

## Study on mycoflora of poultry feed ingredients and finished feed in Iran

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### ABSTRACT

**Background and Objectives:** Unhygienic poultry feedstuffs can lead to nutrient losses and detrimental effect on poultry production and public health. In the present study, mycobiota and colony-forming units per gram in ingredients and finish poultry feed was evaluated with special reference to potentially mycotoxigenic fungi.

**Materials and Methods:** Eighty five samples of corn, soybean meal and poultry finished feed were collected from nine poultry feed factories located in three provinces i.e. Tehran, Alborz and Qom in Iran from October 2014 to January 2015. Samples were cultured on Sabouraud dextrose agar (SDA), *Aspergillus flavus* and parasiticus agar (AFPA) and dichloran rosebengal chloramphenicol agar (DRBC) and incubated at 28 °C for 7-10 days. Purified fungal colonies were identified by a combination of macro- and microscopic morphological criteria. For determining the rate of fungal contamination, samples were cultured on SDA and colony forming units (CFUs) were calculated.

**Results:** A total of 384 fungal isolates belonging to 7 genera of filamentous fungi and yeasts were obtained from corn (124 isolates), soybean meal (92 isolates), and feed before (72 isolates), and after pelleting (96 isolates). The most prominent fungal isolate in corn, soybean meal and feed before pelleting (feed as mash form) was *Fusarium* but in feed after pelleting was *Aspergillus*. Among 5 *Aspergillus* species isolated, potentially aflatoxigenic *A. flavus* isolates was predominant in corn (46.6%), soybean meal (72.7%) and poultry finished feed (75%). CFUs results indicated that 9/22 corn samples (40.9%), none of 22 soybean meal samples, 19/41 finished feed (46.3%) were contaminated higher than the standard limit.

**Conclusions:** Our results indicated that corn, soybean meal and finished feed of poultry feed mill are contaminated with various fungal genera by different levels sometimes higher than the standard limits. Contamination with potentially mycotoxigenic fungi especially *Aspergillus* species may be considered as a human public health hazard.

**Keywords:** Mycoflora, *Aspergillus*, Poultry feed, Mycotoxigenic fungi.

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### INTRODUCTION

Contaminated feeds either in the form of ingredients or as a finished product can transmit fungal pathogens to animal farms and sometimes to human food (1). Fungal contamination in raw materials or finished feed not only induces disease in poultry

flocks but also causes other infectious diseases in birds (2, 3). It also reduces the use of animal of feed consumption and resulting in a drop in productivity. Among microorganisms, fungi have important effects on quality of food. Fungal growth sometimes leads to non-consumption of feed for poultry (4, 5). These fungi grown on feedstuffs that are kept in poor conditions can widely contaminate feed and agricultural products. Each of these can be infected with one or several fungal toxin that results the final product to be contaminated with various toxins (6-8). Certain strains of filamentous fungi such as *Aspergillus*, *Penicillium* and *Fusarium* often arise as fungi causing toxins. These fungi in the feed mills with unfavorable conditions (temperature and moisture) for storage, grows on finished feeds, and produce fungal toxins (9-12). Agricultural products containing fungal contamination or fungal toxins have not good quality and sold at cheaper prices. The growth of these fungi on raw materials as well as poultry feed, may cause detrimental effects such as suppressing the immune system, reduced performance, increase feed conversion ratio, poultry mortality, and economic losses. According to the World Health Organization (WHO), aflatoxins residue has greater share in contamination comparing with other toxins. According to the International Agency for Research on Cancer (IARC) aflatoxins has been introduced as a human liver carcinogens type A (13). Aflatoxins are produced mainly by *Aspergillus flavus* and *A. parasiticus* (10, 14). *A. flavus* is one of the main types of aflatoxin producing fungi and responsible for the contamination of crops before harvest or during storage is in stock. It should be noted all strains of *A. flavus* and *A. parasiticus* are not able to produce aflatoxin. Toxigenic strains cannot produce toxin in all environments (15). The spread of the fungal infections is related to several factors such geographic location, storage conditions, processing of various feeds and moisture. Among the mentioned factors, moisture is the most important factors, so that reducing the moisture of the feed content to less than 12%, fungal growth and aflatoxin production will be stopped (16,17). Aspergillosis is a fungal disease created in poultry farm by *Aspergillus* species, especially *A. flavus* and *A. fumigatus*. Pulmonary aspergillosis is the most common disease in commercial poultry and has been reported in two acute and chronic forms. The disease usually looking for infection, poor nutrition, environmental temperature and relative humid-

ity inappropriate and poisoning caused (3, 18).

