



Research Article

A Research on Mould and Aflatoxin Contamination Levels of Feeds Used in Trout Farms of Adana (Turkey)**

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Abstract. This research was conducted to analyze the mould counts and aflatoxin contamination levels of different types and sizes of feed used in trout farms of Adana. Totaly 33 different feeds supplied from 15 trout farms of Adana (Turkey) were analysed for moulds and aflatoxins (AFB₁, AFB₂, AFG₁ and AFG₂) between July-December 2010. To identify mould counts; the samples were incubated in the aerobic conditions at 25 ± 1 °C for five days by using Dichloran Rose Bengal Chloramphenicol Agar (DRBC). Aflatoxins were determined by using liquid-solid extraction and recoveries were obtained 70.00% to 112.20%. The precision of measured values of mould and aflatoxin analysis methods was calculated with the results of repeatability and reproducibility. The bias, expressing the accuracy value, varies between 4.312 and 12.909. Although moulds were found in 12 of 33 analysed samples (36.30%); aflatoxins were determined 8 out of 33 analysed samples (24.25%). Positive sample's range were 3.08-4.86 log₁₀ kob g⁻¹ moulds and 0.21-0.90 µg AFB₁ kg⁻¹ (ppb) of trout feeds. As a result, this research showed that none of the trout feeds used in trout farms of Adana has aflatoxins above Turkey legal limits.

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Adana'da Alabalık Çiftliklerinde Kullanılan Yemlerin Küf ve Aflatoksin Kontaminasyon Düzeyleri Üzerine Bir Araştırma

Anahtar kelimeler:

Aflatoksin, yem, küf, alabalık

Özet. Bu araştırma, Adana'daki alabalık çiftliklerinde kullanılan farklı tür ve büyüklükteki yemlerin küf ve aflatoksin kontaminasyon düzeylerinin belirlenmesi amacıyla gerçekleştirilmiştir. Adana'daki on beş alabalık çiftliğinden tedarik edilen 33 farklı alabalık yeminin, Temmuz-Aralık 2010 döneminde küf ve aflatoksin (AFB₁, AFB₂, AFG₁ ve AFG₂) analizleri gerçekleştirilmiştir. Küf sayılarını belirlemek için; yem örnekleri Dichloran Rose Bengal Chloramphenicol Agar (DRBC) kullanılarak aerobik koşullarda 25 ± 1 °C'de beş gün inkübe edilmiştir. Aflatoksinler sıvı-katı ekstraksiyon tekniği kullanılarak tespit edilmiş ve geri kazanım oranları 70% ile 112.20% arasında bulunmuştur. Küf ve aflatoksin analiz metodlarının kesinlik değerleri tekrarlanabilirlik ve tekrar üretilebilirlik sonuçlarından hesaplanmıştır. Doğruluk değerini ifade eden bias, 4.312 ile 12.909 arasında değişmektedir. 33 yem numunesinin 12'sinde küf bulunurken (36.30%); 8'inde aflatoksin tespit edilmiştir (24.25%). Pozitif numuneler için aralıklar 3.08-4.86 log₁₀ kob g⁻¹ küf ve 0.21-0.90 µg AFB₁ kg⁻¹ (ppb)'dir. Sonuç olarak, bu araştırma Adana'da incelenen alabalık çiftliklerinde kullanılan alabalık yemlerinin hiçbirisinde Türkiye yasal limitlerinin üzerinde aflatoksin bulunmadığını göstermiştir.

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INTRODUCTION

Due to reduction of fish population in natural resources, aquaculture has been increasing in Turkey along with other countries in the world. Although fish farming in Turkey has extensive water resources it is still not at the expected level, but developments are promising. Adana, which is the center of the current research, is a modest example of increasing the number of trout farms in the provinces and districts in recent years.

In parallel with this increase; trout feed industry has been growing as well. Trout feed is generally; produced from corn, corn gluten meal, wheat, wheat bran, soybean oil, soybean expeller, fish meal and fish oil by formulating for different life stages and presented as compact pellets and in different sizes (Greco *et al.*, 2015). Since their chemical and nutritional properties are related to their activity, approximative compound of trout feed ingredients must be known (Anater *et al.*, 2016).

