aflatoxins, Ochratoxins	Equine leukoencephalomalacia (ELEM), poor performance, liver toxicity
Dogs	Aflatoxins, Ochratoxins, Penitrem A	Neurological disorders, liver damage, tremors, seizures
Cats	Trichothecenes, Ochratoxins, Citrinin	Kidney dysfunction, digestive distress, hematological disorders

5. Impact of Mycotoxins on Sheep Health

Mycotoxins pose significant health risks to sheep, affecting their immune system, metabolism, and overall productivity. Among the most concerning mycotoxins, aflatoxins and ochratoxins are known to cause liver toxicity, immunosuppression, and reduced growth rates in sheep (Yang *et al.*, 2020). Chronic exposure to contaminated feed can lead to digestive disorders, impaired wool quality, and increased susceptibility to infections (El-Sayed *et al.*, 2022). Additionally, zearalenone, a mycotoxin with estrogenic properties, may disrupt reproductive functions, affecting fertility and lambing success (Santos Pereira *et al.*, 2019). Economic losses due to mycotoxin contamination include reduced feed efficiency, lower meat and wool yields, and increased veterinary costs (Fumagalli *et al.*, 2021).

6. Impact of Mycotoxins on Cattle Health

Mycotoxins significantly affect cattle health and productivity, leading to digestive disorders, immune suppression, and reduced milk yield. Aflatoxins, one of the most toxic mycotoxins, are known to cause liver damage, impaired feed efficiency, and carcinogenic effects in cattle (Elgioushy *et al.*, 2020). Chronic exposure to ochratoxins and fumonisins can result in neurological dysfunction, reproductive issues, and metabolic disturbances (Awuchi *et al.*, 2022). Additionally, deoxynivalenol (DON) and T-2 toxins contribute to reduced appetite, weight loss, and increased susceptibility to infections (Duringer *et al.*, 2020).

Economic losses due to mycotoxin contamination in cattle include lower milk production, poor feed conversion rates, and increased veterinary costs. Regulatory agencies, such as the FDA and EFSA, have established strict limits on aflatoxin levels in dairy feed, as residues can transfer into milk, posing risks to human health (Table 2)(Jiang *et al.*, 2021).

7. Impact of Mycotoxins on Horse Health

Mycotoxins pose a significant risk to equine health, affecting digestive function, neurological stability, and immune response. Horses are particularly susceptible to fumonisins, which can cause equine leukoencephalomalacia (ELEM)-a fatal neurological disorder characterized by ataxia, paralysis, and brain lesions (Pinton *et al.*, 2019). Aflatoxins and ochratoxinscontribute to liver toxicity, reduced feed intake, and immunosuppression, leading to poor performance and increased disease susceptibility (Liesener *et al.*, 2010). Chronic exposure to zearalenone may disrupt reproductive health, mimicking estrogenic activity and affecting fertility (Table 2) (Dänicke *et al.*, 2021).

8. Impact of Mycotoxins on Dog Health

Dogs are highly sensitive to mycotoxins, particularly aflatoxins, ochratoxins, and penitrem A, which can cause severe liver damage, neurological disorders, and immunosuppression (Kearley *et al.*, 2024). Tremorgenic mycotoxins, commonly found in moldy food and decomposing organic matter, lead to vomiting, seizures, tremors, and respiratory distress (Pet Poison Helpline). Chronic exposure to contaminated pet food has been linked to hepatic failure and increased mortality rates, emphasizing the need for strict feed quality control (Table 2)(Tegzes, 2025).

9. Impact of Mycotoxins on Cat Health

Cats, though less prone to indiscriminate eating than dogs, can still suffer from mycotoxicosis due to contaminated pet food. Trichothecene mycotoxins have been associated with lethargy, inappetence, and hematological disorders, including pancytopenia and thrombocytopenia (Parent-Massin, 2004). Ochratoxins and citrinin are known nephrotoxins, leading to kidney dysfunction and metabolic imbalances (Tegzes, 2025). Additionally, aflatoxins can cause digestive distress, jaun-

dice, and hepatic failure, particularly in cases of prolonged exposure (Table 2)(Aquino and Corrêa, 2011).