Overall, the components of poultry feed prepared in feed mills with fungi contamination is of great importance, so extensive studies by researchers and scientists has been done around the world on this subject (19-23). The production of poultry products in Iran needs to million metric tons of healthy feedstuffs, therefore, study on the poultry feed contamination is necessary in consideration to climatic conditions (temperature and relative humidity), especially in storage and transportation.

The present comprehensive study was designed for the first time in Iran to assess contamination rate and fungal mycoflora of the main components (corn and soybean meal) as well as finished poultry feed (before and after pelleting) with emphasis on isolation and identification of potentially mycotoxigenic *Aspergillus* species.

## MATERIALS AND METHODS

**Sample preparation.** Survey was conducted in nine feed poultry mill factories from October 2014 to January 2015 in three provinces in Iran. Samples of feed factories were collected from provinces of Tehran, Alborz and Qom that licensed and permit from the Veterinary Authority. Sampling was carried out based on number code 7570, Institute of Standards and Industrial Research of Iran as well as a role of Iranian Veterinary Organization (IVO 2015). Samples were taken from the main raw material feed ingredients including corn and soybean meal, and the final product before entering the "pelletizer" and then ready to feed "pellet form". According to the sampling tables, sample size was determined. Samples were corn (22 samples), soybean meal (22 samples), before (22 samples) and after pelleting (19 samples). Each sample was collected from at least three parts of bulks or bags. The sampling was performed randomly to provide an equal chance of selection. Corn samples from silos or power supply line system production, soybean meal samples of soy silos or warehouse. Feed samples before the pellet form of the mixer device and pellet finished feed samples were collected after pelletizer device. The cup or grape method was performed depending on the type of feedstuffs and factories. Pre-samples were thoroughly mixed to obtain a working sample. At this stage, random samples (1kg) of corn, soybean meal, and

pelleted feed before and after weight for reduction (500 g) prepared in sterile plastic bags were transferred to Mycology Department of Faculty of Veterinary Medicine, University of Tehran.

#### Fungal isolation and identification

Samples of corn, soybean meal, and finished feed were mixed by a high-speed mixer. For dilution, a dilution of  $10^{-2}$  was prepared in 9 ml of sterile distilled water into a test tube. Diluted samples were cultured on Sabouraud dextrose agar (SDA, E. Merck, Germany) plates contained 0.005% chloramphenicol (E. Merck, Germany), Dichloran Rose Bengal chloramphenicol agar (DRBC, Thermo Scientific™ Oxoid™, UK) and *Aspergillus flavus* and parasiticus agar (AFPA, Thermo Scientific™ Oxoid™, UK) and incubated at 28 °C for 7-10 days. Cultures were checked visually and each suspected fungal colony was sub-cultured on SDA plates for final isolation of fungal isolates. Fungal isolates were identified using a combination of macroscopic and microscopic morphological features at the genus level according to standard methods (17, 24). *Aspergillus* isolates were identified at species level according to Pitt and Hocking (17). Different colony characters used to identify fungal genera including *Aspergillus* species were colony size, surface, color (surface and reverse), texture and margins. Microscopic morphology of isolated fungi was studied using slide culture technique.

#### Determination of colony forming unit (CFU).

For this purpose, 25 g of each sample (corn, soybean meal and finished feed before and after pelleting) was added to 225 ml peptone water and mixed well for 30 seconds at 260 rpm. Dilutions of  $10^{-1}$  to  $10^{-4}$  were prepared using tubes containing 9 ml peptone water. Amounts of 10 ml of each sample from each dilution were spread on plates containing SDA and incubated for 3 to 5 days at 25 °C. After this period of time, average colony numbers were counted per gram to determine the quality control of poultry feed. Standard limits were  $5 \times 10^4$  and  $1 \times 10^3$  for ingredient and finished feed respectively, according to the rule of Iranian Veterinary Organization (IVO).

**Statistical analysis.** Data were analyzed according to One-way ANOVA using SPSS 16.0 software (Illinois, USA). Means were compared using Duncan's multiple rang test method. Significant was accepted at the level of  $p < 0.05$ .