Depending on the type of fish grown and the location of the farm; the production of different types of feed and the use of new techniques have become a necessity. For the production of quality, clean and economical feed; providing appropriate conditions is very important. Because; quality feed means trout with high nutritional value and no microbiological or toxicological risks (Kop and Korkut, 2002).

Moulds are defined as nucleated microorganisms (eukaryotes) included in the class of Mycota or Fungi and they are usually referred to as fungi. They can easily grow anywhere as they are plenty in nature and are not prone to develop (Şahin and Korukluoğlu, 2000). They cause an unfavorable sour smell, deterioration, reduction in dry matter, nutrient and economic loss of the product that they grow on; more than that they can produce different types of mycotoxins (Maciorowski *et al.*, 2007; Faria *et al.*, 2017).

In the event of moist content being over 14.5-15.5% in raw material or end product in storage, fungi start to grow. Under certain temperatures and moistures, fungi produce mycotoxins. The fish that are fed with feed stuff that include such elements are observed to lose weight and also die in some cases (Kop and Korkut, 2002).

In the process of metabolic activities for their feeding, fungi are capable of forming an enormous number and variety of materials. The metabolic forms that fungi produce are classified as primary and secondary metabolics depending on formation amounts. Among primary metabolic formations are certain alcohols, organic acids, enzymes and others while secondary metabolic formations include mycotoxins, the most dangerous of which are *Aspergillus* and *Penicillium* (Sahin and Korukluoglu, 2000).

Aflatoxins are the most crucial group and important secondary toxic metabolites produced by species of *Aspergillus* and *Penicillium*. These fungal toxins are hepatotoxic and cause mutagenic and teratogenic diseases in such as liver toxicity, liver cancers and protein lackness in humans and animals (Mohammadi *et al.*, 2018). Aflatoxins grow due to various reasons such as feed or food stuff not being harvested in time, inefficient storage conditions, high levels of moisture in products and low hygiene during processing of feed or food stuff. An average of 25% of products harvested worldwide is contaminated by mycotoxins to varying degrees (Aydın *et al.*, 2003).

Fish are highly sensitive to feedstuff-based toxins. Trouts are particularly affected by aflatoxins caused by feed stuff with fungi. Unsaturated fats that are easily oxidized cause the feedstuff to go bad, which cause Salmonid type of fish to have steatorrhoea hepatitis (fatty liver) and this finally causes anemia (Atesoglu, 1999).

Commonly seen among rainbow trout; aflatoxin intoxication leads to tumor growth in liver. Even if fish consume feeds containing 10-20 ppm levels of aflatoxins; parenchymal cells grow and become irregular (Ekici, 2017). Aflatoxins occur as a result of moulding in feed sacks. Tumor growth in livers of fish that are fed this feedstuff could be lethal. In order to prevent moulding in feed sacks, storages are supposed to be dry, aired, dark and cool. Maximum of 4 or 5 packages of feed sacks should be piled on to each other, each row of packages should not contact and they should be placed on shelves 5-10 cm high above the ground (Aydın, 2007).

This research is designed in order to detect presence of mould and determine aflatoxin amounts of trout feeds in different types and sizes in trout farms of Adana.

MATERIAL AND METHOD

Samples

Between July 2010-December 2010, 33 feed samples (each about 500 g) were supplied from 15 trout farms in Adana (Turkey). Samples were carried to the microbiology and mycotoxin laboratory in a thermos. All samples were milled in the stainless-steel blender (Waring, Connecticut, USA) to provide standard particle size and kept in glass jars and deep freeze till test it. All samples were tested until expiration date of trout feeds (Golge *et al.*, 2013).

