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OCHRATOXIN CONTAMINATION OF CORN AND APPLIED DETOXIFICATION APPROACHES: A CASE STUDY FROM PUNJAB PAKISTAN

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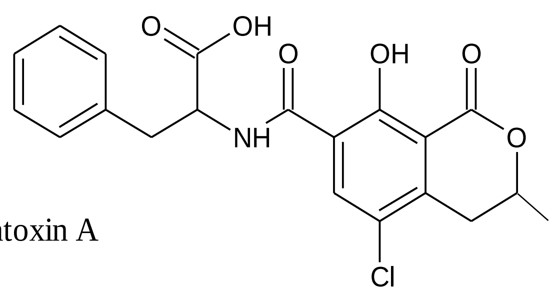
Ochratoxin (OTA) is a potent carcinogen which may pose a tremendous threat to human health when found beyond permissible levels. This study aims at evaluating ochratoxin contamination in corn. The corn samples (n=80) were collected from 4 major districts (Bahawalpur, Faisalabad, Lahore and Rawalpindi) of Punjab Pakistan. The quantification of OTA in collected corn samples was carried out by High Performance Liquid Chromatographic technique. Different methods (physical, chemical and natural/biological) were used for the detoxification of contaminated corn. It was found that 59% of total 80 samples were contaminated with ochratoxin. Among contaminated samples, 26% samples were beyond permissible levels i.e. 5µg/kg OTA in corn samples as set by European Commission Regulation while 33% samples were found contaminated within permissible levels. However, 41% samples were not contaminated with OTA. Highest level 231.49 ± 29.39µg/kg of OTA was found in corn sample of Faisalabad. The corn samples collected during summer season were found more contaminated than winter season. OTA contamination in corn seems to be a very serious issue in Punjab, Pakistan. Different environment-friendly, easy and cheap detoxification approaches were adopted to decontaminate OTA contaminated corn. Maximum detoxification of OTA in corn was found 48.17% by cooking, 64.25% by 0.5% hydrochloric acid and 52.38% by probiotic among physical, chemical and biological methods respectively. Proper handling, suitable storage conditions and proper management may be helpful to avoid ochratoxin contamination in corn and corn products.

Keywords: Ochratoxin, Fungi, Aspergillus, Penicillium, Corn, Contamination, Detoxification, HPLC, Punjab, Pakistan.

INTRODUCTION

Ochratoxin is harmful metabolite formed by numerous fungal species like *Aspergillus* and *Penicillium* (Balendres *et al.,* 2019). Ochratoxins are classified as Ochratoxin A, B and C on the basis of minor chemical structural differences. Ochratoxin A was found to be the more carcinogenic than other types of ochratoxins (Zebiri *et al.,* 2019). The structure of OTA-A is given in Figure 1 (Chen *et al.,* 2018).



Ochratoxin A

Figure 1. Structure of Ochratoxin A.

OTA may cause different diseases like nephrotoxicity, mutagenicity, teratogenecity and immunosuppression in humans (Jarmila *et al.,* 2013). Ochratoxin affects kidney as its target and also triggers nephropathy (Marin-Kuan *et al.,* 2011). A study also reported to induce skin tumor and DNA damage (Kumar *et al.,* 2012). International Agency of Research on Cancer (IARC) declared Ochratoxin as the most probable human carcinogen which was placed in 1993 in group 2B because a huge amount of substantiation of its carcinogenity was revealed in numerous animal studies.

The tolerable intake of 100ng/kg body weight on weekly basis was set by "Joint FAO/WHO Expert Committee on Food Additives" (Benford *et al.,* 2001). Available data is very limited on the nutritional exposure of diverse communities to different mycotoxins. According to JECFA reports, the available records on mycotoxin contamination are insufficient for under developed countries (JECFA, 1999, 2001, 2002, 2007, 2010). The European Union set the Regulatory limits for OTA ranging from 2-10µg/kg in various commodities like cereals and derived products. European Commission

Regulation (EC, 2005) has set the permissible limit of Ochratoxin A in corn samples i.e. 5µg/kg.