## RESULTS

Results of the study fungal flora isolated from ingredients and finished feed in feed mills located in Tehran, Alborz and Qom provinces are presented in Table 1. A total of 384 fungal isolates belonging to 7 genera of filamentous fungi and yeasts were isolated from corn (124 isolates), soybean meal (92 isolates), and feed before (72 isolates), and after pelleting (96 isolates). Fungal genera isolated from corn, soybean meal, and before and after pellets were significantly different ( $p < 0.05$ ). The genus *Fusarium* was the most prominent fungal group isolated from corn (45.1%), soybean meal (30.5%) and before pelleting (61.2%), while the genus *Aspergillus* was the predominant fungal group in samples of pellets (29.1%). Totally, the most frequent fungus isolated from ingredients and finished feed was *Fusarium* (36.4%) followed by *Aspergillus* (21.8%), *Penicillium* (11.4%) and yeast (11.4%) (Fig. 1).

The genus *Scopulariopsis* was isolated from before pelleting samples (5.5%) and *Chrysosporium* only from soybean meal samples (4.3%). *Rhizopus* isolated from only the corn (3.2%) and feed before pelleting (11.1%) and yeasts only on soybean meal (21.7%) and post pellets (25%).

The frequency of the possible mycotoxigenic fungi including *Aspergillus*, *Fusarium* and *Penicillium* and non-toxigenic fungi are shown in Fig. 2 ( $p < 0.05$ ). The ratio of toxigenic/non-toxigenic fungi was shown in corn (87.1/12.9%), soybean meal (60.8/39.2%), before pelleting (72.2/27.8%) and after pelleting (54.1/45.9%). No significant difference was recorded between toxigenic and non-toxigenic fungi in ingredients and final pellet ( $p > 0.05$ ).

As shown in Table 2, *A. flavus* was predominant species in corn (46.6%), soybean meal (72.7%) and feed before and after pelleting (75%). As indicated in Fig. 3, totally, the most frequent isolates from ingredients and finished feed was *A. flavus* (64/3 %) and after that *A. niger* (16.6%) was the second prevalent species followed by *A. fumigatus* (10.7%), *A. ochraceus* and *A. glaucus* (each 2.4%). There was a significant difference between the frequency of *A. flavus* species on ingredients and finished feed in poultry ( $p < 0.05$ ).

As shown in Table 3, colony forming unit (CFU/g) range of isolated fungi was determined in corn ( $3 \times 10^2$  to  $1.2 \times 10^5$ ), soybean meal ( $1 \times 10^2$  to  $4 \times 10^3$ ), before pelleting ( $1 \times 10^1$  to  $5 \times 10^4$ ) and after pelleting ( $1 \times 10^1$

to  $1.2 \times 10^3$ ).

Among 22 corn samples fungal CFUs were higher than the standard limits of Iran in 9 samples (40.9%), while in none of 22 samples of soybean meal, contamination was not higher than the standard limit

(Table 3). As indicated in Table 3, there was a significant difference between fungal CFUs in samples of after pellets ( $p < 0.05$ ). Fungal contamination was higher than the standard limits in 14 (63.6%) samples of feed before pelleting (feed as mash form) and 5

**Table 1.** Determination of fungal mycoflora on ingredients and finished feed in poultry feed mills in Tehran, Alborz and Qom provinces, Iran.

