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Natural Incidence of *Aspergillus* Mycoflora and Mycotoxins in Fresh and Ensiled Maize Fodder

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Authors' Contributions
NQH conceived and designed the study. NS analyzed mycoflora and mycotoxins, and wrote the article. IT statistically analyzed the data. SI supervised the study.

Key words
Ensiling, Maize fodder, Fungi, Total aflatoxins, Ochratoxin A.

ABSTRACT

The present study evaluated *Aspergillus (A)* spp. and mycotoxins in fresh and ensiled maize fodder using a model fermentation system and raw materials from different areas of Punjab, Pakistan. A. *niger, A. flavus* and *A. fumigatus* were the most dominant followed by *A. terrus* and *A. ochraceous* in fresh and ensiled maize fodder. Total fungal counts ranged from 1×10^3 to 4×10^3cfu/ml for all species in both fresh and ensiled maize fodder. Total aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) and ochratoxin A (OTA) analysis showed that $AFB_1$was present with high frequency in fresh (37.5%) and ensiled (41.66%) fodder, with an average concentration of 9.49 and 8.36ng/g respectively. Aflatoxin B_2 was detected in only two samples (16%) (1.2 and 1.3ng/g), aflatoxin G_1 and G_2 were not found. Ochratoxin A was found more frequently in fresh (54.16%) than in ensiled (20.86 %) samples with a mean of 8.06 and 4ng/g respectively. Average values for total aflatoxins (TAFs) and OTA were below the permissible limits as per regulation of European commission (EU), *i.e.* 20ng/g and 10ng/g, respectively.

INTRODUCTION

Maize is most widely grown crop in Pakistan, Major consumption areas are direct in human food, wet milling industry and livestock feed (GoP, 2014-2015). Fresh maize plant fodder is also being used to preserve as silage worldwide. Maize silage has a balance between energy density of grain, fiber and digestibly of green plant that is ideal for ruminant feeding and utilized as feed source over a long time (Cavena *et al*., 2009).

Ensiling of maize fodder entails incorporation of whole plant and its storage is based on principle of preservation. In anaerobic conditions with help of lactic acid bacteria, sugars present in green fodder is converted into lactic acid, which lowers pH to a level at which clostridia and most fungal growth are inhibited (Sparo and Mallo, 2001). However, environmental factors such as insufficient drying, moisture content, heat, insects and other conditions may cause aerobic spoilage (Dos Santos *et al*., 2003). Improper compression and covering, early opening, entry of uncontrolled rodents and insects etc. may result in exposure to oxygen that may affect the quality of silage and promote the growth of mycoflora. As maize silage consists of chopped whole plant, it includes not just grain but high percentage of stalks and stover which is more susceptible for fungal attack. The dominant genera reported in maize silage include *Aspergillus*, *Pencillium*, *Fusarium*, *Mucor*, *Absidia, Monascus, Scopulriopsis* and *Trichoderma* (Velazquez *et al*., 2008). During ensiling, most fungi can be eliminated. However, there are other species such as *Aspergillus fumigatus*, *Penicillium roqueforti*, *P. paneum*, *F. oxysporum* and *Monascus ruber* that are able to tolerate both high levels of organic acids and carbon dioxide in addition to low availability of oxygen (Gallo *et al*., 2015). Fungal spoilage in silage can not only reduced palatability of food but also leads to production of toxic secondary substances like mycotoxins which pose a serious health hazard for dairy animals (Velazquez *et al*., 2008).

