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Research article

# Assessment of mycotoxins (deoxynivalenol, zearalenone, aflatoxin $B_1$ and fumonisin $B_1$) in hen's eggs in Jordan

Tareq M. Osaili [a,b,*], Akram R. Al-Abboodi [c], Mofleh AL. Awawdeh [c], Samah Aref M.AL. Jbour [c]

[a] Department of Clinical Nutrition and Dietetics, College of Health Sciences, University of, Sharjah, P. O. Box 27272 Sharjah, United Arab Emirates

[b] Department of Nutrition and Food Technology, Faculty of Agriculture, Jordan University of Science and Technology, P.O. Box 3030, Irbid 22110, Jordan

[c] Department of Pathology and Public Health, Faculty of Veterinary Medicine, Jordan University of Science and Technology, P.O. Box 3030, Irbid 22110, Jordan

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ABSTRACT

The present study was carried out to evaluate the prevalence of mycotoxins (Deoxynivalenol (DON), Zearalenone (ZEA), Aflatoxin $B_1$ ($AFB_1$) and Fumonisin $B_1$ ($FB_1$)) in local hen's table eggs (white and yolk) as well as their stability upon refrigeration. Two hundred and fifty of fresh table eggs samples collected from Jordan governorates were analyzed using Liquid Chromatography- Mass Spectrophotometry (LC– MS/MS) More than half (67%) of the tested samples were positive for mycotoxins. The mean concentration of $AFB_1$, $FB_1$ and ZEA was 0.5 ± 0.4, 0.5 ± 0.2 and 3.2 ± 1.5 μg/kg, respectively. The overall prevalence of $AFB_1$, ZEA, $FB_1$ was 56.8, 16.0 and 7.6%, respectively. DON was not found in any of the samples. The highest prevalence was observed in Amman (85.7%) followed by Mafraq (78.6%), Karak (75.0%) and Zarqa'a (66.6%). None of the investigated mycotoxins were detected in egg whites. However, the prevalence of $AFB_1$, ZEA, $FB_1$ in egg yolk was 21.3, 16 and 7.6%, respectively. Refrigeration up to 4 weeks did not decrease the mycotoxin concentration significantly. Mycotoxin concentration in all investigated samples in this study were well below both the International and Jordanian acceptable limits. However, continuous exposure may lead to bioaccumulation over a long term and pose a threat to health.

## 1. Introduction

Mycotoxins are secondary products of fungal metabolism. The optimal growth conditions for fungi range between 10 and 40 °C, a pH of 4–8, and a water activity greater than 0.70 (Gock et al., 2003). Thus, improper storage conditions in warm and humid environments could potentially encourage fungal growth and eventually mycotoxin production (Liu et al., 2020). Approximately, 200 mold species produce mycotoxins, the ones which could impact poultry health include Aflatoxins, Zearalenone (ZEA), Ochratoxin A, Fumonisins, Trichothecenes and Deoxynivalenol (DON) (Murugesan et al., 2015). The harmful types have been recorded to target the gastrointestinal tract, causing hepatitis, hemorrhages, hepatic carcinomas, esophageal cancers, result in kidney failure and perturb the normal T-cell, B-cell, macrophage activity thereby compromising the immunity of an individual (Reddy et al., 2010). Aflatoxins, ZEA have been classified as Group 1 and 3 carcinogens, respectively (Iqbal et al., 2014).

A study conducted on the global occurrence/prevalence of mycotoxins in feed and feed raw materials reported that 72% of the samples contained harmful mycotoxins (albeit in acceptable ranges) (Schatzmayr and Streit, 2013). Poultry feed in Nigeria was observed to have an Aflatoxin contamination ranging from 13.5 to 93.1 μg/kg of feed (Kehinde et al., 2014). In another study conducted in Kuwait, the prevalence of Fumonisin, DON and ZEA ranged from 1.4 to 3.2 ppm, 0.17–0.29 ppm, and 46.4 to 67.6 parts per billion (ppb) in poultry feed samples, respectively (Beg et al., 2006). The degree of toxin contamination of feeds varies based on geography, season and feed type (Alam et al., 2012).