Chemicals and Reagents

While Dichloran Rose Bengal Chloramphenicol Agar, sodium chloride, nitric acid and potassium bromide were supplied by Merck (Darmstadt, Germany); acetonitrile and methyl alcohol (HPLC grade) were procured by Sigma-Aldrich (St. Louis, MO, USA). The AflaPrep immunoaffinity columns were produced by R-Biopharm Rhone (Glasgow, Scotland) and supplied from Sincer (Izmir, Turkey). 18.2 MΩ•cm ultrapure water was produced with Millipore Synergy water purification system (Merck, Molsheim, France). AFs standards were produced by R-Biopharm Rhone (Glasgow, Scotland) and supplied from Sincer (Izmir, Turkey), (Aflastandart Solution, catalog no. P22). AFs mix contained 250 ng AFB₁, AFB₂, AFG₁ and AFG₂, respectively in 1 ml of methyl alcohol.

Enumeration of Mould and Calculation

Mould analysis is carried out by using Dichloran Rose Bengal Chloramphenicol Agar (DRBC) and incubating petri boxes under aerobic conditions for five days under 25±1 °C (Anon, 2001). Petri colonies between 10-150 have been enumerated and results have been calculated. Numbers are presented as kob g⁻¹. For calculation the formula below has been used. The calculated results have been converted into log₁₀ kob g⁻¹.

$$N = \Sigma C / [(1 \times n_1) + (0.1 \times n_2) \times d]$$

N = Colony number g or ml

ΣC = Total of colonies in petris

n₁ = Number of petri number in the first dilution

n₂ = Number of petri number in the second dilution

d = Dilution factor of the first numbered petri

Preparation of Standard Solutions

AFs solution was adulterated with methyl alcohol to make concentrations of 10 ng ml⁻¹ for AFs. Different standard levels (0.1, 0.2, 0.4, 1.2, 2, 4, 5 ng ml⁻¹) were obtained by using this mother solution. All aflatoxin standards were refreshed once a month and used to create calibration table of AFs analysis and recovery studies.

Analysis of Feed Samples with Immunoaffinity Column

Aflatest HPLC procedure was applied to extract and clean-up with IAC the AFs from trout feed samples (Anon., 1999). 50 gram of feed was stirred with 5 g NaCl and 100 ml solvent (80% methyl alcohol: 20% water) in a blender and milled and homogenized for 2 min. After homogenization; the extract was filtered with prefolded paper. 10 ml filtrate was mixed with 40 mL ultrapure water. This mixture was filtered again by using glass microfiber filter. 10 ml filtrate was undergone through the immunoaffinity column at a speed of 2–3 ml/min. Then, IAC was washed with 10 ml ultrapure water. Finally, bounded aflatoxins eluted slowly with 1 ml methyl alcohol by pushing air through the IAC to obtain the last drops of eluate in 1.5 ml vial and diluted with 1 ml ultrapure water.

HPLC Equipment and Chromatographic Conditions

Compartments of Agilent 1100 HPLC were; isocratic pump, degasser, autosampler, column oven and fluorescence detector (Agilent, California, USA). HPLC mobile phase contained water–acetonitrile–methyl alcohol (6:2:3, v/v/v) including 0.12 g l⁻¹ potassium bromide and 350 μl l⁻¹ nitric acid (4 M) and the flow speed was 1 ml min⁻¹. ODS 2 HPLC column (250 x 4.6 mm) was used for chromatographic separations. The column temperature was 25 °C, injection volume was 100 μl. Improved chromatography provides the fluorescent responses of AFB₁ and AFG₁ with post column derivatization. Potassium bromide and Kobracell® (R-Biopharm AG, Darmstadt, Germany) were used for derivatization. The fluorescence detector was set to wavelengths of 360 nm for excitation and 430 nm for emission. Total analysis period was about 14 min. and the retention times of AFB₁, AFB₂, AFG₁ and AFG₂ were 10.2, 9.2, 8.2 and 6.5 min., respectively.

Method Verification Procedures

In spite of precision parameter was applied to evaluate the method performance of mould analysis; linearity, precision, accuracy, limits of detection (LOD) and limits of quantification (LOQ) were applied to ensure the method verification of AFs. Linearity was assigned by injecting triplicate AFs standard solutions (0.1, 0.2, 0.4, 1.2, 2, 4, 5 ng ml⁻¹). The linearity was interpreted by linear regression analysis using the least squares method.