Mycotoxins producing fungi invade crops in field and may grow during storage under favorable environmental conditions. The detection of fungi does not necessarily entail the presence of mycotoxins, since mycotoxins production depends on various factors such as presence of toxigenic fungi, chemical composition of the substrates, moisture content, temperature, relative humidity and time course of fungal growth (Roige *et al*., 2009). Occurrence of mycotoxins in nature is a global problem; however geographic distribution of mycotoxins has diverse variation with respect to their occurrence (Lawlor and Lynch, 2005). Mycotoxins are mainly produced by *Aspergillus,*

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Fusarium and *Penicillium* genera. Major mycotoxins are trichothecenes, fumonisins, ochratoxin A, aflatoxins and zearalenone etc. Among these, *Aspergillus* acquires a significant attention due to their wide occurrence and climatic condition of Pakistan favors their production. Aflatoxins and ochratoxin A are produced by *Aspergillus* species. They are carcinogenic, teratogenic, nephrotoxic, mutagenic and immuno-suppressive in nature (Akande *et al*., 2006) and exert lethal health hazards in human and animals (CAST, 2003).

In Pakistan, silage making is in practice for a few decades to achieve higher production parameters and to provide a uniform diet to animals. Pakistan is situated in tropical part of world and climatic conditions are conducive for fungal contamination and mycotoxins production. Furthermore, temperate and tropical climatic conditions of Pakistan plus inadequate feed storage practices provide ideal conditions for *Aspergillus* growth, reduction of nutritional values and mycotoxins production. Silage can be contaminated with more than one fungi and mycotoxins at same time. However, in present study emphasis was given on *Aspergillus* and their toxins due to the fact that it metabolizes and carry over into milk in form of aflatoxin M_1. However, on the other hand, anaerobic environment, adequate substrate and sufficient amount of lactic acid bacteria are required for good quality silage. Epiphytic population of lactic acid bacteria are in low amount that is insufficient for fermentation process. In order to increase population of lactic acid bacteria, microbial inoculant was added before ensiling to enhance the process of fermentation (Bayatkouhsar *et al*., 2012). A rapid decline of the pH is very important during the start of the fermentation process, because it inhibits growth of toxigenic fungi. In view of this comprehensive background, the present study was planned to assess the effect of inoculant application in fresh maize plants collected from various areas of Punjab and ensiled over a period of 60 days. The parameters under study were frequency and colony numbers of *Aspergillus* spp., species differentiation of *Aspergillus*, aflatoxins and ochratoxin A contents in fresh and ensiled maize fodder.

MATERIALS AND METHODS

Samples collection

A total of twenty-four fresh maize fodder samples (5kg each) were collected from main maize producing areas (Chakwal, Rawalpindi, Sargodha, Sahiwal, Rahimyar Khan and Multan) of the province of Punjab. Samples were divided into two portions. Half of the portion of green fodder was wrapped for process of ensiling under anaerobic conditions. Homofermentative microbial inoculants (BIOSTABIL WRAPS) that is a mixture of different homofermentative strains, *Enterococcus faecium* BIO 34 (DSM 3530) and *Lactobacillus plantarum* IFA 96 (DSM19457) was applied before ensiling at a level of 4mg/kg. Population density of bacteria in one gram of inoculant was 1×10^6cfu/g. For ensiling purpose, samples were first packed in airtight envelops and kept in air tight buckets in order to achieve anaerobic conditions. Mini silos were then opened after the 60thday of study. Samples of fresh (n=24) and ensiled (n=24) maize fodders were preserved at -4ºC until *Aspergillus* spp., total aflatoxins (TAFs) and ochratoxin A (OTA) analysis. In addition, the pH of fresh and ensiled samples was also measured before start of analysis.

Mycological analysis

Media preparation

Aspergillus spp. was isolated by using oxytetracyline glucose agar media (OGA).For media preparation, 1.25g of yeast extract, 5g of glucose and 5g of agar were dissolved in 250ml of water and autoclaved for 20 min. Antibiotic like Terramycin capsules (250mg) was added into a little hot (~40-50°C) autoclaved media and slightly shakes the flask in order to inhibit bubble formation. Then the media was poured on autoclaved petriplates and kept at 30ºC for 24 h (Mossel *et al*., 1962).