Ochratoxin is mostly present in moderate or continental climates (Covarelli *et al.*, 2012; Quintela *et al.*, 2013; Toffa *et al.*, 2013; Zhang *et al.*, 2018). Many factors like storage, environmental conditions and fugal species are responsible for ochratoxin contamination in cops. Ochratoxins are very toxic and thermally stable substances (Malir *et al.*, 2016).

The presence of Ochratoxin was reported for the first time in European and North American Countries especially in wheat and barley samples (Krska *et al.*, 2007). Different grains and their derivative products and mainly beer are much more susceptible to this OTA (Zahra *et al.*, 2016). This fungal specie is present in different food and food commodities like cereal based products, beer, dried fruits (figs, apricot, peanut and pine nuts) and spices. OTA occurrence in cereals (Khoshnamvand *et al.*, 2019) may deteriorate its nutritional aspects due to its toxicity and may affect human health (Wan *et al.*, 2020).

In Pakistan, 4th largest cultivated crop is corn after cotton, rice and wheat (Sabahat *et al.*, 2010; Chauhdary *et al.*, 2019). The area of corn crop cultivation is more than 1.0 million hectares while its production is about 3.5 million metric tons. Among 30% of total production, Punjab contributes 39%, NWFP contributes 56%, Sindh and Baluchistan contributes almost 3% of total area (Rahim *et al.*, 2018). The huge economic losses may occur due to presence of ochratoxin in different food entities. Mycotoxins presence in foods is inescapable and its contamination is inclined by different environmental factors. The extent of ochratoxin contamination is erratic and may vary with geographic site, different agricultural practices the susceptibility of food entities to fungal attack during preharvest and postharvest storage periods. Corn, rice and wheat are at high risk of ochratoxin contamination. Physical, Chemical and natural/biological detoxification techniques may be used to detoxify diverse strains of ochratoxins (Fuchs *et. al.*, 2008).

Ochratoxins have drawn the attention of scientific community in recent years and hence the awareness about various mycotoxins among the common people is increased. The main objective of current study is to evaluate the contamination levels of ochratoxins in corn of major districts of Punjab, Pakistan and detoxification of the contaminated corn samples by various cheap, safe and easy methods.

MATERIAL AND METHODS

This study was conducted by the collaboration of College of Earth and Environmental Sciences, University of the Punjab, Lahore, FBRC, PCSIR Laboratories Complex, Lahore and Kinnaird College, Lahore in April 2018 to March 2019.

***Collection of Samples*:** The corn samples were collected from various districts of Punjab and brought to laboratory for quantitative determination. Corn samples were collected from different local shops on the basis of their physical appearance, adulteration and storage conditions. The temperature range during sample collection was between 25-40ºC and 13-22ºC alongwith humidity of 45-69% and 40-55% in summer (April-September) and winter (October-March) seasons, respectively. The suitable plan of sampling (Trucksess, 2005) was adopted and sampling procedure of AOAC no. 977.16 was followed. One kg corn sample was collected by seed probe from 2-3 different places of corn container diagonally. The collected corn seed samples were then passed through sample divider and obtained 200g of corn after carefully homogenizing the sample. Each corn seed sample was again mixed thoroughly and grinded to fine powder in a grinding and sub-sampling mill (Romer Labs) to a particle size of <0.4mm before analysis (Nisa *et al.*, 2013). The grinded corn samples were kept in polyethylene bag at -20 ºC until used.

Four major districts (Bahawalpur, Faisalabad, Lahore and Rawalpindi) of Punjab were selected for the collection of samples (Figure 2).

***Chemicals*:** All the chemicals used during analyses were of analytical or HPLC purity grade. Acetonitrile used for extraction and for mobile phase was of Merck (Darmstadt, Germany).