10. Discussion

Effective quality control measures for raw ingredients and proper feed storage conditions are essential in minimizing mycotoxin contamination in animal feed. Several strategic interventions reduce the risk of high-dose exposure, ranging from physical decontamination methods (such as screening, drying, heat treatment, and raw material purification) to chemical applications (including acids, alkalis, salts, and oxidizing agents). Additional techniques involve binding agents (either mineral-based or microbial-derived from cell wall components) and biological treatments, such as enzymes or microorganisms capable of degrading toxins. Novel strategies, such as ozone exposure and advanced carbon-based nanoparticles, further enhance detoxification efforts.

Beyond conventional detoxification methods, species-specific impacts of mycotoxins must be considered when designing mitigation strategies. Sheep, for example, are highly susceptible to aflatoxins and ochratoxins, which can lead to iver toxicity, immunosuppression, and reproductive disorders. In cattle, chronic exposure to fumonisins and deoxynivalenol results in reduced milk yield, metabolic disturbances, and feed inefficiency. Horses, particularly sensitive to fumonisins, may develop equine leukoencephalomalacia (ELEM), a fatal neurological disorder. Dogs and cats, as monogastric animals, exhibit severe hepatic and renal dysfunction when exposed to aflatoxins and ochratoxins, with symptoms ranging from vomiting and lethargy to neurological impairment (Duringer *et al.*, 2020, Elgioushy *et al.*, 2020, Tegzes, 2025).

These methods aim to lower mycotoxin intake upon feed consumption, mitigating their harmful effects on livestock compared to untreated feed. However, despite their effectiveness in reducing specific mycotoxin levels, no single approach can entirely eliminate all toxins or provide uniform detoxification under varying contamination levels. The efficiency of these interventions is highly dependent on multiple factors, including mycotoxin structure, toxicity mechanisms, animal species, immune status, feed processing parameters, environmental influences, and cost feasibility. As advancements in feed safety technologies continue, comprehensive mycotoxin control from farm to consumer may become increasingly attainable.

11. Conclusion

Preventing mycotoxin production is the most effective strategy to reduce animal exposure risks. However, post-harvest interventions remain critical, including mycotoxin adsorbents or enzyme-based solutions targeting specific toxins in feed. For efficient monitoring, rapid commercial test kits enable screening of individual or multiple mycotoxins, while advanced laboratory diagnostics provide quantitative analysis of diverse mycotoxin profiles through streamlined workflows.

Integrating a holistic control framework-incorporating Good Agricultural Practices (GAP), Good Manufacturing Practices (GMP), Good Hygiene Practices (GHP), quality assurance protocols, and Hazard Analysis and Critical Control Points (HACCP)-ensures feed security and toxin mitigation across the entire food production chain. Additionally, species-specific monitoring protocols should be implemented, ensuring that high-risk animals, such as dairy cattle and equines, receive targeted screening for mycotoxin exposure. Emerging biotechnological solutions, including genetic resistance breeding and microbiome-based detoxification, offer promising avenues for long-term mitigation.

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Histological Characterization of the Brain and Eye in Drosophila Melanogaster: A Model for Neurodegenerative Research

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Brain histology
Retinal morphology
Hematoxylin-eosin staining

Abstract

Background: Drosophila melanogaster, a key model in genetic and neurological research, offers advantages due to its short life cycle (∼10 days), high fertility (400–500 offspring per cycle), and ethical feasibility for experiments. It has been widely used in studies of Alzheimer's, diabetes, and Parkinson's disease, yet limited histological analysis of its head structures has been conducted in Iran. This study aims to characterize its brain and ocular histology to enhance its application in neurodegenerative disease modeling.
Material & Methods: Drosophila melanogaster specimens were anesthetized at temperatures below 4°C, then sex-separated under a dissecting microscope. Heads were dissected from the thorax, ensuring optimal penetration of Carnoy's fixative. Samples underwent fixation, dehydration, paraffin embedding, and serial sectioning. Histological staining was performed, and imaging was conducted using Dino-Lite digital microscopy, processed via Dino Capture software (Version 2).
Results: Histological analysis showed that the eye's external surface consists of mosaic-shaped photoreceptor cells, beneath which lies the retinal layer. Below the retina, the lamina was identified. The brain contains two hemispheres, each with lamina, medulla, lobula plate, and lobula. Lobular architecture revealed dense neuronal nuclei surrounding each lobe, with interspersed fibers and centrally distributed neuroglial matrix. Neuronal fiber degeneration led to vacuolated spaces, mainly within lobular cores, indicating neurodegenerative changes.
Conclusion: Considering the distinct histological characteristics of Drosophila melanogaster especially the prominent staining properties of its head structures it appears to be a highly suitable laboratory model for studying neurodegenerative diseases and evaluating the impact of various factors on disease induction and recovery.