Sample preparation

For each sample, 5g of sample was homogenized with 45ml of trypticase salt solution and shake for 15 min. After shaking, 10 fold dilutions were made and 1ml of each dilution sprayed in a Petri dish (90 mm diameter) under laminar flow hood containing OGA media. The tentative identity of isolates was determined by macro- and microscopic examination (Mansfield and Kuldau, 2007; Raper and Fennell, 1965). After identification of fungi, cultures were preserved on potato dextrose media (PDA). Colony forming unit was determined by using following formula:

$$\text{Colony Forming unit } \left(\frac{\text{cfu}}{\text{ml}}\right) = \frac{\text{No. of colonies} \times \text{dilution factor}}{\text{Volume of sample inoculated (ml)}}$$

Mycotoxins analysis

For analysis of total aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) and ochratoxin A (OTA) standard methods using Immunoaffinity chromatographic sample extract cleanup and HPLC with fluorescence detection, following international official standards were used as described by Sultana *et al*. (2013) and Khalil *et al*. (2013).

Sample preparation

Fresh and ensiled maize fodder samples (25g) were homogenized with 100ml of methanol: water (80:20; v/v) and placed in an orbital shaker for 1 h. Mixture was

filtered through a Whattman No.1 filter paper. Then, 8ml of Phosphate buffer saline (pH =7.4) was added to 4ml of sample extract. The pH was adjusted to 7.0 for TAFs and 7.4 for OTA with 0.1mol/HCl or 0.1 mol/NaOH. The aliquot was then loaded on a AflaStar®Immunoaffinity column or OchraStar®Immunoaffinity column (Romer Labs, Austria). The flow rate was adjusted at 1ml/min. The column was then washed with 20ml distilled water. Aflatoxins and OTA were eluted with 3ml of methanol at flow rate of 0.5ml/min. Purified extracts were transferred into capped glass vials and evaporated by using an Evap system (Romer Labs, Inc. MO, USA) under vacuum at 60°C. Mycotoxins residue were then re-dissolved in Toluene: Acetonitrile (95:5 v/v; TAF) and toluene: acetic acid (99:1 v/v; OTA), respectively.

Analysis by high performance liquid chromatography (HPLC)

After re-dissolving, TAFs and OTA were analyzed using a Shizmadu LC20 AT system equipped with UV detector and photochemical post column derivatization cell. Flow rate of isocratic mobile phases for both toxins *i.e*. TAFs {Acetonitrile: methanol: water (20:20:60; v/v/v)} and OTA {acetonitrile/water/acetic acid (51:47:2, v/v/v)} was 0.8 ml/min. Temperature of column was maintained at 40ºC. Total aflatoxins and OTA were detected at wavelengths of 365nm and 333nm with injection volume of 20 µl, respectively.

RESULTS AND DISCUSSION

The present study was conducted to evaluate the growth and mycotoxins production of different *Aspergillus* spp. in a model system consisting of fresh (n=24) and ensiled maize fodder (n=24). The raw materials for this study were collected from the main maize growing areas (Chakwal, Rawalpindi, Sargodha, Sahiwal, Multan and Rahimyar khan) of Punjab, Pakistan. Moreover, inoculant was applied before ensiling and a drop in pH (4.0) was observed in ensiled samples at the third day of the experimental fermentation. The prime importance of inoculant is to support epiphytic bacteria with additional population for quick acidification process and instant decline in pH for nutrient preservation (Bayatkouhsar *et al*., 2012). Present finding revealed quick decline of pH values, which is an indicator of good quality silage having a pH value of 4.2 or lowers. For pH maintenance, various factors are being involved including water soluble carbohydrate of fresh fodder, buffering capacity, dry matter content and types of epiphytic bacteria on fresh fodder (Kadivar and Stapleton, 2003).