***Standard Solution Preparation*:** Stock solution of Ochratoxin (25 ppb) was obtained from Neogen Corporation, North America and diluted in acetonitrile. Standard was stored at −20°C in freezer. The stock solution was used for the preparation of working standard solution by dilution with 20:80 v/v acetonitrile/water (Irakli *et al.*, 2017).

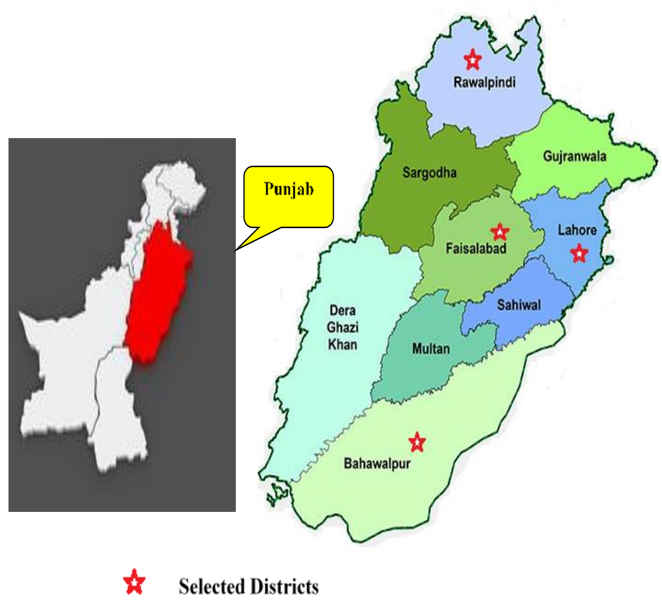


Figure 2. Map Showing Districts of Punjab.

***Instrumentation*:** Agilent 1200 system (Agilent Technologies, Urdorf, Switzerland) was used for the reversed phase-HPLC outfitted with a 20 µL loop with Rheodyne injector valve and quaternary pump (Irakli *et al.*, 2017). The HPLC was connected with DAD (Diode array detector) and FLD (Fluorescent detector) in series. The recording and valuation of HPLC chromatograms was done with Agilent Chemstation software (Agilent Technologies; version B.04.01).

***Procedure*:** The corn sample (5g) was taken in conical flask and 50 mL of 70% acetonitrile was added (Firdous *et al.*, 2012). The sample solution was shaken on wrist action shaker for about 30 minutes. After extraction of ochratoxin the solution was filtered by filter paper. The collected extracted was gain filtered by using Whatman 4 filter paper. The extracts were filtered with syringe filters of 0.5 microns before injecting sample extracts in HPLC C18 column. Twenty microlitres extracted sample solution was injected into HPLC assembled system. The mobile phase in the study was prepared from acetonitrile and water (45:55 v/v). The permissible level for OTA in corn was set by European Commission, 2005 i.e. 5µg/kg.

***Detoxification of OTA contaminated samples*:** Detoxification of ochratoxin was carried out on naturally contaminated samples of corn. Highly contaminated sample of corn was selected for detoxification purpose. Physical (washing, washing with hot water, cooking), Chemical (20% citric acid, 10% acetic acid, 5% sodium bicarbonate, 0.5% hydrochloric acid) and natural/biological (ginger, garlic, black seed oil, probiotics) methods were used for the detoxification of contaminated corn samples (Hussain and Ali, 2012; Nisa *et al.,* 2013; Vijayanandraj *et al.*, 2014; Aiko *et al.,* 2016; Majeed, 2018; Chlebicz, A. and Śliżewska, K., 2020). Fifty grams of contaminated samples were treated by useful ingredients/methods to detoxify positive samples. The reduction in ochratoxin contamination was then verified by HPLC analysis. Detoxification approaches given in this study are easily applicable, cheap and environment and human friendly.