1. Introduction

Drosophila melanogaster, a species within the Drosophila genus and Drosophilidae family, is commonly known as the fruit fly or vinegar fly (Sang, 2001). Wild-type Drosophila exhibits yellow-brown coloration, brick-red eyes, and black transverse bands on the abdomen. This species demonstrates sexual di-

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morphism, where females reach approximately 2.5 mm in length, while males are slightly smaller with a darker dorsal surface. Males can be distinguished from females by coloration differences, as well as the presence of a distinct black patch on the abdominal surface and sex combs_a row of dark bristles on the forelegs (Steven *et al.*, 2016).

Drosophila melanogaster has a diploid chromosome number (2n = 8), making genetic investigations more straightforward. The species exhibits XY sex determination, with males being the heterogametic sex. This organism is widely used in laboratory research due to its short life cycle (∼10 days), high reproductive rate (400–500 offspring per cycle), and ethical feasibility for experimentation. Moreover, its double-stranded DNA structure enables robust molecular analyses (Adams *et al.*, 2000).

The pioneering geneticist Thomas Hunt Morgan introduced Drosophila melanogaster as an essential model for genetic research in 1933 (Sang, 2001). Since then, Drosophila has been extensively used to study Alzheimer's disease, diabetes, and Parkinson's disease, revealing mechanistic insights into neurodegeneration (Huang *et al.*, 2019). Histological sections of Drosophila brains have also facilitated the modeling of Parkinson's disease (Drobysheva *et al.*, 2008). Additionally, neurodegenerative effects such as neuronal deterioration in Alzheimer's disease have been studied using Drosophila (Sunderhaus and Kretzschmar, 2016).

Despite the wide application of Drosophila in neurological research, histological investigations of its head structures remain limited, particularly in Iran. This study aims to characterize the histology of the brain and eye in Drosophila, establishing its potential as a laboratory model for neurodegenerative disease research.

2. Materials and Methods

2.1. Sample Preparation

Prior to specimen collection, Drosophila melanogaster flies were anesthetized by exposure to temperatures below 4°C. Male and female flies were carefully separated under a dissecting microscope before regaining consciousness. The heads were dissected from the thorax, ensuring optimal penetration of the fixative solution through the connective passage.

2.2. Fixation & Tissue Processing

Samples were fixed using Carnoy's solution, prepared as follows:

60 mL absolute ethanol
30 mL chloroform
10 mL glacial acetic acid

Specimens were immersed in Carnoy's fixative for 4 hours to ensure proper tissue preservation. Subsequent dehydration was performed using two ethanol baths (100%), each for 30 minutes. Following dehydration, specimens were air-dried at room temperature, then immersed in methyl benzoate for 1 hour before being embedded in molten paraffin and processed into paraffin blocks.

3. Sectioning & Staining

Histological sections were prepared using a rotary microtome (DS4055), generating serial sections of 7 m thickness. Staining was performed following standard histotechnical procedures (Morovvati and Kalantari-Hesari, 2019).

3.1. Imaging

Images of stained sections were captured using a Dino-Lite digital microscope and processed using Dino Capture software (Version 2).

4. Results

Histological examination of Drosophila melanogaster head sections revealed distinct structural correlations between ocular and cerebral components.

4.1. Eye Morphology

The external surface of the eye comprises mosaic-shaped photoreceptor cells, each functioning as an independent visual unit. Beneath these mosaic cells, the retinal layer was identified, with bundles of nerve fibers connecting to individual photoreceptor units (Fig. 1(B)).

4.2. Brain Organization

Directly beneath the retina, the lamina layer was observed. Generally, the Drosophila brain consists of two hemispheres, each subdivided into:

- Lamina
- Medulla
- Lobula plate
- Lobula (Fig. 1(A), 1(C)).

5. Neurohistological Features

The lobular structures exhibited a dense aggregation of neuronal nuclei, primarily surrounding each lobule, while bundles of neural fibers and glial matrix were concentrated centrally. Degeneration of neuronal fibers

resulted in the appearance of vacuolated spaces, particularly within central lobular regions (Fig. 1(C), 1(D)).