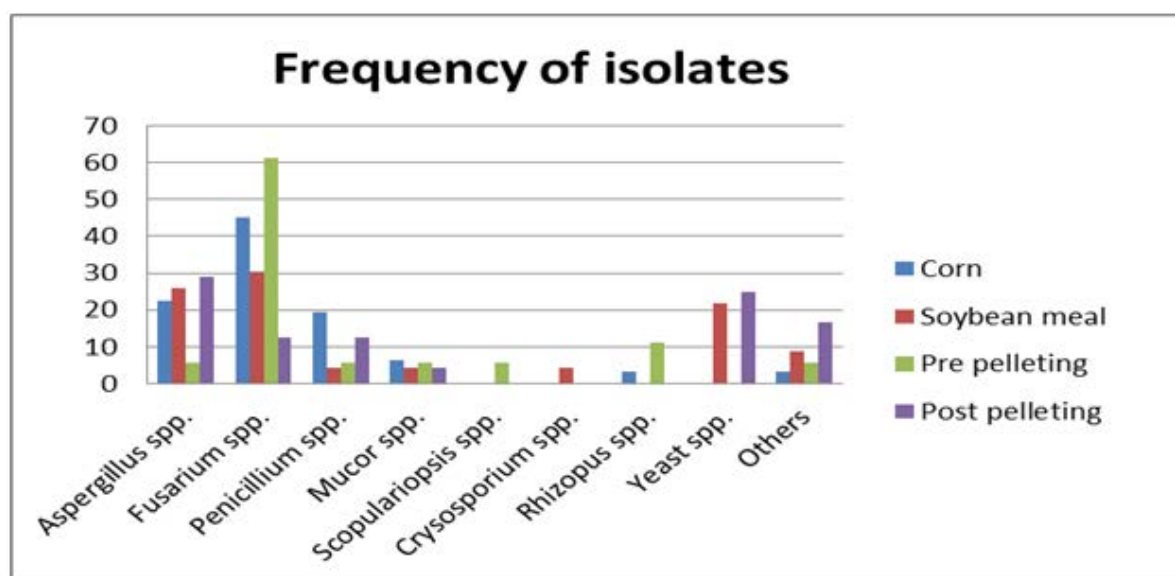
Fungi	Samples	Ingredients				Finished feed				Total	F <sup>c</sup>
		Corn		Soybean meal		Pre-pelleting		Post-pelleting			
		22 <sup>a</sup>	F <sup>b</sup>	22 <sup>a</sup>	F <sup>b</sup>	22 <sup>a</sup>	F <sup>b</sup>	19 <sup>a</sup>	F <sup>b</sup>		
<i>Aspergillus</i> spp.		28	22.5	24	26	4.0	5.5	28	29.1	84	21.8
<i>Fusarium</i> spp.		56	45.1	28	30.5	44	61.2	12	12.5	140	36.4
<i>Penicillium</i> spp.		24	19.3	4.0	4.3	4.0	5.5	12	12.5	44	11.4
<i>Mucor</i> spp.		8.0	6.4	4.0	4.3	4.0	5.5	4	4.1	20	5.2
<i>Scopulariopsis</i> spp.		0	0	0	0	4.0	5.5	0	0	4.0	1.0
<i>Chrysosporium</i> spp.		0	0	4.0	4.3	0	0	0	0	4.0	1.0
<i>Rhizopus</i> spp.		4.0	3.2	0	0	8	11.1	0	0	12	3.1
Yeast spp.		0	0	20	21.7	0	0	24	25	44	11.4
Others <sup>d</sup>		4.0	3.2	8.0	8.7	4.0	5.5	16	16.6	32	8.3
Total		124	100	92	100	72	100	96	100	384	100

<sup>a</sup>Number of samples

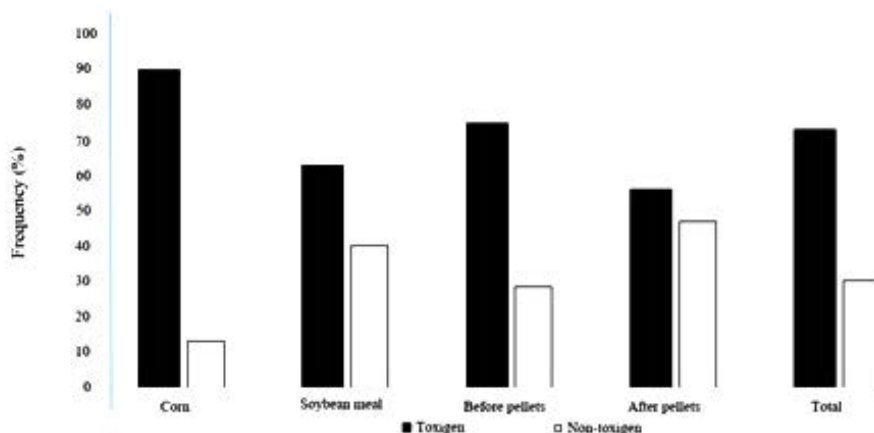
<sup>b</sup>Frequency percentage of each fungus to all fungi isolated from each sample