***Statistical analysis*:** The quantities of OTA in corn samples were statistically investigated using SPSS software (IBM, SPSS Statistics 20, USA). Mean and standard deviation was calculated and all results are given in mean± standard deviation. Two way Analysis of variance (ANOVA) was used for comparing groups by applying least significant difference test (α=0.05). The results at $P<0.05$ were considered to be statistically significant (Steel *et al*., 1997).

RESULTS AND DISCUSSION

In current study, presence of ochratoxin in corn samples, collected from different districts of Punjab province was analyzed by HPLC (Figure 3). The corn samples (n=80) were collected i.e. 40 samples in each summer and winter. It was analyzed that 47 samples out of total 80 (59%) were found contaminated. Among these total samples, 26% samples were found contaminated beyond permissible levels. However, 33% samples were contaminated and found within permissible levels while 41% samples were non-contaminated (Table 1).

The study conducted in Sierra Leone showed that ochratoxin was found as a co-contaminant in different foodstuffs as in corn, dried red pepper and smoked dried fish in appreciable quantities (Ringot *et al.*, 2006; Karbancıoglu *et al.,* 2008; Terra *et al.*, 2013). Ochratoxin A upto 25% and 0.293ng/g was detected in corn based breakfast cereals (Coronel *et al.,* 2012). In another study sample of corn flour (0.8%) demonstrated an amount of 64µg/kg of OTA which exceeded the permissible limits of legislation from many countries (Sekiyama *et al.*, 2005).

In a study conducted on corn samples obtained from different

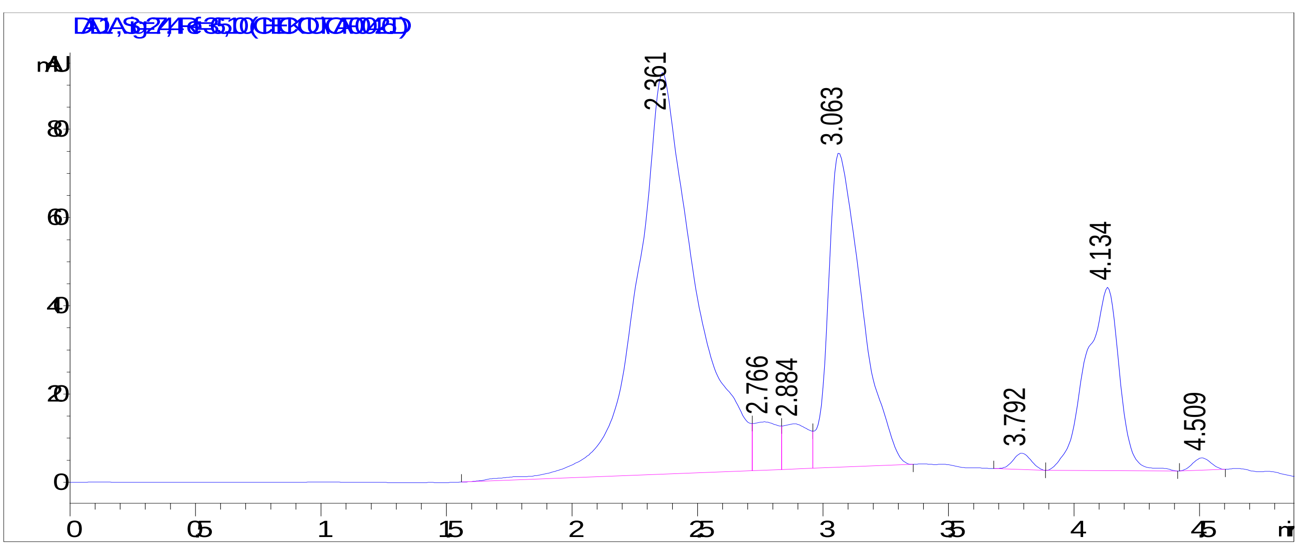


Figure 3. Chromatogram of Ochratoxin.