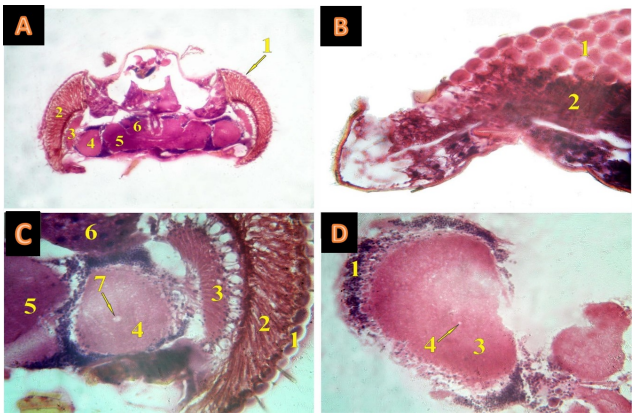

Fig. 1. Histological Sections of the Eye and Brain in Drosophila melanogaster. Hematoxylin & Eosin (H& E) staining, magnification 100X and 1000X. A) Mosaic-shaped photoreceptor cells (1), retina (2), lamina (3), medulla (4), lobula plate (5), lobula (6). B) Mosaic-shaped photoreceptor cells (1), retina (2). C) Mosaic-shaped photoreceptor cells (1), retina (2), lamina (3), medulla (4), lobula plate (5), lobula (6), vacuolated spaces due to neuronal degeneration (7). D) Aggregation of neuronal nuclei (1), neural fibers and neuroglial matrix (2), vacuolated spaces due to degenerative changes (3)

6. Discussion

The brain structure of Drosophila melanogaster consists of distinct layers, including mosaic-patterned photoreceptor cells, retina, lamina, medulla, lobula plate, and lobula. The histological examination conducted in this study using light microscopy confirms findings previously reported through electron microscopy, demonstrating the consistency of Drosophila's neuroanatomical organization (Huang *et al.*, 2019; Drobysheva *et al.*, 2008; Sunderhaus and Kretzschmar, 2016).

One of the most striking observations in this study was the clear structural separation between the retinal layers and underlying neuronal compartments . The mosaic-shaped photoreceptor cells function as individual visual processing units , each connecting to nerve fiber bundles. Degenerative changes within the lobular regions resulted in vacuolated spaces , a characteristic commonly associated with neurodegenerative diseases (Sunderhaus and Kretzschmar, 2016).

Given Drosophila melanogaster's short life cycle (∼10 days), high reproductive rate (400–500 offspring per cycle), and genetic accessibility, it is widely used as a model organism for studying neurodegeneration, aging, and genetic disorders. Previous studies have successfully applied Drosophila to model Alzheimer's disease, diabetes, and Parkinson's disease, utilizing its high tissue staining affinity and rapid phenotypic manifestation (Huang *et al.*, 2019; Drobysheva *et al.*, 2008). The present findings further validate its suitability for histopathological studies on disease progression, neuronal degeneration, and therapeutic interventions.

7. Future Directions

To expand upon these findings, future research should focus on:

1. Advanced neuronal mapping techniques, such as immunohistochemistry and confocal microscopy, to assess apoptotic and oxidative stress markers.
2. Comparative studies with mammalian models, investigating parallels between Drosophila's retinal degeneration and human neuropathologies.
3. Potential neuroprotective interventions, evaluating how drug candidates influence Drosophila's brain structure and neuronal integrity.

8. Conclusion

In conclusion, Drosophila melanogaster's distinct histological characteristics, genetic flexibility, and staining affinity establish it as an ideal experimental model for investigating neurological disorders. These findings strengthen its relevance in neurodegenerative disease modeling, facilitating mechanistic studies and therapeutic advancements.

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ORIGINAL RESEARCH PAPER

Evaluation of Biogenic Amine Contents of Iranian Kilka Fish Meal

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Abstract

The present study was conducted to evaluate the biogenic amine contents of Iranian Kilka fish meal (KFM). A total of 10 composed samples of KFM were provided during two months sampling period from rendering units of two industrial fish meal plants in Gilan province. The biogenic amine contents including tryptamine, phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine and spermine for each composed samples of KFM were measured by reversed phase high performance liquid chromatography equipped with C_{18} Luna column. The data were analyzed in a completely randomized design. Each sample was examined in 6 replication. The results of this study indicated that the biogenic amine contents including tryptamine, phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine and spermine had highly significant differences ($P<0.01$) among the KFM samples and their average values were 19.6, 18.3, 116.8, 145.9, 64.7, 76.4, 60.2 and 52.1 microgram per gram, respectively. The average values for biogenic amine contents of the KFM samples were highly significantly lower than those of their maximum limited allowances ($P<0.01$). According to the results of this experiment, it seems that KFM can be used in poultry diets without any concern.

