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RESEARCH ARTICLE

Investigation of Ochratoxin A Level in Feed Used in Bovine Feeding in Ardahan Province

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ABSTRACT

This study's objective is to determine the quantity of Ochratoxin A (OTA) in the feed given to cattle in the Ardahan region. The research was carried out on 30 randomly selected bovine farms in the Ardahan region. Cattle farms were visited in February, March, and April 2023. Thirty samples (10 meadow grass, 10 hay and 10 crushed barley) were collected each month, one sample from each farm visited. In all, 90 feed samples were examined for OTA within the three-month period. The amount of OTA in feed samples was determined by using an ELISA kit. The study did not detect OTA in the meadow grass, hay and crushed barley collected in February. In hay samples, 0.19 μg/kg was detected in March, 0.21 μg/kg in April, and in crushed barley 0.20 μg/kg in March and 0.21 μg/kg OTA in April. In this study, the highest amount of OTA was determined in meadow grass (0.28 μg/kg) collected in April. The difference between the average OTA amounts detected in meadow grass in March and April was statistically significant ($p<0.05$). It was seen that the amount of OTA detected in the meadow grass, hay and crushed barley was below the maximum limit (5.0 μg/kg) set by the Turkish Food Codex Contaminants Regulation. In this study, it was concluded that meadow grass, hay, and crushed barley given to cattle in February, March, and April in Ardahan region contain ochratoxin A at a level that does not pose a risk to animal and human health.

Keywords: Ardahan, Cattle, Feed, Mycotoxin, Ochratoxin A.

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Ardahan Yöresinde Sığır Beslenmesinde Kullanılan Yemlerde Okratoksin A Düzeyinin Araştırılması

ÖZ

Bu araştırmanın amacı Ardahan yöresinde sığırlara verilen yemlerde Okratoksin A (OTA) miktarını belirlemektir. Araştırma Ardahan yöresinden rastgele seçilen 30 sığır işletmesinde yürütüldü. Sığır işletmeleri 2023 yılı Şubat, Mart ve Nisan aylarında ziyaret edildi. Her ziyaret edilen işletmeden bir numune olmak üzere her ay 30 numune (10 adet çayır otu, 10 adet saman ve 10 adet arpa kırması) toplandı. Üç aylık dönemde toplam 90 yem örnekleri OTA yönünden incelendi. Yem örneklerinde OTA miktarının belirlenmesinde ELİSA kiti kullanıldı. Araştırmada Şubat ayında toplanan çayır otu, saman ve arpa kırmasında OTA tespit edilmedi. Saman örneklerinde Mart ayında 0,19 μg/kg, Nisan ayında 0,21 μg/kg, arpa kırmasında ise Mart ayında 0,20 μg/kg, Nisan ayında 0,21 μg/kg OTA tespit edildi. Bu araştırmada en yüksek OTA miktarı Nisan ayında toplanan çayır otunda (0,28 μg/kg) belirlendi. Mart ve Nisan ayında çayır otunda tespit edilen ortalama OTA miktarları arasındaki fark istatistiksel olarak anlamlı ($p<0,05$) bulundu. Çayır otu, saman ve arpa kırmasında tespit edilen OTA miktarı Türk Gıda Kodeksi Bulaşanlar Yönetmeliğinin belirlediği maksimum limitin (5,0 μg/kg) altında olduğu görüldü. Bu araştırmada Ardahan yöresinde Şubat, Mart ve Nisan ayında sığırlara verilen çayır otu, saman ve arpa kırmasının hayvan ve insan sağlığı için risk oluşturmayacak düzeyde okratoksin A içerdiği sonucuna varıldı.

Anahtar kelimeler: Ardahan, Mikotoksin, Okratoksin A, Sığır, Yem.