Table 1. Ochratoxin Analysis in Corn Samples of Punjab Districts.

Sr.	Bahawalpur	Sr.	Faisalabad	Sr.	Lahore	Sr.	Rawalpindi
			OTA (µg/kg) ± SD (SUMMER SAMPLES)				
1.	5.75±0.15	21.	21.72±0.60	41.	30.81±1.67	61.	5.86±0.08
2.	10.13±0.06	22.	2.75±0.49	42.	5.69±0.42	62.	11.29±0.86
3.	ND	23.	ND	43.	2.17±0.04	63.	ND
4.	2.41±0.52	24.	3.12±0.02	44.	1.05±0.52	64.	44.41±2.14
5.	ND	25.	3.47±0.36	45.	ND	65.	0.685±0.04
6.	ND	26.	1.98±0.74	46.	1.0±0.02	66.	ND
7.	ND	27.	231.49±29.39	47.	6.34±0.03	67.	15.75±0.47
8.	12.1±0.11	28.	7.36±0.08	48.	1.27±0.02	68.	0.72±0.46
9.	3.72±0.43	29.	6.15±0.03	49.	0.96±0.24	69.	8.75±0.63
10.	ND	30.	15.23±0.44	50.	ND	70.	ND
			OTA (µg/kg) ± SD (WINTER SAMPLES)				
11.	ND	31.	ND	51.	ND	71.	ND
12.	3.18±0.11	32.	ND	52.	ND	72.	ND
13.	ND	33.	ND	53.	ND	73.	5.29±0.16
14.	10.04±0.22	34.	ND	54.	ND	74.	0.25±0.05
15.	2.80±0.05	35.	14.23±0.36	55.	ND	75.	0.71±0.03
16.	ND	36.	ND	56.	5.16±0.06	76.	2.23±0.04
17.	ND	37.	ND	57.	0.52±0.26	77.	ND
18.	1.03±0.07	38.	2.33±0.23	58.	0.36±0.23	78.	4.12±0.17
19.	ND	39.	4.35±0.01	59.	ND	79.	ND
20.	0.99±0.18	40.	1.56±0.12	60.	ND	80.	5.09±0.33

*ND= Not Detected, **SD= Standard Deviation

store houses of 15 districts of Punjab for the estimation of ochratoxin levels, ochratoxin A was absent in all samples (Iram *et al.*, 2014). The contamination of ochratoxin may be due to high temperature, high humidity and improper storage conditions of shops, godowns and stores. In current study OTA was found in higher concentration 10.13µg/kg, 231.49µg/kg, 30.81µg/kg and 44.41µg/kg in corn samples collected in summer (April-September) while 10.04µg/kg, 14.23µg/kg, 5.16µg/kg and 5.29µg/kg in corn samples collected in winter (October-March) from Bahawalpur, Faisalabad, Lahore and Rawalpindi of Punjab Province of Pakistan, respectively (Table 1). Overall thirteen corn samples of both Faisalabad and Rawalpindi while eleven corn samples of Lahore and ten corn samples of Bahawalpur were found contaminated with ochratoxin.

It has been recognized that the infectivity of mycotoxins in different cereals may be result of unsuitable storeroom conditions (Zahra *et al.*, 2016). The highest levels of OTA in corn samples may be affected by climatic or poor storage conditions. Maize is generally cultivated during humid summer season in Pakistan. These high temperature and humid environment support the attack of fungi (*Aspergillus* and *Penicillium*) with highest OTA production. The reason for high levels of ochratoxins found in raw corn samples may be due to inappropriate storage circumstances as in villages after harvesting, farmers store corn in mud bins that absorb maximum moisture during moist and rainy season. Likewise, in current study the contamination of OTA was found more in summer as compared to winter season (Figure 4).