Results of present findings further demonstrated that *A. niger* was the most dominant specie followed by *A. fumigatus, A. flavus*, *A. terrus* and *A. ochraceous* in fresh and ensiled maize fodder, respectively (Table I). Results were further evaluated for total fungal colonies (cfu/ml). Highest fungal count was observed for *A. terrus (*5×10^3cfu/ml) and *A. niger (*2.7×10^3) in ensiled and fresh maize fodder respectively. Among areas high mean fungal densities were observed for *A. flavus* in Rawalpindi (4×10^3cfu/ml) and *A. terrus (*4.25×10^3cfu/ml) in Rahimyarkhan (Table II).

Results of recent study indicated that samples of fresh as well as ensiled maize fodder were found positive for *Aspergillus* spp. There is a presumption that ensiled maize fodder is less affected by spoilage microorganism due to low pH and acidic conditions. Fungi can grow under wide-ranged environmental conditions like 10-40ºC, 4-8 of pH and a water activity of 0.7. High temperature and low water activity are required for growth of *Aspergillus* spp. *Aspergillus* spp. usually found dominant in warm environment (Reyneri, 2006). Pereyra *et al*. (2008) also reported that *Aspergillus* spp. can survive under microaerophiilic conditions, which were well adapted in acidic environment.

Table I.- Incidence (%) of *Aspergillus* spp. in fresh and ensiled maize fodder from various areas of Punjab.

Locations (sub-areas)	*Aspergillus niger* (%)		*Aspergillus fumigatus* (%)		*Aspergillus flavus* (%)		*Aspergillus terreus* (%)		*Aspergillus ochraceous* (%)	
	Fresh	Ensiled	Fresh	Ensiled	Fresh	Ensiled	Fresh	Ensiled	Fresh	Ensiled
Chakwal	2.5×10^3	1.5×10^3	3×10^3	1.5×10^3	3.5×10^3	1×10^3	1.5×10^3	1×10^3	2.5×10^3	2.5×10^3
Rawalpindi	1×10^3	1.5×10^3	3.3×10^3	2×10^3	4×10^3	1.5×10^3	ND	1×10^3	1×10^3	1.5×10^3
Sargodha	1×10^3	1×10^3	4×10^3	4×10^3	1.5×10^3	2×10^3	4×10^3	1.5×10^3	2×10^3	3×10^3
Sahiwal	3×10^3	1×10^3	2×10^3	1×10^3	1×10^3	1.5×10^3	1×10^3	1×10^3	3×10^3	1.5×10^3
Rahimyar Khan	1.5×10^3	21×10^3	2×10^3	2×10^3	2×10^3	1.5×10^3	1×10^3	ND	2×10^3	3.5×10^3
Multan	ND	1×10^3	2×10^3	1.5×10^3	2×10^3	1×10^3	ND	2×10^3	5×10^3	1.5×10^3
Mean	1.8×10^3	5×10^3	2.7×10^3	2×10^3	2×10^3	1.5×10^3	2×10^3	1.5×10^3	2.5×10^3	2×10^3

ND, not detected.

Table II.- Total fungal density (cfu/ml) of *Aspergillus* spp. In fresh and ensiled maize fodder from various areas of Punjab.

Sampling Areas	*Aspergillus terreus* (cfu/ml)		*Aspergillus niger* (cfu/ml)		*Aspergillus flavus* (cfu/ml)		*Aspergillus ochraceous* (cfu/ml)		*Aspergillus fumigatus* (cfu/ml)	
	Fresh	Ensiled	Fresh	Ensiled	Fresh	Ensiled	Fresh	Ensiled	Fresh	Ensiled
Chakwal	2.5×10^3	1.5×10^3	3×10^3	1.5×10^3	3.5×10^3	1×10^3	1.5×10^3	1×10^3	2.5×10^3	2.5×10^3
Rawalpindi	1×10^3	1.5×10^3	3.3×10^3	2×10^3	4×10^3	1.5×10^3	ND	1×10^3	1×10^3	1.5×10^3
Sargodha	1×10^3	1×10^3	4×10^3	4×10^3	1.5×10^3	2×10^3	4×10^3	1.5×10^3	2×10^3	3×10^3
Sahiwal	3×10^3	1×10^3	2×10^3	1×10^3	1×10^3	1.5×10^3	1×10^3	1×10^3	3×10^3	1.5×10^3
Rahimyar Khan	1.5×10^3	21×10^3	2×10^3	2×10^3	2×10^3	1.5×10^3	1×10^3	ND	2×10^3	3.5×10^3
Multan	ND	1×10^3	2×10^3	1.5×10^3	2×10^3	1×10^3	ND	2×10^3	5×10^3	1.5×10^3
Mean	1.8×10^3	5×10^3	2.7×10^3	2×10^3	2×10^3	1.5×10^3	2×10^3	1.5×10^3	2.5×10^3	2×10^3

