

Influence of Storage Condition and Period Time on Fungal Contamination and Mycotoxin in Ruminant Feed

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Received on 29/8/2020 and Accepted for Publication on 7/3/2021.

ABSTRACT

Contamination of animal feeds with mycotoxins in livestock farms is a severe matter. This study aimed to evaluate mycotoxigenic fungi, type of toxins, and toxin concentrations and contamination levels of Aflatoxin, Ochratoxin, and T2-toxin in ruminant feed collected from three different sites (a department laboratory, field 1, and field 2) and two periods of time (January and March). Also, four different genera were isolated and diagnosed from the animal feed based on the macroscopic morphology as well as on microscopic characteristics. The genera were *Aspergillus* spp., *Penicillium* spp., *Fusarium* spp., and *Rhizopus* spp. The result shows significant differences in fungal count among location storages, and among period times, too. Significant differences ($P < 0.05$) also has found for mycotoxin concentration while storage in different locations. Additionally, animal feed collected from different sites and different periods contained lower limits of mycotoxins than the standard limits.

Keywords: Feed quality, mycoflora, mycotoxin, and ruminant feed.

INTRODUCTION

Ruminant production is a significant part of the national economy and plays an important role in providing humans with a good quality of food (Hamasalim, 2009). The livestock sector's role in agricultural production is well known, and its contribution to poverty alleviation in rural areas is enormous (Abedullah *et al.*, 2009). Commercial mixed feeding stuff is a basic aspect of modern animal production. Among other elements, they include mixtures of homegrown cereals and imported commodities. The use of these ingredients ultimately results in the final mixed

feed being infected with fungi (Chelkowski, 1991). Once animal feed products are infected with molds, the secondary metabolites of these molds called mycotoxins create a major risk of contamination (Čonková *et al.*, 2006; Kabak *et al.*, 2006). The term mycotoxin refers to a group of chemical diverse secondary fungal metabolites (Binder, 2007; Fujimoto, 2011). For instance, it is produced by species such as *Aspergillus*, *Penicillium*, and *Fusarium* genera (Sweeney and Dobson, 1998; Bryden, 2004). Ochratoxin, fumonisins, aflatoxins, T-2 toxin, zearalenone, and deoxynivalenol are the most common mycotoxins (Didwania and Joshi, 2013). Mycotoxins when consumed

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in contamination with feed may show negative effects on health and production than when consumed alone (Denli and Okan, 2006; Yegani et al., 2006). Fungi typically grow between 10 and 40 °C, ranging from 4 to 8 in pH and above 0.70 in water activity (Lacey, 1991). Mycotoxins were found in various food sources from many parts of the world and are commonly recognized as one of the most harmful pollutants in animal feed and diet (Okoli *et al.*, 2007). There are differences of opinion about the role of fungi and mycotoxins in animal problems, mainly due to their impact on animal health and production (Seglar and Mahanna, 1995). The effect of mycotoxins on animals includes hepatotoxicity, oncogenesis, immunotoxicity, genotoxicity, and nephrotoxicity (Dierheimer, 1998; Kurata and Ueno, 1984). Contamination can occur both in the field and during storage and is largely dependent upon environmental factors (Bryden, 2012; Marquardt, 1996). Once consumed above a certain concentration by humans or animals, mycotoxins cause a toxic reaction called mycotoxicosis (Binder, 2007; Sweeney and Dobson, 1998; Bryden, 2004). The effects of mycotoxin intake vary from decreased body weight gain and fertility and immune suppression (Oswald and Comara, 1998), leading to increased susceptibility to disease and parasites to disease and death.

The objectives of this study were; first to identify fungi in different environment conditions (different temperature and humidity). Second, focus on the fungal species of mycotoxicological interest and analyze the natural occurrence of Aflatoxin, Ochratoxin, and T-2 toxin and the effects of the interactions of storage condition and period time on fungal growth and mycotoxin production.