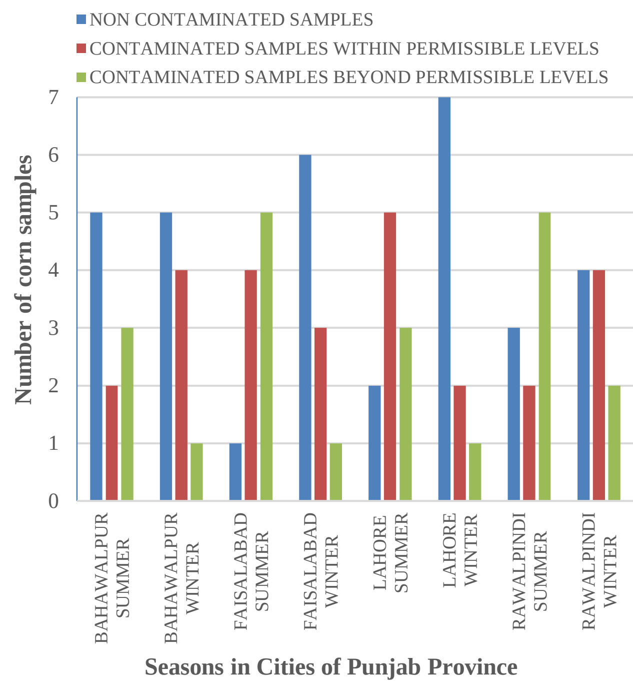


Figure 4. Season-wise comparison of OTA contamination in samples collected from different cities.

In current study 50% corn samples of both Faisalabad and Rawalpindi districts were found contaminated with OTA beyond permissible levels during summer season while 40% and 30% corn samples of Bahawalpur and Lahore district, respectively were found contaminated beyond permissible levels during summer season. During winter 10% contamination was found in corn of Bahawalpur, Faisalabad and Lahore district while 20% contamination was found in corn of Rawalpindi beyond permissible levels. Overall corn samples collected from Rawalpindi district were more contaminated with OTA than other districts of Punjab. In comparison of the study conducted by Majeed *et al.,* (2013), it was observed that 50% of total analyzed corn samples were found contaminated with OTA collected from three districts of Punjab. Similarly, study reported from Canada showed 30% contamination of the breakfast corn samples analyzed (Roscoe *et al.,* 2008).

The results (Table 2 and 3) indicated that for OTA it was observed that p value for difference in mean for different cities was less than 0.05 which is significant (Mukhtar *et al.,* 2016).

Concerning the occurrence of ochratoxin in various samples of yellow corn, barseem hay, wheat bran and poultry feed, it was found that contamination of OTA during hot weather was relatively higher than in winter weather. 100% contamination was found during humid and summer season as high temperature and high humidity are favorable for fungal growth (Abdou *et al.,* 2017). Table 3 depicts the pairwise comparison of seasons i.e. summer and winter. It was found that the contamination of OTA in both seasons is significant as p value is less than 0.05 which was significant.

In comparison with present research, OTA presence was checked in 40 different samples and found that 3 (60%) of corn flour, 1 (20%) of corn flakes, 1 (20%) of wheat flour, 1 (20%) of white flour, 3 (60%) of bread and 2 (40%) of biscuits were contaminated with OTA. The highest levels of OTA were in biscuit as 360 ngg^{-1}. 75% samples were beyond permissible levels as suggested by European Union regulations (Majeed *et al.,* 2018).

The mean difference between the seasons for OTA was also significant. In detailed analysis using LSD (Least significant difference), it was observed that the mean difference in between Bahawalpur and Faisalabad and Bahawalpur and Rawalpindi were significant but Bahawalpur and Lahore was not significant (Figure 5).

OTA levels reduction was checked by water washing, ordinary cooking and pressure cooking of rice. It was found that the rice cooked by pressure cookers had significantly lower levels of OTA (59 to 75%) than in the raw polished and water-washed rice (Park *et al.,* 2005).