ND, not detected.

Table III.- Natural prevalence of total aflatoxins and ochratoxin A in fresh and ensiled maize fodder from various areas of Punjab.

	Area	Aflatoxin B_1			Aflatoxin B_2			Ochratoxin A		
		Mean (ng/g)	Positive (%)	Range (ng/g)	Mean (ng/g)	Positive (%)	Range (ng/g)	Mean (ng/g)	Positive (%)	Range (ng/g)
Fresh fodder	Chakwal	2.95	25.0	<0.1-11.5	0	0	0	6.54	75.0	<0.1-11.28
	Rawalpindi	1.33	25.0	<0.1-5.29	0	0	0	4.78	75.0	<0.1-7.17
	Sargodha	4.37	100.0	1.54-13.4	0	0	0	6.15	25.0	<0.1-24.6
	Sahiwal	7.85	50.0	0.1-28.2	0	0	0	-	25.0	<0.1-0.41
	Rahimyar Khan	<0.1	-	-	1.2	49.00	<0.5-3	6.74	100.0	<0.1-8.61
	Multan	5.43	25.0	0.1-21.44	1.2	47.00	<0.5-4	--	25.0	<0.1-4.1
Cumulative contamination		**9.49**	**37.5**	**0.1-28.2**	**1.2**	**16.06**	**0.5-4**	**8.06**	**54.16**	**<0.1-24.6**
Ensiled fodder	Chakwal	2.57	25.00	<0.1-10	<0.5	0	0	<0.1	0	<0.1
	Rawalpindi	6	25.00	<0.1-12	0	0	0	3.77	50.00	<0.1-4.92
	Sargodha	2.43	50.00	0.1-7	0	0	0	7.79	50.00	<0.1-8.2
	Sahiwal	6.59	75.00	1.2-24	0	0	0	<0.1	-	-
	Rahimyar Khan	5.58	50.00	<0.1-20	1.5	450	<0.5-4	3.95	25.00	<0.1-15.5
	Multan	1.3	25.00	<0.1-5	1.12	400	<0.5-3	-	-	-
Cumulative contamination		**8.36**	**41.66**	**<0.1-24**	**1.31**	**16.06**	**<0.5-4**	**4.00**	**20.86**	**<0.1-15.5**

Total fungal densities in all samples of fresh and ensiled maize fodder ranging from $1.5x10^3$-$5x10^3$ cfu/ml that was in agreeble limit as per recommendation of quality standard of 1×10^4 cfu/ml defined by Good Managemental Practices (GMP, 2005).

Prevalence was considerabaly high but fungal biomass was far below than agreeble limits of good managemental practices. Fungi can grow in field, during sowing and harvesting but proper preventive measures like proper compression and exclusion of air may reduce their fungal biomass and inhibit growth due to acidic conditions. Another possible explanation was that in ensiled fodder, homofermentative bacterial inoculant was applied that consisting of *Lactobacillus plantrum*. Several studies have shown that some strains of *Lactobacillus plantrum* have antifungal activity and considered as natural biological antagonists. They produce phenyllactic acid, which may inhibit growth of *Aspergillus niger* and *Aspergillus flavus* (Dalie *et al*., 2010).