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INTRODUCTION

Various toxins are synthesized by some species of fungi in foods and feedstuffs. These toxins are called mycotoxins (Tao et al. 2018). Ochratoxins, a type of mycotoxin, are secondary metabolites synthesized by some fungi of the genus Penicillium and Aspergillus (Battacone et al. 2010). *Penicillium verrucosum, Penicillium nordicum and Aspergillus ochraceus*are the most important species synthesizing ochratoxin.According to their chemical structure, ochratoxins are divided into three groups: ochratoxin A, ochratoxin B and ochratoxin C. The most toxic of these is ochratoxin A (OTA) (Wang et al. 2022). OTA is a white-colored, odorless, heat-resistant, water-soluble, crystallized solid molecule. Its chemical structure is formed by dihydroisocoumarin, which binds to beta-phenylalanine via 7-carboxyl (Tao et al. 2018).

When food containing OTA is consumed by humans and animals, severe health problems occur. The liver and kidneys are especially sensitive to this toxin. Furthermore, mutagenic, immunotoxic, teratogenic, genotoxic, embryotoxic, and carcinogenic effects have been reported (Turkoglu and Keyvan 2019; El-Sayed et al. 2022; Miguel Alfonso et al. 2022; Wang et al. 2022). OTA causes chronic kidney damage (Balkan Endemic Nephropathy) in humans. It is also reported to induce liver and kidney tumors. For this reason, it is included in the 2B carcinogen group by the International Agency for Research on Cancer (IARC) and the World Health Organization (WHO) (Bernardini et al. 2014; Stoev 2021; El-Sayed et al. 2022).

The susceptibility of animal species to OTA is associated with the structure of the digestive tract. In single-stomach animals, nearly all the toxin is absorbed, whereas in ruminants, microbial decomposition occurs in the rumen. It undergoes peptide hydrolysis microbial enzymes. Following this hydrolysis, phenylalaninand dihydroisocoumarin are released. The degradation products are also known as ochratoxine α. Ochratoxin α is less poisonous than OTA (Boudra et al. 2007; Marin et al. 2013; Miguel Alfonso et al. 2022). The toxins absorbed through the gastrointestinal tract bind strongly to serum proteins (particularly albumin). This relationship determines its half-life in the bloodstream and varies depending on the animal species (Battacone et al. 2010). OTA and ochratoxin α are mostly excreted in urine and feces (Boudra et al. 2007).

The types of fungi in the contaminated agricultural products vary depending on the geographic area. *Penicillium verrucosum* generally reproduces in temperate areas, while *Aspergillus ochraceus* synthesizes ochratoxin by reproducing in warm climates (Battacone et al. 2010). The toxin inhibits protein synthesis in cells. It also leads to mitochondrial lesions and DNA damage, lipid peroxidation and oxidative stress. As a result of the toxic effect of the toxin on DNA, mutagenic, genotoxic, and carcinogenic effects occur (Marin et al. 2013; Miguel Alfonso et al. 2022). OTA was detected in 76% of tumorous kidneys that underwent nephrectomy in humans (Malir et al. 2021).

OTA can be found in numerous agricultural products. Cereals, wine, beer, coffee, and grapes are some of them. OTA is synthesized when suitable conditions (such as humidity, temperature, and oxygen) are created in contaminated nutrients. It passes into the meat and milk of animals fed with contaminated feedstuffs. The OTA content in animal products presents a risk to human health (Battacone et al. 2010; Miguel Alfonso et al. 2022; Wang et al. 2022). Moreover, they are very heat resistant and do not decompose with normal milk processes (such as pasteurization and UHT). It is necessary to apply at least 250ºC of heat for a few minutes to decompose the toxin (Tao et al. 2018). Therefore, most countries have a maximum limit for OTA in nutrients. This limit is 5 ppb for non-processed cereals, 3 ppb for cereals, and 2 ppb for beer and grape juice by the European Commission. It is applied by the Food and Agriculture Organization as 5 μg/kg for barley, wheat, and rye (Wang et al. 2022). According to the Turkish Food Codex Contaminants Regulation, the maximum level of OTA in untreated cereals is 5 μg/kg (Anonymous 2011).