Table 2. Pairwise Comparisons of Cities for Significant Difference in Ochratoxin Distribution.
Dependent Variable: OTA

(I) Cities	(J) Cities	Mean Difference (I-J)	Std. Error	Sig.[d]	95% Confidence Interval for Difference[d]	
					Lower Bound	Upper Bound
BWP	FSD	-13.179[*,b,c]	0.604	0.000	-14.372	-11.986
	LHR	-0.160[b,c]	0.604	0.792	-1.353	1.033
	RWP	-2.649[*,b,c]	0.604	0.000	-3.842	-1.457
FSD	BWP	13.179[*,b,c]	0.604	0.000	11.986	14.372
	LHR	13.019[*,b,c]	0.604	0.000	11.826	14.212
	RWP	10.530[*,b,c]	0.604	0.000	9.337	11.722
LHR	BWP	0.160[b,c]	0.604	0.792	-1.033	1.353
	FSD	-13.019[*,b,c]	0.604	0.000	-14.212	-11.826
	RWP	-2.490[*,b,c]	0.604	0.000	-3.682	-1.297
RWP	BWP	2.649[*,b,c]	0.604	0.000	1.457	3.842
	FSD	-10.530[*,b,c]	0.604	0.000	-11.722	-9.337
	LHR	2.490[*,b,c]	0.604	0.000	1.297	3.682

*. The mean difference is significant at the .05 level. b. (I) An estimate of the modified population marginal mean.
c. (J) An estimate of the modified population marginal mean. d. Least Significant Difference (equivalent to no adjustments).

Table 3. Pairwise Comparisons of Seasons for Significant Difference in Ochratoxin Distribution.
Dependent Variable: OTA

(I) Season	(J) Season	Mean Difference (I-J)	Std. Error	Sig.[d]	95% Confidence Interval for Difference[d]	
					Lower Bound	Upper Bound
SUMMER	WINTER	9.999[*,b,c]	0.427	0.000	9.155	10.842
WINTER	SUMMER	-9.999[*,b,c]	0.427	0.000	-10.842	-9.155

*. The mean difference is significant at the .05 level. b. (I) An estimate of the modified population marginal mean.
c. (J) An estimate of the modified population marginal mean. d. Least Significant Difference (equivalent to no adjustments).

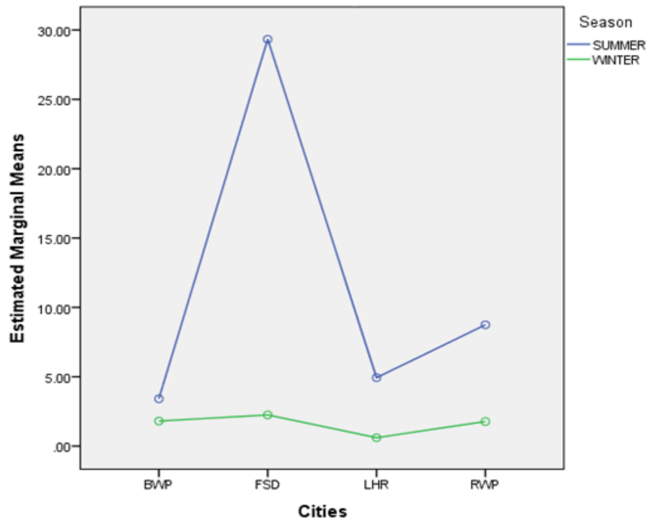


Figure 5. Estimated Marginal Means of OTA.

Different physical, chemical and biological methods were adopted to reduce OTA levels in corn. Normal cooking reduced OTA in corn sample upto 48.17% in the current study. Detoxification of OTA in corn by different ways is given in Table 4.

Table 4. Detoxification of contaminated corn samples by various methods.

