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Efficacy of Ammonization to Eliminate Common Mycotoxins

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ABSTRACT

Mycotoxin is a worldwide problem threatening animal health and performance as well as public health. The objective of this experiment was to test the effect of ammonization on elimination of common mycotoxins in laying hen compound feed (CF) and dairy cattle total mixed ration (TMR). The CF for laying hens and TMR for dairy cows were contaminated with commonly occurring mycotoxins [aflatoxin B_1 (AFB_1), ochratoxin A (OTA), and zearalenone (ZEA)] at 25 times of their accepted legal limits (20 ppb, 200 ppb and 500 ppb, respectively). They were then subjected to ammonization with ammonium bicarbonate (NH_4HCO_3) at 50°C for 24 hours under the atmospheric pressure. Mycotoxin levels were analyzed using the LC-MS/MS technique. The elimination levels in CF and TMR were 53% and 54% for AFB_1; 31% and 31% for OTA and 22% and 22% for ZEA, respectively. In conclusion, ammonization was effective in destroying common mycotoxin, at an order of AFB_1 > OTA > ZEA.

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Introduction

Mycotoxins are secondary metabolites that are produced by certain fungi such as *Aspergillus, Penicillium and Fusarium* and that stimulate the toxic response in animals when ingested even at low concentrations (Bennet, 1987; Moss, 1991). The common mycotoxins that most capable to contaminate the feedstuffs and food materials among the thousands of mycotoxins are aflatoxins (AFB), ochratoxin A (OTA), and zearalenone (ZEA) (Ismaiel and Papenbrock, 2015). Mycotoxins deliver detrimental effects in animals including mortality, production loss, and feed conversion inefficacy as well as hepatotoxic, nephrotoxic, immunotoxic, cancerogenic, and genotoxic effects (Bitay et al., 1979; Smith et al., 1992; Dierheimer,1998; Gabal and Azzam, 1998; Wild and Gong, 2012).

Different methods have been developed to eliminate the negative impacts of mycotoxins. These methods can be classified as: physical methods such as irradiation, heating, extraction, and adsorption (Jalili et al., 2010). Adsorptive agents are feasible and practical under the farm conditions. In some studies, mineral compounds as adsorptive agents demonstrate efficient adsorbing effects *in vitro*, in contrast, some of them are not able to adsorb mycotoxins efficiently *in vivo* (Jaynes et al., 2007). It was also shown that the adsorptive agents were selectively bound to mycotoxins (Gregorio et al., 2014). Chemical and physical properties of adsorbents and mycotoxins, such as surface phenomenon, size and distribution of the porous, total charge, mycotoxins polarity, shape, size and low surface area are another factor for the effectiveness of binders and adsorbents for mycotoxins (Huwing et al., 2001). Biological methods include fungi (*i.e.*, nontoxigenic *Aspergillius* spp.), bacteria (*i.e.*, *Actinomycetales* spp.), and enzymes (*i.e.*, laccase and manganese peroxidase) (Alberts et al., 2009; Wu et al., 2009; Wang et al., 2011; Kong et al., 2012). There are some limitation factors on the elimination of mycotoxins such as required long incubation periods for effective detoxification and complicated extraction procedures to obtain the active extracts (Ji et al., 2016).

Similar to treatment with common chemicals such as sodium bisulfite and calcium hydroxide (Samarajeewa et al., 1990), ammonization is considered chemical methods, based on the principle to convert the mycotoxins into the non-toxic or less toxic metabolites by the oxidation or

hydrolyze of the lactone ring present in their structure, through the binding of chlorine to furan ring and breaking down the double bonds in the lactone ring (Swenson, 1981). Ammonization occurs by two step chemical reactions: first, hydrolysis of the lactone rings and then decarboxylation (Lee et al., 1974; Grove et al., 1984). In ammonization, the lactone ring in the aflatoxins is the main target and opened by aminolysis and ammonia forms an amide by binding to the carboxyl terminal end, due to in the acidic nature, of this group. In the latter step, remains a β-keto acid after the removing of ammonia from the amide moiety. Presence of the H_2O, H^+ or OH^- ions in the environment or presence of the heat cause to the decarboxylation of the β-keto acid and to remove the CO_2 from the structure. After this step, formed new structure is called aflatoxin D_1, which is 2000-20000 times less toxic than AFB_1 (Hawortha et al., 1989).