Material and methods

Samples and storage conditions: Ten labeled replications of ruminant feed stored in three different sites; a department laboratory (Department of Animal Sciences, University of Sulaimani, Iraq), field 1, and field 2. After

one month, one sample from each replication was tested for the detection of fungi and toxins. The experiment repeated after two months from the same labeled replications. As the ruminant feed was stored in the normal condition in two fields, the temperature and humidity fluctuated, which were between (3.2–12.7 °C) and (67 %) respectively in January while the temperature between (10.7–21.3 °C) and humidity 57 % in March. But the ruminant feed stored in the laboratory, the temperature between (17.1–24.3 °C) and humidity 41 %. All samples were examined for mycological and mycotoxins detection. Feed samples intended for mycological analysis were normally immediately analyzed upon arrival or stored in paper bags for 2-3 days at room temperature (22-25 °C). The remaining parts of the feed samples intended for analysis of mycotoxins were deposited at -18 °C. Accordingly, the samples were removed from the freezer and kept at room temperature for one hour before the study was started.

Mycological analysis: The dilution plating method was used to measure the fungal flora in the feed samples. In short, one gram of finely ground feed was thoroughly mixed with 9 ml of sterile distilled water, followed by 10-fold serial dilutions up to 108. An aliquot (0.1 ml) of each dilution has been spread over the surface of a 15 ml Potato Dextrose Agar petri dish. The dishes were agitated, permitted for 5-7 days to set and incubate at 28 °C. Selecting the plates containing 10-30 colonies has chosen the correct dilution factor. To test the laboratory procedure and precision, all the serial dilutions and the cultures in plates were prepared in triplicate and considered the mean of fungal colonies. Three Petri dishes per sample were set out altogether. All the colonies were recorded and cultivated in cases where many fungi were isolated from a single sample. The findings were expressed as units per gram forming a colony (cfu/g).

Identification of fungi: Dilute plate technique was used in fungi counting, while feed parts were used for

mycotoxin assessment, three standard Aflatoxin, Ochratoxin, and T2-toxin kits were subjected to and determined by ELISA method. According to Nelson *et al.* (1983), Pitt and Hocking (1997), and Samson *et al.* (2004), the fungi were identified. Identification of the fungal species was based on the isolates' macroscopic and microscopic properties.

Sample preparation for toxin measurement: Feed samples were prepared for mycotoxin measurement using an ELISA method. Five grams of a feed sample was diluted with 70 % methanol in a ratio of 1:5 (5 gm of feed + 25ml of 70% methanol), used for Aflatoxin and T2 toxin determination. While for ochratoxin detection, 10 gm feed was diluted with 50 % methanol in a ratio of 1:4 (10 gm of feed + 40ml of 50% methanol).

Mycotoxins analysis

Ochratoxin analysis: Connect 10 gm of ground feed to 40 ml of 50% methanol/water and shake it up. The extract was then filtered through a Whatman filter by pouring at least 5 ml and collecting the sample filtrate. To test each sample, one red marked mixing well was used, plus 5 red marked wells for controls. In each red marked mixing well, 100 ml of the conjugate is added. Thus, 100 ml of control and ruminant feed samples were transferred to the red marked mixing antibody-coa. Mixed for 10-20 seconds by pushing the microwell holder back and forth over a flat surface without splashing reagents from the wells, incubated for 10 minutes at room temperature, then. Shake the substance of the antibody well and fill it with distilled water and dump it out. Repeat this step five times and then turn the well upside-down until the remaining water is drained. 100 ml of the substrate was primed and piped into the wells and incubated for 10 minutes. After that, 100 ml of red stop solution was pipetted into each well. Lastly, using a 650 nm filter, wiping the bottom of the microwells and reading in a microwell reader. Finally, use the EL301 reader and use the Neogen Veratox software for

Windows to measure results.

Aflatoxin and T2 toxin analysis: The method for analysis of aflatoxin and T2 toxin are the same as mentioned above (Ochratoxin analysis), the only differences are; to connect 5 gm of ground feed to 25 ml of 70% methanol/water and shake it up. The rest is just the same.