The main source of livelihood in Ardahan province is cattle breeding. Local livestock complete the year by feeding approximately half in grasslands and pastures and the other half in shelters. The period in the shelter environment coincides with the winter and spring seasons. During these periods, cattle are fed stocked meadow grass. In addition, hay and crushed barley obtained by harvesting cereals (especially barley) are among the important forage items. It is very important to human health to feed cattle with safe food. Because animal products (meat and milk) produced from cattle are a major source of nutrients for humans (Doğan and Doğan 2020). The contamination of animal products with toxins gives rise to serious human health problems. Therefore, no substances harmful to human health should be present in animal products. Animal products containing OTA may be a source of toxins for people (Wang et al. 2022). These toxins go into breastfeeding mothers' milk and negatively affect the baby's health. They also build up in tissues and organs, like muscles and the liver, which are important sources of nutrients for humans (Battacone et al. 2010).

Penicillium verrucosum, a fungus that synthesizes OTA, can grow in agricultural products grown in temperate climates (Clark and Snedeker 2006). The Ardahan region has a cool and moderate climate. In addition, the food harvested during summer (August) is stored and kept until winter. This waiting period strengthens the possibility of OTA synthesis in stock feed materials. Literature reviews have found no research on the presence of OTA in livestock feeds in the Ardahan region. The objective of this investigation was to assess the amount of OTA contained in

certain feed materials given to cattle that are fed indoors during winter and spring in the Ardahan region.

MATERIALS and METHODS

The study began with the approval of the Ministry of Agriculture and Forestry (letter dated 07.11.2022 and numbered E-29486769-325.99-7632633). The research material consisted of certain feeds that were fed to cattle in the province of Ardahan. 30 cattle enterprises that are actively operating in the region were identified for this purpose. The determination of enterprises did not take into account the size of the enterprise and the criteria for animals (number, breed, age, and gender). These farms were visited in mid-February, March, and April 2023 (day 15). In each month visited, 30 samples were collected, one feed sample from each farm. During the three-month study period, 90 (30 meadow grass, 30 hay, and 30 crushed barley) feed samples were collected. Approximately 50 grams of the feed materials served to the cattle were taken and the samples were placed in sterile and sealed bags. After being brought to the laboratory in cold conditions, the samples were stored at -18 °C until analysis.

ELISA kit (Cat. No: CSB-EFD027449, CusaBio, U.S.A) was used to determine the OTA level in feed samples. Sample preparation and analysis were conducted according to the method described by the manufacturer. In this method, 5 grams of crushed samples were placed in a beaker. 25 ml of 40% ethanol solution was added to it. It was then stirred in the vortex for two minutes. 5 mL of the resulting mixture was centrifuged at 4000 rpm for 5 minutes. After centrifugation, 500 µL of the supernatant was taken and was thoroughly mixed with 500 µL of the sample dilution solvent. Then, 50 µL of the resulting mixture was taken and used for analyzes. The standards and well counts for the samples were determined prior to starting the analysis. 50 ml of standard was added to the standard wells and 50 µL of sample was added to the sample wells. Then, 50 µL of HRP-conjugateand 50 µL of antibody were added to each well. The plate was coated with a new adhesive strip and mixed thoroughly. After that, it was left to incubate at 25°C for 30 minutes. The plate was washed four times with the washing solution after incubation. The plate was placed upside down after the last wash, the solution inside was diverted, and drained with a clean paper towel. Then 100 µL of TMB substrate was added to each well and mixed well. The plate was left to incubate at 25°C for 15 minutes, protected from light. After incubation, 50 µL of stop solution was added to each well and the optical density of the sample and standards were measured at 450 nm in an ELISA reader (BioTek ELx800, USA). The amount of OTA was calculated by comparing the optical density of the samples with the optical density of the standards (Yılmaz and Aksu Elmalı 2016).

Statistical evaluation of the data obtained in this study was done using IBM SPSS 20.0 software. The data was checked for normality using the Shapiro-Wilk test. The comparison of the average OTA amount by month was conducted using an independent sample T-test. The results were presented in the form of an average ($\bar{X}$) and a standard deviation (SD). $P<0.05$ was found to be statistically meaningful for this study.