Methods	Type	Initial Concen-tration (µg/kg)	Final Concen-tration (µg/kg)	% Reduction
Physical	Washing with water		150.12	35.15
	Washing with hot water		144.11	37.75
	Cooking		119.98	48.17
Chemical	20% Citric acid	231.49	84.83	63.35
	10% Acetic acid		121.69	47.43
	5% Sodium bicarbonate		125.65	45.72
	0.5% Hydrochloric acid		82.74	64.25
Natural /Biological	Ginger paste (5%)		118.75	48.70
	Garlic paste (5%)		119.86	48.22
	Black seed oil (10%)		116.92	49.49
	Probiotics (Lactobacillus Bacteria)		110.24	52.38

There are so many chemicals reported for ochratoxins detoxification in different studies but it is noted that use of chemicals may destroy essential nutrients in food commodities which in turn reduces nutritional value (Awad *et al.*, 2010). Maximum detoxification of OTA was observed by using 0.5% hydrochloric acid i.e. 64.25%.

The lactic acid bacteria were activated in MRS Broth and incubated in OTA solution. The evaluation for OTA detoxification was done thereafter. It was found that about 90% reduction of OTA was successful by strain *Lactobacillus casei* while about 70% reduction of OTA was found by *Lactobacillus gasseri* (Hathout *et al.*, 2014). However, in current study probiotics reduced OTA upto 52% in contaminated corn sample (Figure 6).

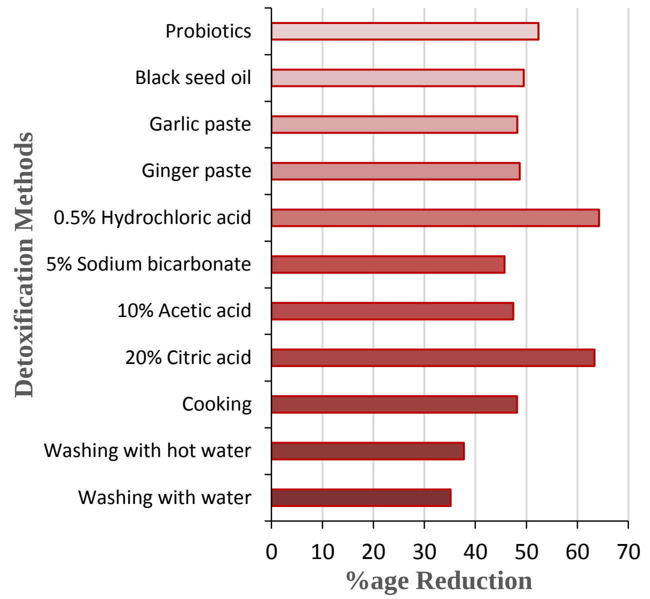


Figure 6. %age reduction levels of OTA by different methods.

Physical and chemical detoxification approaches have many limitations such as nutrients loss, time consuming and ineffective and change organoleptic properties of food. However, the use of natural/biological methods is safe for the detoxification of ochratoxin contamination in corn samples (Wang *et al.*, 2019; Agriopoulou *et al.*, 2020).

***Conclusion*:** The rising apprehension on food protection has led all countries to give attention on food protection. Corn and corn products are of special concern because before exporting them the clearance of contamination is needed. Ochratoxins may be a big threat of deterioration of corn samples in stock. It is really needed to control ochratoxin contamination in corn and corn products by proper monitoring and measurement at both domestic and international levels. In this regard vigilant handling and proper storage of corn crop are necessary in avoiding contamination of ochratoxin. The results of present studies showed high levels of Ochratoxin in corn samples which are highly alarming. Overall contamination of OTA in corn samples of Punjab, Pakistan was 59% which really needs attention. The scrutiny on continuous basis is direly needed to pass up any dangerous and hazardous health circumstances and awareness must be given to inspect contamination of corn and corn products. The proper handling is needed during pre and post harvesting practices to decrease chances of ochratoxin contamination. Proper management and good manufacturing practices (GMP) can help for the crop protection.

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