Statistical Analyses: Collected data were analyzed by a two-factor factorial analysis in a completely randomized design (CRD). Repeated measures data, such as storage condition and location were analyzed using the MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC). The statistical model included fixed effects of treatment, time, and treatment by time and the least-squares mean \pm standard error of the means was reported. The differences among means were tested by Duncan's multiple range test according to $P < 0.05$ significance (Steel *et al.*, 1996).

Results

The frequency of fungi genera and mycotoxin that existed in the 30 ruminant feeds samples are shown in table 1. The low count of fungi in the first period (January 2019) while the high count of fungi in the second period (April 2019). Also, significant differences ($P < 0.05$) were fungi count determined in ruminant feed storage in two ruminant fields and lab as control. Also, ruminant feed inspected of the first period contained a low concentration of Aflatoxin, Ochratoxin, and T2 toxin (1.80, 6.08 and 105.49 $\mu\text{g/kg}$ respectively) while compared ruminant feed checked off the second period contained a high concentration of Aflatoxin, Ochratoxin, and T2 toxin (2.75, 7.01 and 108.87 $\mu\text{g/kg}$ respectively).

Table 1: Mycobiota and mycotoxin contamination of ruminant feed stored in the two periods

Measurements	Storage condition	
	First period (After one month)	Second period (After three months)
Fungal contamination CFU/gm	2.556×103b± 0.503	3.222×103a± 0.741
Aflatoxin (µg/kg)	1.803b± 0.256	2.752a± 0.493
Ochratoxin (µg/kg)	6.078b± 0.194	7.006a± 0.447
T2 toxin (µg/kg)	105.490b± 4.679	108.866a± 5.585

a-b Means followed by different superscripts within a row are significant ($P < 0.05$).

The fungal contamination and mycotoxin concentrations of the ruminant feed were compared in accordance with the lab storage and ruminant field, shown in table 2. A significant difference in the fungal count was shown among the three-location storage of ruminant feed. The ruminant feed storage in field 2 appeared to have a higher count of fungal (5.000×10^4 cfu/gm). But, the ruminant feed storage in the lab as control had a lower count of fungal (0.833×10^4 cfu/gm). Significant differences ($P < 0.05$) between ruminant feed for mycotoxin concentration while stored in a different location. The feed collected in field 2 recorded higher Aflatoxin concentration while the feed store in the lab contained low levels of Aflatoxin. There was a significant difference ($P < 0.05$) in Ochratoxin concentration which appeared in all feeds collected in field and lab as control; higher Ochratoxin concentration value was detected in field 2 ($7.68 \mu\text{g/kg}$) but a lower concentration was detected in lab feed ($5.66 \mu\text{g/kg}$). The same table shows the mean value for the T2-toxin concentration of ruminant feeds. Generally, T2-toxin

concentration was higher in feed collected in field 2 ($127.55 \mu\text{g/kg}$) while feed collected in field 1 showed amid concentration ($98.98 \mu\text{g/kg}$) and feed store in the lab as control showed rather low concentrations of T2-toxin ($95.00 \mu\text{g/kg}$).

Table 2: Mycobiota and mycotoxin assessment in ruminant feed stored of the lab and two fields.

Measurements	Locations		
	Lab (Control)	Field 1	Field 2
Fungal contamination CFU/gm	0.833×10^3 $c \pm 0.167$	2.833×10^3 $b \pm 0.307$	5.000×10^3 $a \pm 0.365$
Aflatoxin (µg/kg)	$1.120 c \pm$ 0.029	$2.060 b \pm$ 0.228	$3.653 a \pm$ 0.408
Ochratoxin (µg/kg)	$5.662 c \pm$ 0.134	$6.283 b \pm$ 0.282	$7.680 a \pm$ 0.416
T2 toxin (µg/kg)	$95.003 c \pm$ 0.417	$98.983 b \pm$ 0.496	$127.547 a \pm$ 1.629

a-c Means followed by different superscripts within a row are significant ($P < 0.05$).