Concerning mycotoxin contamination, nine samples of fresh maize (37.5%) contained AFB_1,with an average of 9.49ng/g (range of <0.1-28.2ng/g). This was quite

similar with the frequency (41.66%) found in the ensiled batches of maize fodder, which contained AFB_1 at a mean level of 8.36ng/g (0.1-24ng/g). AFB_2 was detected in only two samples, each one of fresh and ensiled maize fodder (1.2 and 1.3ng/g). AFG_1 and AFG_2 was not found in any sample. Ochratoxin A contamination was found in 13 (54.16%) samples of fresh maize fodder with a mean of 8.03ng/g (0.1-24.16ng/g). In the ensiled batches, 20.86% were positive for OTA, with an average of 3.98ng/g (0.1-15.50ng/g) (Table III). Mean levels for AFB_1 and OTA were below the Europeon Union regulatory limits of 20ng/g (aflatoxins) and 10ng/g (OTA) (Europeon Commission, 2005). However, some samples slightly exceeded these maximum values.Overall the values found for aflatoxins and for OTA were not alarmingly high, although the frequency of toxigenic *Aspergillus* species was quite high in these maize samples, both as fresh produce and after experimental model ensilage.

In the present study, samples collection were carried out during month of july. Maize is usually grown during hot weather and harvested in humid summer in Punjab. These conditions promote attack of fungi with maximum production of TAFs and OTA. Climate in most areas of Punjab remains hot, where high temperature (30 to 48 °C) and relative humidity ranging from 25% to 50%, prevail in summer (Chaudhary *et al*., 2009). Environmental conditions of Punjab with its warm temperature are favorable for proliferation of toxigenic fungi (Saleemullah *et al*., 2006; Iqbal *et al*., 2011). Stress factors such as shortage of water, insect infestation, and other pests attack can also enhance mycotoxins production (Sanchis and Magan, 2004; Milani, 2013). explained the stabilty of TAFs in maize silage and showed that acidic conditions can not completely inhibit toxins production. Moreover, a study conducted by Pereyra *et al*. (2008) shows that occurrence of aflatoxins was higher in silage than to fresh fodder that was similar to present findings in which prevalence was higher in silage (41.66%) than fresh fodder (37.5 %).

Total aflatoxins and OTA were prevalent in warm and humid environment and gain much importance due to their carcinogenic, teratogenic, immunosuppresive, nephrotoxic and mutagenic effects. Total aflatoxins metabolized in liver and excreted in bile. It increases the apparent protein requirement of cattle and pose as potent cancer causing agent (Chohan *et al*., 2016). It has been classified as class 1A carcinogen by international agency of research on cancer (IARC, 2002). Total aflatoxin also have carryover effect in milk in form of AFM_1 that have adverse effects on animal as well as on human (CAST, 2003). Ochratoxin A, a well known nephrotoxin (class 2B carcinogen) has been associated with fatal human kidney disease referred to as balkan endemic nephropathy. It aslo increased the incidence of tumors of upper urinary tract in human as well as in animals (JECFA, 2001).

Present study has provided baseline in identifying problem of mycoflora and mycotoxins contamination in fresh and ensiled maize fodder that affects quality of silage. In maize silage, grains basically become the source of aflatoxins contamination in addition to compound feed. Although the levels noticed in maize silage are not significantly higher but contribution from feed may exert negative impact on animal's health. Sometimes mycotoxins in combination may exert synergistic, additive and anatagonistic effects on animal. Proper silage making and use of inoculant (homofermentative type) maintains stability of silage and prevent it from spoilage. Mycotoxins contamination of silage seems to be unavoidable to a certain degree during processing and storage of silage. Moreover, to prevent spoilage of silage, care must be taken during feed out phase as there is chance of oxygen penetration that may lead to silage spoilage. Furthermore, good managemental practices during silage making plays an important role in reduction of fungal count and mycotoxins contamination.

Statement of conflict of interest

The primary data is available with us and may be provided when required. The authors declare that there is no conflict of interest. We are giving copyright reserved to Journal.

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