According to IUPAC Orange Book Section 18.4.3.17; to calculate the LOD and LOQ of the Aflatoxins, blank trout feed samples were spiked 0.1 ng ml⁻¹ for AFB₁, AFG₁, AFB₂ and AFG₂, respectively. Spiking was applied in ten replicates. Finally; these formulas were used to calculate LOD and LOQ: LOD= 3 x standard deviation, LOQ= 10 x standard deviation (Curie, 1995).

For recovery practice; blank trout feed samples were spiked at 1 ng ml^{-1} for all AFs. Spiking was applied in total twenty replicates by two person. Blank and spiked samples were extracted, cleaned-up and injected in vials to the HPLC by the same analyst.

The accuracy was calculated with trueness (bias). To determine trueness; recovery datas were used. This formula was used to calculate trueness: $\text{Bias} = [(X_i - X_t) / X_t] \times 100$

X_i : Expected amount, X_t : Determinated amount.

The precision of international standard mould analysis method was calculated from intra-day repeatability with limit of intra-day repeatability ($r=2.8 \times \text{RSD}$) and inter-day reproducibility with limit of inter-day reproducibility ($R=2.8 \times \text{RSD}$) by ten-replicated analysis for each person (total three person) of the samples, respectively. For the precision of AFs; total twenty samples spiked with AFs at 1 ng ml^{-1} were analysed on two different days by two persons. The precision was evaluated in terms of intra-day repeatability with limit of intra-day repeatability ($r=2.8 \times \text{RSD}$) and inter-day reproducibility with limit of inter-day reproducibility ($R=2.8 \times \text{RSD}$).

RESULTS AND DISCUSSION

Method Verification Results

In mould analysis, reproducibility and repeatability relative standard deviations obtained with verification studies were found to be suitable according to "validation parameters acceptance criteria" stated in AOAC sources.

The linearity range for AFs, the correlation coefficient (r^2), linear regression equation, LOD and LOQ for each aflatoxin are presented in Table 1. The calibration curves were linear with correlation coefficients (r^2) of 0.9999, 0.9999, 0.9992 and 0.9995 for AFB₁, AFB₂, AFG₁ and AFG₂, respectively. In addition, RSD of calibration curves were 0.1110, 0.0801, 0.2436 and 0.1977 for AFB₁, AFB₂, AFG₁ and AFG₂, respectively. The LODs and LOQs values were 0.104-0.140 $\mu\text{g kg}^{-1}$. These values were similar to those calculated previously results for AFs (Fu et al., 2008; Kabak, 2012; Golge et al., 2013).

Table 1. Linearity, correlation coefficient, linear regression equation of calibration curve, LOD and LOQ values for AFs.

Çizelge 1. Aflatoksinlerin kalibrasyon eğrisinin linearite, korelasyon katsayısı, LOD ve LOQ değerleri.

Toxin name	Linearity range ($\mu\text{g l}^{-1}$)	r^2	LOD ^a ($\mu\text{g kg}^{-1}$)	LOQ ^b ($\mu\text{g kg}^{-1}$)
AFB ₁	0.1 – 5	0.99994	0.104	0.111
AFB ₂	0.1 – 5	0.99999	0.104	0.110
AFG ₁	0.1 – 5	0.99920	0.105	0.115
AFG ₂	0.1 – 5	0.99951	0.113	0.140

r^2 : correlation coefficient

^a Limit of detection

^b Limit of quantification

The recovery results and precision (intra-day repeatability as limit of intra-day repeatability (r) and inter-day reproducibility with limit of inter-day reproducibility (R) of the AFs analytical method and precision (intra-day repeatability as limit of intra-day repeatability (r) and inter-day reproducibility with limit of inter-day reproducibility (R) of the mould analysis method are showed in Table 2.

Table 2. The recoveries, precision (repeatability, reproducibility), trueness (bias) of AFs.

Çizelge 2. Aflatoksinlerin geri kazanım oranları, kesinlik (tekrarlanabilirlik, tekrarüretilebilirlik), doğruluk değerleri.