The objective of this experiment was to test the efficacy of the ammonization procedure on destruction of commonly occurring mycotoxins (AFB_1, OTA, and ZEA) in laying hen compound feed (CF) and dairy cattle total mixed ration (TMR).

Material and Methods

Samples, Contamination, and Ammonization

Commercial compound feed for laying hens (CF) (11.09 MJ/kg, 17% crude protein, 4% crude fiber, 3.4% crude fat and 12.5% crude ash) and total mixed ratio for dairy cattle (TMR) (19.4% grass hay, 19.2% barley silage, 24.0% corn silage, 9.7% rolled barley grain, 27.8% protein-energy concentrate on a dry matter basis) were dried at 60°C for three days and then ground to pass 1 mm screen. The mycotoxins were purchased (A6636: 1 mg aflatoxin B_1, Z2125: 25 mg zearalenone, and 32937: 5 mg ochratoxin A, Sigma-Aldrich, Taufkirchen, Germany).

The feedstuffs were subjected to contamination of mycotoxins at 25-fold of the legal limits [(AFB_1, OTA and ZEA are 20 µg/kg, 200 µg/kg and 500 µg/kg, respectively (Amtsblatt der Europäischen Union, 2006) (Table 1). The mycotoxins at the contamination level were suspended within 1.500 L distilled water. The feeds were split into two group: the control group and the contaminated group (800 g). After spraying mycotoxin cocktail in plastic containers, the contaminated feeds were subjected to the drying process at 60°C for three days. The feedstuffs were shaken every three hours during the drying process to achieve homogeneous contamination. Contaminated CF and TMR were split into two groups: the contaminated group and the contaminated plus ammonized group (400 g). The ammonization process was applied at the dose of 10 g/kg NH_4HCO_3 (Toxifarm Dry, Farmavet International, Manisa, Türkiye). While the ammonization at 50°C for 24 hours in oven, the lids of plastic containers were slightly secured in order to prevent the gas escape as NH_4HCO_3 gasifies as soon as contact with air and heat. The containers were shaken every three hours during the ammonization process.

The feeds of the control, contaminated and ammoniated groups were subsampled 8 bags, each weighing 50 g for analyses of mycotoxins using the liquid chromatography/mass spectrometry (LC/MS 6420, Agilent, Santa Clara, CA).

Before analyses of mycotoxins, the feed samples were extracted by weighing 2 g feed samples in the falcon tube and adding 10 ml extraction-1 solution [79% acetonitrile (HPLC grade), 20% distilled water and 1% formic acid (HPLC grade)]. The samples were centrifuged for 5 min at 4500 rpm after stirring process at 250-300 rpm for one hour. At the end of the centrifugation, 1 ml extraction-2 solution [79% distilled water, 20% acetonitrile (HPLC grade) and 1% formic acid (HPLC grade)] were added to 1 ml supernatant (this process ensured both the solvation and 10 times distillation of mycotoxins) and filtered through 0.45 µm filter and transposed to another falcon tube. The obtained specimens were analyzed by taking into the vials.

Statistics

The data were subjected to one-way ANOVA using the PROC. GLM procedure (SAS, Statistical Analysis System, Version 9.0, Cary, NC, 2002). The linear model was as follows: $Y_{ij}= \mu + G_i + e_{ij}$, where Y: response variable, µ: population mean, G: group (i: control, contaminated and ammoniated), and e: experimental error (i: in group, j: in sample). Difference between-groups was evaluated with the LSD option. Statistical difference among groups was considered significant at $p<0.05$ and the group values were presented as least square mean ± standard error.

Results

Intra- and Inter-Assay Variation

When the control feed groups were considered, the intra-assay variations were 13.5%, 39.0% and 45.5% and inter-assay variations were 16.7%, 63.8% and 46.0% for AFB, OTA, and ZEA, respectively.