RESULTS

The amount of OTA in feed collected in the Ardahan region in February, March, and April is presented below in Table 1.

As seen in Table 1 above, OTA was not detected in the meadow grass, hay and crushed barley collected in February. OTA was 0.20 µg/kg in meadow grass samples collected in March and 0.28 µg/kg in April. The difference in mean OTA levels detected in meadow grass in March and April was statistically significant ($p<0.05$). The quantity of OTA in the hay samples was 0.19 µg/kg in March and 0.21 µg/kg in April. For barley crushing, it stood at 0.20 µg/kg in March and 0.21 µg/kg in April. In hay and crushed barley collected in April, the amount of OTA increased compared to March. However, the increase was considered statistically non-significant ($p>0.05$).

Table 1. Amount of OTA in Feed by Months (µg/kg).

Samples	February		March		April		P-value
	n	$\bar{X} \pm$ SD	n	$\bar{X} \pm$ SD	n	$\bar{X} \pm$ SD	
Meadow grass	10	0 ± 0 *	10	0.20 ± 0.03 [a]	10	0.28 ± 0.06 [b]	0.003
Hay	10	0 ± 0 *	10	0.19 ± 0.03 [a]	10	0.21 ± 0.03 [a]	0.29
Barley Crushed	10	0 ± 0 *	10	0.20 ± 0.04 [a]	10	0.21 ± 0.04 [a]	0.63

*: It was not detected in the range of test susceptibility limits.

a, b: Those who have different letters on the same line were statistically significant in the $p<0.05$ range.

DISCUSSION

The effects of OTA have been investigated on a number of animal species. It is reported that when applied to female mice at a dose of 1 mg/kg for one week, it disrupts egg development and causes decreases in the number of offspring (Jia et al. 2020). Moreover, it is pointed out that tumors of the kidneys, liver and mammary glands are the causal agents of female mice (Tao et al. 2018) and kidney tumors in male mice (Clark and Snedeker 2006). In a study, degenerative changes in neuron and glial cells were found in mice fed with feeds containing 10 ppm OTA, adenocarcinoma in the intestines at the 19th month and in the lungs at the 21st month (Stoev 2021). In another study, pigs fed with feed containing OTA showed a decrease in sperm motility and a significant reduction in height on the 40th day following ingestion of toxins (Solti et al. 1999).

Cattle are subject to OTA with raw materials. Susceptibility to the toxin is related to ruminal function. One study indicates that the uptake and adverse effects of OTA increase in cattle fed high-sugar feeds (Pantaya et al. 2016). Healthy livestock with improved rumen function can decompose up to 12 mg/kg of the toxin. Exposure of calves whose ruminal function is undeveloped to the toxin threatens the health of calves. Because calves have a greater absorption of toxins than adult bovine animals. The long half-life of the binding toxin in serum proteins (about 77 hours) increases toxicity. It is reported that 1 mg/kg OTA administered to calves with undeveloped ruminal function results in deaths within 24 hours (Battacone et al. 2010).

A number of studies have been carried out to determine the level of OTA in foods and feedstuffs. In 82 commercial cattle fattening feeds collected from different regions of Türkiye, the highest amount of OTA (4.04 µl / kg) was determined in the Marmara region and the lowest (0.76 µl /kg) was determined in the Aegean region (Akkaya and Bal 2013). In a study conducted in the Sivas region, it is reported that the amount of OTA in December, February, and April is 0.025, 0.024, and 0.024 ppm in compound feeds and 0.022, 0.020, and 0.021 ppm in barley samples respectively (Yılmaz and Aksu Elmalı 2016).