The effects of interactions between locations and period time significantly ($P < 0.05$) affected fungal contamination and mycotoxin concentration in the ruminant feed (Table 3). The highest value of fungal contamination resulted at second period/field 2 (5.667×10^4) but the lowest value resulted in the second period while stored in the lab. Then, the highest value of Aflatoxin, Ochratoxin and T2 toxin concentration resulted at the second period though the feed stored in the fields and the lowest value resulted in the ruminant feed while stored at the lab as control (1.177 , 5.530 and $95.520 \mu\text{g/kg}$) respectively.

Table 3: fungal contamination and mycotoxin concentration of ruminant feed affected by period time and location.

	First Period (After one month)				Second period (After three months)			
Location	Fungal contamination CFU/gm	Aflatoxin (µg/kg)	Ochratoxin (µg/kg)	T2 toxin (µg/kg)	Fungal contamination CFU/gm	Aflatoxin (µg/kg)	Ochratoxin (µg/kg)	T2 toxin (µg/kg)
Lab (Control)	1.000×103e ± 0.000	1.063d± 0.023	5.793c± 0.220	94.487e± 0.687	0.667×103e± 0.137	1.177d± 0.024	5.530c± 0.154	95.520e ± 0.360
Field 1	2.333×103d ± 0.321	1.583c± 0.093	5.667c± 0.128	97.930d± 0.288	3.333×103c± 0.333	2.537b± 0.156	6.900b± 0.035	100.037 c± 0.196
Field 2	4.333×103b ± 0.134	2.763b± 0.133	6.773b± 0.139	124.053b ± 0.881	5.667×103a± 0.638	4.543a± 0.154	8.587a± 0.159	131.040 a± 0.531

^a-eMeans followed by different superscripts within interactions are significant (P < 0.05).

Identification characteristics of fungal of ruminant feed collected in different locations and two periods as detected in table 4. Based on the standard method of identification for ruminant feed, four genera were isolated which were *Fusarium* spp., *Penicillium* spp., *Aspergillus* spp., and *Rhizopus* spp. While, based on morphological characteristics, the first fungal of *Aspergillus* spp. groups can be defined as colonies of a hairy, velvety, milky-creamy color, but later converted to a black colony of fungi with yellowish color at the other side, and septate with unbranched conidiophores, Then radiate head was formed as a result of double sterigmata cover for whole vesicles.

The isolation of *Penicillium* spp. created branched septate hyphae with flask-formed sterigmata, the conidia is unbranched with a penicillate or blue color appearance, white-creamy powdery surface but later turned blue-green with whitish reverse side and edges in the agar. The *Fusarium* spp. had a hairy growth with buttery color, and then turned to rosy (pink) with a yellowish at the opposite side and septate branched conidiophore with rectangle conidia. Finally, *Rhizopus* spp produced Septate hyphae with enlarged in a cup shape. Then, can be described as a colony of a soft powdery growth that later turned black.

Table 4: Identification characteristics of fungal of ruminant feed storage in the three locations.

Serial No of Isolates	Macroscopic characteristics and texture	Microscopic Characteristic	isolated
1	Velvety, hairy, milky- creamy color, but later converted to a black colony of fungi with yellowish color at another side	Septate with unbranched conidiophores, then radiate head was formed as a result of double sterigmata cover for whole vesicles	Aspergillus spp
2	White-creamy powdery surface but later turned blue-green with whitish reverse side and edges in the agar.	Branched septate hyphae with flask-shaped sterigmata, the conidia are unbranched with a penicillate or blue color appearance.	Penicillium spp
3	Hairy growth with creamy color that later turned to pink with a yellowish at the opposite side	Septate with branched conidiophore and rectangle conidia.	Fusarium spp
4	Soft powdery growth that later turned black	Septate hyphae, unbranched sporangiospores are from the foot of rhizoids that enlarged in a cup-shaped form with the mycelial region	Rhizopus spp

Discussions

This study discovered that the fungal count in the ruminant feed was affected by period time with storage conditions. Higher fungal counts and the mycotoxin concentration were obtained in the ruminant feed determined in the second period. On the other hand, low fungal counts were examined in the ruminant feed while stored in the first period. In the field, fungal contamination of animal feed can occur during the handling or storage of harvested crops or feed if the environmental conditions are suitable for fungal growth (FAO, 2013).