Toxin name	Amount ($\mu\text{g kg}^{-1}$) ($n=20$)	Recovery (%)	Repeatability ^a RSD (%) for first person	Repeatability ^a RSD (%) for second person	Reproducibility ^b RSD (%)	Bias ^c RSD (%)
AFB ₁	1	93.17	0.193	1.061	0.585	7.296
AFB ₂	1	105.79	0.129	0.087	0.129	5.482
AFG ₁	1	88.13	0.454	0.409	0.414	13.507
AFG ₂	1	90.89	0.395	0.291	0.356	10.011

^a Repeatability was calculated by analysis of ten replicate samples at one concentration level of each toxin on the same day by two persons.

^b Reproducibility was calculated by analysis of ten replicate samples at one concentration level of each toxin on the different two days by two persons.

^c Bias was calculated by analysis of ten replicate samples at one concentration level of each toxin on the on the different two days by two persons.

Table 3. The precision (repeatability, reproducibility) of mould analysis method.

Çizelge 3. Küf analiz metodunun kesinlik (tekrarlanabilirlik, tekrarüretilebilirlik) değerleri.

Intra-day repeatability ^a (S _r) for first person	Intra-day repeatability ^a (S _r) for second person	Intra-day repeatability ^a (S _r) for third person	Inter-day reproducibility ^b (S _R)
0.039	0.036	0.031	0.048

a Repeatability was calculated by analysis of ten replicate samples at two concentration levels and two parallels on the same day by three persons.

b Reproducibility was calculated by analysis of ten replicate samples at two concentration levels and two parallels on the different two days by three persons.

Analysis of Trout Feed Samples

Mould analysis results of trout feeds are summarized on Table 4. Out of 33 feed samples, 21 samples (63.6%) were found not to contain mould on the other hand in 12 samples (36.3%) had mould. Results of the feed samples that contained mould varied between <10 to and 4.86 log₁₀ kob g⁻¹ were very similar to other values determined by Greco *et al.* (2015). Extrude, pres-pelet growing and stem feeds did not include mould. Highest concentration of mould production was seen in growth feeds with 3 mm diameter. The obtained results indicate that feed type and diameter are considerable in mould growth.

Table 4. Mould analysis results of feed samples.

Çizelge 4. Yem örneklerinin küf analizi sonuçları.

Feed No	Feed Type	Feed Diameter (mm)	Period of purchase	Mould Amount (log ₁₀ kob g ⁻¹)
1	Growth	4	July 2010	3.94
2	Growth	3	July 2010	4.00
3	Fry	2	July 2010	3.79
4	Growth	2	August 2010	4.20
5	Growth	3	August 2010	3.90
6	Growth	3	August 2010	4.86
9	Growth	8	August 2010	4.00
10	Fry	2	August 2010	3.49
14	Growth	3	September 2010	3.08
18	Growth	3	October 2010	3.30
25	Growth	2	November 2010	4.15
27	Growth	4	November 2010	3.78

When aflatoxin B₁ levels are considered, as it is the most toxic group among aflatoxins; 25 samples were reported to have aflatoxin B₁ under limit of detection (LOD). 8 feed samples (24.25%) on the other hand, have various levels of aflatoxin B₁ between 0.21 ppb and 0.90 ppb. Although these results confirm the previous studies by Kaymak 2000; Yaroglu and Gul, 2007; Merako, 2010); AFB₁ levels in our study were smaller than other studies (Altug and Beklevik, 2003; Goncalves *et al.*, 2018).

Kaymak (2000) studied aflatoxin analysis of 59 feed samples from different feed factories in Turkey. According to the results of his analysis, 47.45% of the feed samples did not contain aflatoxin while 52, 50% had various levels of aflatoxins. The values he found are not above Turkey's limits like our study. At the same time, he concluded that technologies used in feed production (Extrusion, Pelletization) are not a defining factor whether to increase or decrease aflatoxin contents.