Table 1. Effects of the ammonization procedure on mycotoxin levels (µg/kg) in laying hen compound feed (CF) and dairy cow total mixed ration (TMR) upon contamination.

	Groups*		
Feed	Control	Contaminated	Ammonized
Laying Hen CF			
AFB_1	1.61±0.05^c	324.71±2.89^a	152.73±1.36^b
OTA	5.47±0.51^c	1831.61±51.28^a	1267.27±29.11^b
ZEA	4.73±1.58^c	3045.20±94.68^a	2383.82±135.13^b
Dairy Cow TMR			
AFB_1	1.89±0.12^c	146.59±2.59^a	67.95±1,64^b
OTA	12.28±2.24^c	877.65±20.36^a	608.04±18.05^b
ZEA	2.44±1.10^c	2827.88±233.81^a	2192.87±147.09^b

*The different superscripts among the groups differ ($P<0.05$). The feedstuffs were subjected to contamination of mycotoxins at 25-fold of the legal limits [aflatoxin (AFB_1), ochratoxin A (OTA), and zearalonol (ZEA) are 20, 200, and 500 µg/kg, respectively (Ambsblatt der Europäischen Union, 2006). The ammonization process was applied at the dose of 10 g/kg NH_4HCO_3 (Toxifarm Dry, Farmavet International, Manisa, Türkiye).

Contamination and Ammonization

The mycotoxin levels in the laying hen CF and dairy cow TMR when they were not contaminated (control), contaminated with mycotoxins about 25-fold of legal limits (contaminated), and ammoniated after contamination were summarized in Table 1. The levels of AFB_1, OTA and ZEA in the control laying hen CF were much lower than the legal limits determined by the international standards (20, 200, and 500 µg/kg, respectively), which were 1.61, 5.47, and 4.73 µg/kg, respectively (Table 1). Experimental contamination for AFB_1, OTA and ZEA were achieved by 65%, 37%, and 24%, respectively, of the intention, which were 325 (500), 1832 (5000), and 3045 (12500) µg/kg, respectively (Table 1). Significant decreases in concentration of AFB_1 (-53%), OTA (-31%) and ZEA (-22%) were obtained in the contaminated laying hen CF after the ammonization process. However, the ammonization process was not fully successful to decrease the mycotoxin levels below to the legal limits.

The levels of AFB_1, OTA and ZEA in the control dairy cow TMR were much lower than the legal limits determined by the international standards, which were 1.89 (20), 12.28 (200), and 2.44 (500 µg/kg, respectively (Table 1). Experimental contamination for AFB_1, OTA, and ZEA were achieved by 29%, 18%, and 23%, respectively, of the intention, which were 147 (500), 878 (5000), and 2828 (12500) µg/kg, respectively (Table 1). Significant decreases in concentration of AFB_1 (-54%), OTA (-31%), and ZEA (-22%) were obtained in the contaminated dairy cow TMR after the ammonization process. However, the ammonization process was not fully successful to decrease the mycotoxin levels below to the legal limits.

Discussion

Studies involving the ammonization process at various concentrations and different temperatures under the pressure in a laboratory setting for destructions of AFs and OTA are available (Brekke et al., 1979, 1977a, 1977b; Jensen et al., 1977; Bagley, 1979; Norred, 1982; Price et el., 1982; Lee et al., 1984; Koltun, 1986; Norred et al., 1991; Kwon et al., 1997).

The data obtained from the LC-MS/MS analysis demonstrated that a significant degradation in the mycotoxin levels on the AFB_1, OTA and ZEA contaminated laying hen CF and dairy cow TMR (Table 1). The degradation levels for AFB_1, OTA and ZEA were 54%, 31% and 22%, respectively, in the laying hen CF and 53%, 31% and 22%, respectively, in the dairy cow TMR. The data confirms that the ammonization mechanism affects the epoxy terminal end in the lactone ring of the resorcylic acid lactone structure of ZEA similarly at a short period (24 hrs) to the AFB_1 and OTA. The decomposition rates were not fully successful to decrease the levels under the legal limits.