1. Introduction

Although fish meal is the best animal protein source for poultry (Leeson and Summers, 2001, 2005) but one of the most important problems regarding to fish meal utilization in poultry diets is its contamination to biogenic amines (Keirs and Bennett, 1993; Friday *et al.*, 1999: Barnes *et al.*, 2001; Jaw *et al.*, 2012; Ruiz-Capillas *et al.*, 2015). Biogenic amines are naturally occurring low molecular weight organic bases characterized by the presence of an amine group (Tamim *et al.*, 2002; Tamim and Doerr, 2003; Fusi *et al.*, 2004). These substances are found at low levels in all organisms such as plants, animals and microorganisms (Tamim *et al.*, 2002). These compounds in small amounts are necessary for cellular growth, development and differentiation (Bardócz *et al.*, 1995; Sousadias and Smith, 1995; Mogridge *et al.*, 1996; Smith *et al.*, 1996; Phuntsok *et al.*, 1998). Also, biogenic amines contribute in protein, DNA and RNA synthesis (Mogridge *et al.*, 1996; Fusi *et al.*, 2004). In poultry, biogenic amines are present in all cells specially lung cells (Eaton and Fedde, 1978, 1980) and regulate the activity of poultry immune system (McCorkle and Taylor, 1993) and enhanced hypothalamic biogenic amine levels in broiler chicks showing advanced sexual maturation (Davison and Kuenzel, 1991).

Biogenic amines are produced by decarboxylation of amino acids (Friday *et al.*, 1999; Tamim *et al.*, 2002; Tamim and Doerr, 2003; Jaw *et al.*, 2012; Figueiredo

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et al., 2014; Wójcik *et al.*, 2024) or by amination and transamination of aldehydes and ketones (Friday *et al.*, 1999; Wójcik *et al.*, 2024). The most important biogenic amines include putrescine, cadaverine, spermine, spermidine, histamine, tryptamine, β-phenylethylamine, tyramine, dopamine, phenethylamine, ethanolamine, cysteamine, aminopropanol, β-alanine, gama-aminobutylate (GABA), serotonin and 5-adenosylmethioninamine (Sander *et al.*, 1996; Phuntsok *et al.*, 1998; Tamim *et al.*, 2002; Tamim and Doerr, 2003). Biogenic amines are found in silages such as corn silage and alfalfa silage (Phuntsok *et al.*, 1998; Fusi *et al.*, 2004), ruminal bacteria (Phuntsok *et al.*, 1998), protein sources (Phuntsok *et al.*, 1998), spoiled meat products (Tamim and Doerr, 2003), fermented poultry carcasses (Sander *et al.*, 1996), egg (Figueiredo, *et al.*, 2014) and animal protein sources such as fish meal, meat and bone meal and poultry by-product meal (Keirs *et al.*, 1994; Tamim *et al.*, 2002; Tamim and Doerr, 2003). Putrescine is derived from decarboxylation of arginine *via* ornithine and acts as a precursor of spermine and spermidine. Cadaverine is produced from lysine decarboxylation, spermine and spermidine by degradation of putrescine, histamine from decarboxylation of histidine, tryptamine *via* decarboxylation of tryptophan, β-phenylethylamine from decarboxylation of phenylalanine, tyramine by decarboxylation of tyrosine, dopamine from dopa degradation, phenethylamine from degradation of phenylethylamine, ethanolamine by decarboxylation of serine, cysteamine from decarboxylation of cysteine, aminopropanol by decarboxylation of threonine, β-alanine *via* decarboxylation of aspartic acid, gama-aminobutylate (GABA) from decarboxylation of glutamic acid, serotonin by decarboxylation of 5-hydroxytryptophan and 5-adenosylmethioninamine from degradation of 5-adenosylmethionine (Keirs and Bennett, 1993). Currently, biogenic amines are used as indicators of foods such as egg, meat and meat products freshness and hygienic quality (Tamim and Doerr, 2003; Fraqueza *et al.*, 2012; Hutarova *et al.*, 2013; Figueiredo, *et al.*, 2014; Wójcik *et al.*, 2024) and high concentrations of different biogenic amines have long been used as indicators of spoilage (Tamim *et al.*, 2002).