In cattle fed with OTA, secretion of toxins through the milk is of great importance to calves and human health (Turkoglu and Keyvan 2019; Miguel Alfonso et al. 2022; Wang et al. 2022). In one study, it was reported that the average OTA amount of 105 milk samples (35 pasteurized, 35 raw milk, and 35 UHT) was 85 ng/L in UHT milk, 137 ng/L in raw milk, and 135 ng/L in pasteurized milk (Turkoglu and Keyvan 2019). In 40 milk samples collected from milk tanks in Burdur region, the amount of OTA is stated to be in the range of 2-270 ng/L (Keyvan et al. 2018). In one Egyptian study, the average amount of OTA in 10 samples of raw milk was found to be 5.134 ppb (the lowest 0.34 ppb and the highest 13 ppb) (Younis et al. 2016). Three of the 264 milk samples collected at 132 farms in winter and summer (1.1%) contained OTA (Boudra et al. 2007). In a study conducted in Sudan, it is stated that 77.78% of the feed samples given to dairy cattle contained 0.22-1.59 µg/kg and 20% of the milk samples contained 2.73 µg/kg OTA (Elzupir et al. 2009).

OTA was not detected in meadow grass, hay, and barley samples collected in February in this research. OTA levels in meadow grass samples were 0.20 µg/kg in March and 0.28 µg/kg in April. The OTA content of the hay samples was 0.19 µg/kg in March and 0.21 µg/kg in April. The mean level of OTA in crushed barley was 0.20 µg/kg in March and 0.21 µg/kg in April. The results obtained from this study are similar to those reported by Akkaya and Bal (2013), Yılmaz and Aksu Elmalı (2016), Türkoğlu and Keyvan (2019), Keyvan et al. (2018), Younis et al. (2016), Boudra et al. (2007), and Elzupir et al. (2009). In this study, the highest level of OTA was detected in meadow grass collected in April. The difference between the average OTA amounts detected in meadow grass in March and April was statistically significant ($p<0.05$). It is thought that the increase in the amount of OTA in meadow grass in April is influenced by the air temperature and the increased humidity as a result of the melting of the snow. Because in the region, meadow grasses are stored in bales and outdoors. The detection of OTA in these grass piles can be explained as the fact that the fungal species synthesizing OTA are contaminated and that suitable conditions (heat, humidity, oxygen, etc.) are formed for their reproduction.

In this study, it was found that the amount of OTA in the hay and barley crushing samples collected in April was higher than in March. However, this increase was found to be statistically insignificant ($p>0.05$). In April, hay and barley samples had low levels of OTA compared to meadow grass. The low level of OTA in hay samples is mainly due to the way straw is obtained. Because straw is obtained by drying and crushing the stem of cereals (especially barley in the region) thoroughly. Also, in the locality hay is stored in sacks. As a result, it may be argued that straw is drier (at lower moisture) than meadow grass. It may be said that the low moisture content of hay limits fungal growth and toxin synthesis. The low level of toxins in barley crushing may be due to storage conditions. In the Ardahan region, barley crushed is either put in sacks and closed or stored in a closed environment in warehouses. The anaerobe environment created by these storage methods is thought to negatively impact fungal reproduction. Because mushrooms are mandatory aerobics. These storage conditions explain the low OTA content in barley crushed.

CONCLUSION

In the Ardahan region, OTA was not detected in the meadow grass, hay and crushed barley used in cattle feeding in February. OTA was 0.20 μg/kg in meadow grass samples collected in March and 0.28 μg/kg in April. In hay samples, 0.19 μg/kg was detected in March, 0.21 μg/kg in April, and barley crushed 0.20 μg/kg in March and 0.21 μg/kg OTA in April. Concentrations of OTA detected in the meadow grass, hay and crushed barley were found to be below the maximum level set by the Turkish Food Codex Contaminants Regulation. In this research, it was concluded that the meadow grass, hay and crushed barley given to cattle in February, March, and April in the Ardahan region did not pose a risk to human and animal health. It is recommended to take measures to prevent fungal growth in feed materials and to provide the necessary training to the breeders about not using moldy feeds in cattle nutrition.

Conflict of Interest: The author declares that there is no conflict of interest.

Ethics Committee Information: This study was approved by the Ministry of Agriculture and Forestry (letterdated 07.11.2022 and numbered E-29486769-325.99-7632633).

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