This result agrees with Hamasalim *et al.*, (2016) who reported that three fungal genera were isolated in the ruminant feed analyzed, and the kind of feed affected on a number of fungal in the feeds. According to the

International Organization for Standardization (2008), the total fungal count of food and animal feed should be below 104 CFU/g. Contamination of feeds with mycotoxin could be prevented by monitoring the environmental condition affecting fungal production, by regularly cleaning the storage systems by regulating the grain's physical conditions, and by using anti-cracking additives and mold inhibitors (Datsugwai, *et al.*, 2013).

The results showed Aflatoxin was found in all samples with significant differences ($P < 0.05$) in aflatoxin concentration for ruminant feed while collected in a different location and period times. This result agrees with The European Commission for Standardization (2006), aflatoxin concentration of animal feed should be below 20 µg/kg. The concentration of Ochratoxin in ruminant feeds

was significantly different ($P < 0.05$). This result agrees with The European Commission for Standardization (2006), Ochratoxin concentration of animal feed should be below 50 $\mu\text{g/kg}$. According to the result, T2-toxin showed significant differences in ruminant feed, but T2-toxin concentration was lower than the maximal standard limits (250 $\mu\text{g/kg}$).

In our study, *Aspergillus* spp, *Rhizopus* spp *Penicillium* spp, and *Fusarium* spp were the main fungal genera isolated from the ruminant feed samples. This finding is in line with the findings stated by Oliveira *et al.* (2006) who demonstrated that *Penicillium* spp., *Aspergillus* spp., and the *Fusarium* spp. were the three most common fungi isolated from Brazilian feeds likewise.

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Conclusion

In general, the genera of fungi isolated, and their occurrence was *Aspergillus* spp., *Penicillium* spp., *Rhizopus* spp., and *Fusarium* spp. in ruminant feeds collected from different sites and two different periods of times, also, detected concentration of each Aflatoxin, Ochratoxin, and T-2 toxin. Ruminant feed collected in the lab and ruminant field under study were lower than the maximal standard limits of mycotoxin (Aflatoxin, Ochratoxin, and T-2 toxin). Finally, discovered the environment, period time, and storage conditions that affected mycoflora growth and mycotoxin concentration in the ruminant feed.

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تأثير ظروف التخزين والفترة الزمنية على التلوث الفطري والسموم الفطرية في علف المجترات

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تاريخ استلام البحث 2020/8/29 وتاريخ قبوله 2021/3/7.

ملخص

يُعدُّ تلوث أعلاف الحيوانات بالسموم الفطرية في مزارع الماشية أمراً خطيراً، وكان الهدف من هذه الدراسة هو تقييم الفطريات المسببة للسموم، ونوع السموم، وتركيزات السموم ومستويات التلوث للأفلاتوكسين، والأوكراتوكسين، وتوكسين T2 في علف المجترات التي تم جمعها من ثلاثة مواقع مختلفة (مختبر القسم، الحقل 1، والحقل 2) وفي فترتين زمنيتين (كانون اول واذار). وايضا، تم عزل وتشخيص أربعة أجناس مختلفة من العلف الحيواني على أساس التشكل العياني وكذلك على الخصائص المجهرية. وكانت الأجناس هي *Aspergillus spp.* و *Penicillium spp.* و *Fusarium spp.* و *Rhizopus spp.* تظهر النتيجة فروقا ذات دلالة إحصائية في العد الفطري بين مستودعات الموقع، وبين أوقات الفترات أيضاً. وتم العثور على فروق ذات دلالة إحصائية ($P < 0.05$) لتركيز السموم الفطرية أثناء التخزين في مواقع مختلفة. إضافة إلى ذلك، كانت الأعلاف الحيوانية التي تم جمعها من مواقع مختلفة وفترات مختلفة تحتوي على حدود سموم فطرية أقل من الحدود القياسية للسموم الفطرية.

الكلمات الدالة: جودة العلف، ميكوفلورا، السموم الفطرية، علف الحيوانات المجترة.