<sup>c</sup>Frequency percentage of each fungus to all fungal isolates

<sup>d</sup>Including sterile hyphae or unknown fungus



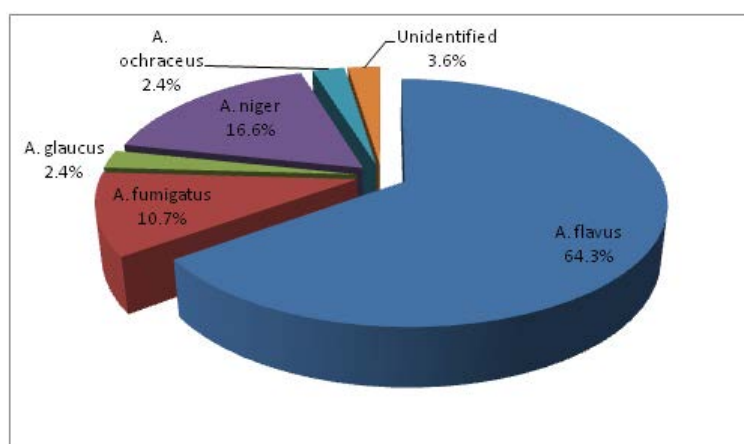
**Fig. 1.** Distribution and frequency of fungi on ingredients and finished feed in poultry feed mills in Tehran, Alborz and Qom provinces, Iran.



**Fig. 2.** Frequency of potentially mycotoxigenic fungi on ingredients and finished feed in poultry feed mills in Tehran, Alborz and Qom provinces, Iran.

**Table 2.** *Aspergillus* species isolated from ingredients and finished feed in poultry feed mills in Tehran, Alborz and Qom provinces, Iran.

Samples	Ingredients		Finished feed				Total		
	No	%	No	%	No	%			
<i>Aspergillus</i>									
<i>A. flavus</i>	14	46.6	16	72.7	3	75	21	75	54
<i>A. fumigatus</i>	7	23.3	2	9.1	0	0	0	0	9
<i>A. glaucus</i>	0	0	2	9.1	0	0	0	0	2
<i>A. niger</i>	5	16.6	1	4.5	1	25	7	25	14
<i>A. ochraceus</i>	2	6.7	0	0	0	0	0	0	2
Unidentified	2	6.7	1	4.5	0	0	0	0	3
Total	30	100	22	100	4	100	28	100	84



**Fig. 3.** The frequency of total *Aspergillus* species on ingredients and finished feed in poultry feed mills in Tehran, Alborz and Qom provinces, Iran.

**Table 3.** Colony forming unit per gram of ingredients and finished feed in poultry feed mills in Tehran, Alborz and Qom provinces, Iran.

Sample	No.	Total CFU	Means of CFU	Minimum of CFU	Maximum of CFU	CFU (higher than Standard limit)		CFU (lower than Standard limit)	
						No.	%	No.	%
Corn <sup>1</sup>	22	332400	1.510×10 <sup>4</sup>	3×10 <sup>2</sup>	1.2×10 <sup>5</sup>	9	40.9	13	59.1
Soybean meal <sup>1</sup>	22	13320	0.060×10 <sup>4</sup>	1×10 <sup>2</sup>	4×10 <sup>3</sup>	0	0	22	100
Pre-pelleting <sup>2</sup>	22	105310	4.786×10 <sup>3</sup>	1×10 <sup>1</sup>	5×10 <sup>4</sup>	14	63.6	8	36.4
Post-pelleting <sup>2</sup>	19	5750	0.302×10 <sup>3</sup>	1×10 <sup>1</sup>	1.2×10 <sup>3</sup>	5	26.3	14	73.7

<sup>1</sup>Limit of standard for ingredients ( $5 \times 10^4$ )

<sup>2</sup>Limit of standard for finished-feed ( $1 \times 10^3$ )

(26.3%) samples of feed in the form of pellets. The mean percentage of fungal CFUs in ingredients and finished feed are shown in Fig.4. There was a significant difference ( $p < 0.05$ ) in fungal CFUs between corn and soybean meal and finished feed.