The use of animal by- product meals such as fish meal, poultry by- product meal, meat and bone meal, blood meal and feather meal in poultry diets allows the opportunity for the presence of biogenic amines. The toxicity of high levels of biogenic amines resulting from the presence of spoiled animal by- products has been suggested as a causative factor for reduction in poultry performance and a condition named "necrotic cellular debris" (Barnes *et al.*, 2001). The consumption of high amounts of biogenic amines in poultry resulted in reduced feed intake, reduction of growth and performance, reduced feed efficiency, the occurrence of proventricular lesion, gizzard erosion, gizzard and intestinal lesions, intestinal epithelial cells necrosis, increasing feed passage rate and thus transmission of undigested feed from gastrointestinal tract and presence of too much mucosa in upper parts of intestine (Harry *et al.*, 1975; Harry and Tucker, 1976; Keirs and Bennett, 1993; Keirs *et al.*, 1994; Friday *et al.*, 1999; Barnes *et al.*, 2001; Tamim *et al.*, 2002; Tamim and Doerr, 2003).

In Iran, Kilka fish (*Clupeonella engrauliformis*) is obtained from the Caspian sea in Golestan, Mazandaran and Gilan provinces. The most part of the catch is processed into fish meal by different processing methods. Now, there are 9 rendering plants in Iran that produce Kilka fish meal by processing whole Kilka fish, which is mostly used in poultry and cold water fish diets. Although some studies have been conducted on biogenic amine of fish meal in other countries (Harry *et al.*, 1975; Jaw *et al.*, 2012; Ruiz-Capillas *et al.*, 2015), but the biogenic amine contents of Kilka fish meal produced in Iran has not been described quantitatively. Due to lack of information, this study was carried out to determine the biogenic amine contents of Iranian Kilka fish meal.

2. Materials and Methods

The KFM samples were provided during two months sampling period from rendering units of two industrial fish meal plants in Gilan province. The samples collected in each six days were mixed together and 10 composed samples were prepared so that samples #1, #2, #3, #4 and #5 were taken from plant A and samples #6, #7, #8, #9 and #10 were taken from plant B. The raw material source of all KFM samples was whole Kilka fish. After grinding and mixing the samples, all samples were stored at -20°C until further analysis.

The biogenic amine contents of the KFM samples were determined by reversed phase high performance liquid chromatography equipped with C_{18} Luna column as described by Tamim *et al.* (2002). Briefly, perchloric acid was used to extract the biogenic amines from a 30 gram of ground-up sample. The extract was then made alkaline by saturated sodium bicarbonate solution. Amines in extract were then dansylated with dansyl chloride and extracted from the aqueous solution by diethyl ether. The dansylated biogenic amines were analyzed by reversed-phase high performance liquid chromatography using a Shimadzu 6-A system with a C_{18} Luna column. Water and methanol in a gradient elution program were used to elute the amines, which were detected by a fluorescence detector at an excitation of 365 nm and emission of 520 nm. Each sample was examined in six replicates. The data were analyzed in a completely randomized design using the GLM procedure of SAS (SAS, 1999). Comparison of means was

conducted by Duncan's multiple range test. Comparison of the average of biogenic amine contents of the KFM samples with their maximum limited allowances was conducted using two-sided t-test (Zar, 1996).