AFB_1 and AFG_1 contain a double bond on the C8-9 point of the furan rings while AFB_2 and AFG_2 do not contain a double bond on the C8-9 of the furan rings. These features of the AFs create the concentration differences in the ozonation method, which is a detoxification/elimination method based on oxidation. For instance, AFB_1 and AFG_1 require low concentrations while AFB_2 and AFG_2 require high concentrations (McKenzie et al., 1997). Likewise, in the microwave application, the double bond on the C8-9 point of furan ring causes wavelength differences (Atalla et al., 2004; Diao et al., 2015). These concentration differences arising from the double bond are not thought to occur in the ammonization method.

The lactone ring in the OTA is irreversibly cleaved opened in the alkaline environment (Müller, 1983). LD_{50} dose for a metabolite produced by the ammonization of the epoxy terminal in the lactone ring has been determined to be 200 µg/egg (Chelkowski et al., 1981), which confirms decreased OTA levels upon the ammonization process in the present study (Table 1).

ZEA is in the resorcylic acid lactone structure and contain an epoxy terminal end in the lactone ring (Kuiper-Goodman et al., 1987). In agreement with the present study, ZEA degradation was observed upon the ammonization process in the earlier studies (Bennet et al., 1980; Chelkowski et al., 1981). However, these levels are not satisfied despite a long processing time for destruction (Bennet et al., 1980; Chelkowski et al., 1981).

The decomposition of mycotoxins by ammonization were found most effective for degradation of AFs and OTAs. In addition, the temperature, pressure and processing time were thought to be critical for evaporate of ammonia and breaking down the double bond in the lactone ring (Brekke et al., 1979, 1977a; Chelkowski et al., 1981; Norred, 1982; Price et al., 1982; Lee et al., 1984; Koltun, 1986).

1% solution of ammonium bicarbonate in water has approximately 7.8 pH at 25°C. In addition, Sutter and Mazzotti (2017) have shown that NH_4^+ and HCO_3^- activity existed in the NH_4HCO_3-H_2O binary system by contrast with the contact of NH_4HCO_3 with air. In this situation, an extra alkaline environment may be created in the presence of water. However, this effect is low for the hydroxylation of the epoxy terminal end in the lactone rings. In a study conducted by Gomma (1987) has shown that 5% sodium bicarbonate provide 10% degradation in aflatoxins. In the present study, dry form of NH_4HCO_3 used for detoxification process. In this case, no an extra alkaline environment was created for hydroxylation.

In our experimental design, the water attached to the feedstuffs was evaporated at 60°C for 3 days. This process creates DM > 88%. Up to 5% ammonium concentration requires 10-20% moisture content in the feedstuffs to provide an effective aflatoxin degradation depending on the temperature and time (Samarajeewa et al., 1990). The total water, both releasing from the NH_4HCO_3 and attached to the feedstuffs, is not sufficient for hydroxylation reaction in the lactone ring.

In the ammonization process, the lactone ring in the mycotoxins is opened by aminolysis and ammonia forms an amide by binding to the carboxyl terminal end, due to in the acidic nature of this group. The opening of the lactone ring is a reversible reaction in the acidic environment (Piva et al., 1995). In the latter step, remain a β-keto acid after the removing of ammonia from the amide moiety. Presence of the H_2O, H^+ or OH^- ions in the environment or presence of the heat cause to the decarboxylation of the β-keto acid and to remove the CO_2 from the structure. In addition, thermal decomposition of NH_4HCO_3 between 35-60°C

releases $NH_{3(g)}$, $H_2O_{(g)}$ and $CO_{2(g)}$ to the environment. In the present case, the CO_2, released both from NH_4HCO_3 and mycotoxins, may acidify the environment. This reaction may convert the formed metabolite to the original state.

In conclusion, mycotoxin degradation by ammonization method was confirmed. However, the degradation rate was not satisfactory, reaching below their legal limits. Future studies should perform different doses at different temperatures while identifying new products occurring in addition to nutrients in case they are lost and/or denatured.

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