## DISCUSSION

In the present study, mycoflora and fungal contamination rate of feed ingredients and finish poultry feed were evaluated. The results of various studies in determining the natural mycobiota of ingredients and finish poultry feed have shown that different fungal genera and species could be isolated (18, 21, 25-27). Orsi et al. (28) studied the mycoflora of corn and showed that *Fusarium*, *Aspergillus* and *Penicillium* were the main fungi isolated. These results are in accordance with our results indicating that these fungal genera are abundance in corn, soybean meal as ingredients of poultry feed. We showed some potentially mycotoxigenic fungi were decrease in processing especially *Fusarium* and *Penicillium*, but *Aspergillus* was increasing after steam pelleting in poultry feed. Many researchers determined that *Aspergillus* was the main mycoflora of pellets of poultry feed (5, 15, 19, 20, 28-30). In the present study, the most dominant fungus in the feed before pellet was *Fusarium* followed by *Aspergillus* and *Penicillium* and in pellet feed, *Aspergillus* followed by *Penicillium* and *Fusarium*. We found *Aspergillus* frequency was the most prevalent in mycoflora pellet form in processing in feed mill. The toxigenic fungal contamination of the raw materials occurs during the pre-harvest and/or the postharvest periods, they are exposed during production, process-

ing, transportation, and storage (31). Our result are in accordance with Dalcerio et al. (21) and Accensi et al (20) confirmed *Aspergillus flavus* over than in poultry feed and *A. niger* was the second prevalent species followed by *A. fumigatus*. *A. flavus* predominates in all kinds of poultry feed in the tropical climate (17, 32-34). The thermo-resistant of *A. flavus* causes of abundantly in poultry feed and the presence in pellet feed may be an indicative of their predominance potentially to produce of aflatoxins. Azarakhsh et al. (35) showed that *A. flavus* was the most prevalent species followed by *A. niger* and *A. fumigatus* in the broiler feeds in Kermanshah province in West of Iran. This difference may be due to the fact that processing stage (temperature, time, etc.) at pelletizer significantly affects the viability of fungal conidia and variable degree of resistance of fungal conidia of various fungi because conditions determines the predominance of mycobiota in feed poultry. According to quality control of samples and standard limits of feed ingredients ( $5 \times 10^4$ ) and the finished feed ( $1 \times 10^3$ ) in Iran, hazardous contamination higher than standard limits were observed in poultry feed examined in the present study. This contamination may arise from the lack of proper drying of corn in farm operations, transportation and inappropriate storage of silage. Since Iran is a major importer of corn from other countries, the risks of fungal infection and toxin formation must be considered. The low level of fungal CFUs on soybean meal may be account for sounds conditions at harvest, heat treatment, addition of antifungal compounds in soybean meal and providing good storage conditions. In pellets, fungal CFU was relatively low because processing operations in the process pellet which can greatly reduce the chance of contamination. In Iran only a few studies have been

conducted on the CFUs contamination of poultry feed commodities and finished feed. Mayahi et al. (36) reported that contaminations of all samples of fish meal, soybean meal, wheat and maize were lower than standards ( $5 \times 10^5$ ) level at that time. Astoreca et al. (26) determined the CFU/g in poultry feed ingredients and showed that corn was the most contaminated feed ingredient ( $4 \times 10^4$  to  $8 \times 10^4$  CFUs/g), while soybean had the least contamination. Also grounded feed (before pelleting) was contaminated higher than pellet poultry feed which is in agreement with the results of our study. These authors showed low level of fungal contamination in raw materials for poultry feed that was good quality indicator of consumption of such materials for humans or animals. Dalcero et al. (21) reported that feed preparing in the form of pellets or crumbles at a high temperature in the poultry feed factories significantly affected the growth of fungal colonies showed that poultry feed pelleting process significantly reduced the fungal counts in the final product which is in agreement with our results.

## CONCLUSION

Our results indicated that corn, soybean meal and finished poultry feed were contaminated by various degrees with different fungal genera especially potentially mycotoxigenic fungi belonging to *Aspergillus*, *Fusarium* and *Penicillium*. According to widely distributed *Aspergillus* spores in the environment, the risk of feeds pellet contamination by this fungus is very high as evidenced in the present study. We showed that some feed ingredients and finished feeds were contaminated with hazardous amounts of fungi higher than the standard limits. These results indicate the need for continuous assessment of the mycological status of finished feed production. The present study showed that untreated such ingredients feed are important vehicles into contaminant poultry feed in Iran. It is therefore, advised to routinely treat ingredients and finished feed with fungal growth inhibitors and necessary of authorizing the code of Hazard Analysis and Critical Control Points (HACCP) for production in poultry factories.

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