Altug and Beklevik (2003), in their research, detected total aflatoxins in 20 feed samples between 21.2 - 42.4 µg kg⁻¹, in 22 samples between 5.0-20.0 µg kg⁻¹ out of 85 trout feeds samples and concluded that in the rest of the 43 samples, no aflatoxins were traced. They discussed that fish feeds should be checked at fish farms periodically, product moisture and storing techniques should be followed, and similar studies should be carried out in larger scale under control. All these points are reported to prevent possible losses.

Yaroglu and Gul (2007), reached the conclusion in their study that fish feed in the province of Erzurum are sold out without waiting long and this prevents them from being exposed to high levels of moulding infestation and as a result, aflatoxin contamination levels observed to be lower.

Merako (2010), collected 27 samples from three different trout feeds use for three different periods (starter, fingerling, growing) in June, July and August 2010 to determine aflatoxin and heavy metal levels. According to aflatoxin analysis results: aflatoxin was found below the detection limit in all of 27 trout feeds.

Greco *et al.* (2015), analysed 28 trout feeds to determine fungal contamination and the occurrence of mycotoxins in Mexico. While fungal count range was <10 to 4.56 log₁₀ kob g⁻¹, the frequency of *Aspergillus* and

Penicillium were found as 3.6% and 21.4% respectively. On the other hand, Aflatoxin B₁ was determined in 50% of feeds as mean 2.82 ppb.

Goncalves *et al.* (2018), analysed 41 feed samples within the scope of BIOMIN mycotoxin survey programme during 2014. Aflatoxins, zearalenone, deoxynivalenol, fumonisin and ochratoxin A analysis in the all samples. Totally; 76% of the samples had more than one mycotoxin, 59% of the samples were contaminated by AFs.

As seen in Table 5; AFB₁ levels in feed samples increased significantly in the period of September to December. Kaymak (2000), searched aflatoxin amounts considering periods and he stated that the highest values were observed in November.

Table 5. Aflatoxin B₁ contents of feed samples based on periods ($\mu\text{g kg}^{-1}$).

Çizelge 5. Farklı zamanlarda yem örneklerinin Aflatoksin B₁ düzeyleri ($\mu\text{g kg}^{-1}$).

Sample No	July	August	September	October	November	December
1	ND	0.21	ND	ND	ND	ND
2	ND	ND	0.30	ND	ND	ND
3	ND	ND	0.63	ND	ND	ND
4	ND	ND	0.32	ND	ND	ND
5	ND	ND	0.21	ND	ND	ND
6	ND	ND	ND	ND	ND	0.90
7	ND	ND	ND	ND	ND	0.70
8	ND	ND	ND	ND	ND	0.80

ND: Not detected

Furthermore, in three feed samples that did not contain aflatoxin, moulding was observed due to unsuitable storage conditions (high levels of moisture and temperature and others). With the aim of determining the type of mould, research was carried out and microscopic analysis of moulds presented that they are Penicillium type.

CONCLUSION

According to Turkey's legislation about aflatoxin level in feeds; maximum acceptable level is $10 \mu\text{g kg}^{-1}$ (Anon., 2014). None of trout feeds that were analyzed in studies were above aflatoxin limits. Major reasons for this could be attributed to the fact that majority of raw materials that trout feeds factories use are imported from abroad and these exported materials are strictly investigated before entering the country and also fish flour, an indispensable raw material of fish feed rations, is also exported to a great extent and due to the technologies that are used in production, toxin growth is considerably low.

In conclusion, it should be taken into consideration that under right conditions in feed storages, mould infestation growth will be a risk; therefore precautions against mould growth should be planned and taken in stages from transport to consumption in business. With this purpose, contamination must be prevented during transport to business, to keep feed packages closed and under right temperatures and moisture conditions to consider the production and expire date on the packages and not to store in great amounts. Pellet feeds should be preferred more as they are harder and are possible to store for an extended period of time. Also, it is vital to ask for analysis reports from dealers before purchasing feeds and to consume feeds in three months.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

DECLARATION OF AUTHOR CONTRIBUTION

Ozgur Golge and Aysegul Aktüre discussed the research concept and designed the experiment. Rozelin Aydın and Aysegul Aktüre carried out the experiment. Ozgur Golge wrote the manuscript with the support of other researchers.

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