3. Results and Discussion

The biogenic amine contents of each KFM samples are shown in Table 1. The tryptamine content showed significant differences (P<0.01) among the KFM samples and its average value was 19.6 μg/g and varied between 10.7 to 29.7 μg/g. The phenylethylamine content had significant differences (P<0.01) among the KFM samples and its average value was 18.3 μg/g and varied between 9.5 to 25.6 μg/g. The putrescine content showed significant differences (P<0.01) among the KFM samples and its average value was 116.8 μg/g and varied between 76.4 to 168.7 μg/g. The cadaverine content had significant differences (P<0.01) among the KFM samples and its average value was 145.9 μg/g and varied between 86.5 to 188.4 μg/g. The histamine content showed significant differences (P<0.01) among the KFM samples and its average value was 64.7 μg/g and varied between 41.8 to 85.5 μg/g. The tyramine content had significant differences (P<0.01) among the KFM samples and its average value was 76.4 μg/g and varied between 68.2 to 91.2 μg/g. The spermidine content showed significant differences (P<0.01) among the KFM samples and its average value was 60.2 μg/g and varied between 49.2 to 71.6 μg/g. The spermine content had significant differences (P<0.01) among the KFM samples and its average value was 52.1 μg/g and varied between 41.2 to 61.3 μg/g. The highest levels of biogenic amines were observed for cadaverine and putrescine, respectively. The reason for these results is that most biogenic amines are derived from degradation of essential amino acids by bacterial activity (Tamim *et al.*, 2002; Tamim and Doerr, 2003; Jaw *et al.*, 2012; Wójcik *et al.*, 2024). Because the highest levels of essential amino acids in fish meal are for lysine and arginine (NRC, 1994), it is logical that the highest levels of biogenic amines are also achieved for cadaverine and putrescine. The average values for biogenic amine contents of the KFM samples were significantly (P<0.01) lower than those of their maximum limited allowances. Ruiz-Capillas *et al.* (2015) assessed the biogenic amine levels in one sample of Capelin fish meal and found that the cadaverine, putrescine and histamine levels in Capelin fish meal were 208, 164 and 46 μg/g, respectively. They also reported that the biogenic amines that presented the highest levels in Capelin fish meal were cadaverine followed by putrescine, and biogenic amine levels in the fish meal studied were low when compared to the toxic levels observed in these kinds of products which are in agreement with the results obtained in the present study. It has been reported that the biogenic amine contents are low during the first 24 to 36 hour of raw material storage prior to rendering and increase rapidly thereafter so if raw materials are processed in short time after collection, the risk of product contamination with biogenic amines is not too high (Tamim and Doerr, 2003). Because the raw material used for production of KFM samples in this experiment processed within maximum 8 hours after collection, their biogenic amine contents were significantly (P<0.01) lower than those of their maximum limited allowances. According to the results of this study, it seems that KFM can be used in poultry diets without any concern.

Table 1
The biogenic amine contents (μg/g) of Kilka fish meal

Sample No.	1	2	3	4	5	6	7	8	9	10	Average	Maximum Limited Allowance[1]
Tryptamine	11.3^{h}	21.6^{d}	24.5^{c}	29.7^{a}	15.8^{f}	22.1^{d}	18.5^{e}	27.2^{b}	10.7^{h}	14.6^{g}	19.6	46^{**}
Phenylethylamine	10.1^{ef}	19.7^{c}	24.2^{b}	25.6^{a}	13.4^{d}	23.9^{b}	20.5^{c}	25.3^{a}	9.5^{f}	10.8^{e}	18.3	28^{**}
Putrescine	87.6^{g}	144.9^{c}	168.7^{a}	153.2^{b}	76.4^{h}	121.8^{e}	117.4^{e}	129.2^{d}	92.3^{f}	76.5^{h}	116.8	285^{**}
Cadaverine	125.9^{e}	179.6^{b}	188.4^{a}	166.1^{cd}	119.3^{e}	167.6^{cd}	160.3^{d}	171.6^{c}	86.5^{g}	93.7^{f}	145.9	363^{**}
Histamine	50.9^{f}	78.7^{b}	85.5^{a}	73.4^{c}	55.8^{e}	79.7^{b}	66.1^{d}	71.4^{c}	43.7^{g}	41.8^{g}	64.7	144^{**}
Tyramine	68.2^{e}	88.5^{a}	91.2^{a}	77.9^{bc}	71.7^{de}	81.4^{b}	69.3^{e}	74.9^{cd}	69.5^{e}	71.4^{de}	76.4	118^{**}
Spermidine	54.3^{f}	71.6^{a}	67.2^{bc}	68.4^{b}	51.3^{g}	64.1^{d}	58.6^{e}	65.2^{cd}	52.1^{fg}	49.2^{g}	60.2	172^{**}
Spermine	48.4^{f}	61.3^{a}	55.9^{cd}	53.2^{e}	44.1^{g}	57.6^{bc}	54.8^{de}	58.9^{ab}	45.6^{g}	41.2^{h}	52.1	164^{**}

[1] The asterisk shows highly significant difference between the average value for KFM and maximum limited allowance value (P<0.01).
$^{a-h}$ In each row, means with different superscripts are highly significantly different (P<0.01).

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