In poultry, mycotoxins could be carried over from the feed to the bird (Oliveira et al., 2003). The bird could further transfer it to the egg. Hens that were fed with polluted feeds containing more than 3300 ppm of Aflatoxin $B_1$ ($AFB_1$) were reported to produce contaminated eggs (Wolzak et al., 1985). Similarly, when Japanese quails were fed between 25 to 100 μg of $AFB_1$, the egg content of $AFB_1$ and Aflatoxin $M_1$ was 0.08 and 0.37 μg/kg, respectively, respectively (Oliveira et al., 2003).

Mycotoxin contaminated eggs could have devastating impact on public health. Eggs are commonly consumed due to their high nutrient, protein content and ease of digestibility (Réhault-Godbert et al., 2019). The distribution/presence or absence of mycotoxin in the yolk and white could vary depending on the ability of the bird to detoxify the toxin, presence of toxin binders in feed, in addition to toxin molecular weight,

* Corresponding author.
*E-mail addresses:* tosaili@just.edu.jo, tosaili@sharjah.ac.ae (T.M. Osaili).

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carry over rate, lipid solubility, and pKa values (Čolović et al., 2019; Costamagna et al., 2019; Gallo et al., 2015).

As Jordan, has a very tropical climate, it is imperative that mycotoxins are present in hen feed which could then further be transferred to eggs. Thus, the aim of this study was to analyze the prevalence of mycotoxins namely DON, ZEA, $AFB_1$ and $FB_1$ in table eggs in Jordan and study the impact of refrigeration on mycotoxin concentrations in table eggs.

## 2. Materials and methods

### 2.1. Sample collection

Two hundred and fifty hen egg samples were collected over a period of six months in the year 2017 (Figure 1) from different marketplaces in Jordan governorates (12 governorates) according to the following equation (Eq. 1):

$$N = \frac{(z - score)^2 * P * Q}{D^2} \tag{1}$$

where, N: sample size required, population size = infinite, Precision: 3.84 => square of 1.96 (α-error = 0.05), P = prevalence of mycotoxins (20%) (Herzallah, 2009), Q = 1- P, D = allowable error or required precision (the survey estimate to be within 5% of true level 95% of the time)

$N = (3.84) \times 0.2 \times 0.8/ (0.05)^2$

The calculated sample size was 246 samples, however; 250 samples were collected.

The samples were placed in appropriately labelled bags in an icebox and transported to the Pharmaceutical Research Center at Jordan University of Science and Technology, Jordan. They were stored at refrigeration temperatures ($4 \pm 1$ °C) until analysis (Pourhoseingholi et al., 2013).

### 2.2. Chemicals, reagents, and instruments

Methanol (MeOH, HPLC grade), acetonitrile (ACN, HPLC Gradient grade), Hexane (HPLC grade), Chloroform AR stabilized with ethanol, Formic acid (LC grade), Glacial acetic acid (analytical grade), Sodium Chloride (NaCl) (99%) was obtained from Fisher Scientific, United Kingdom. $AFB_1$ and $FB_1$ (1 μg, ≥98% assay) were purchased from Sigma-Aldrich Steinheim, Germany. DON (5 mg) and ZEA (10 mg) (ChemCruz; Santa Cruz Biotechnology Inc., Dallas, United States of America) were purchased. The solid phase extraction column ISOLUTE/ $C_{18}$ (100 mg/10 ml–1 ml XL) and LC column (Zorbax (4.6 * 150 mm, 3.5 μm), Agilent, California, USA) was purchased from Biotage (Uppsala, Sweden). Magnesium Sulfate anhydrous was obtained from Sigma-Aldrich Steinheim.

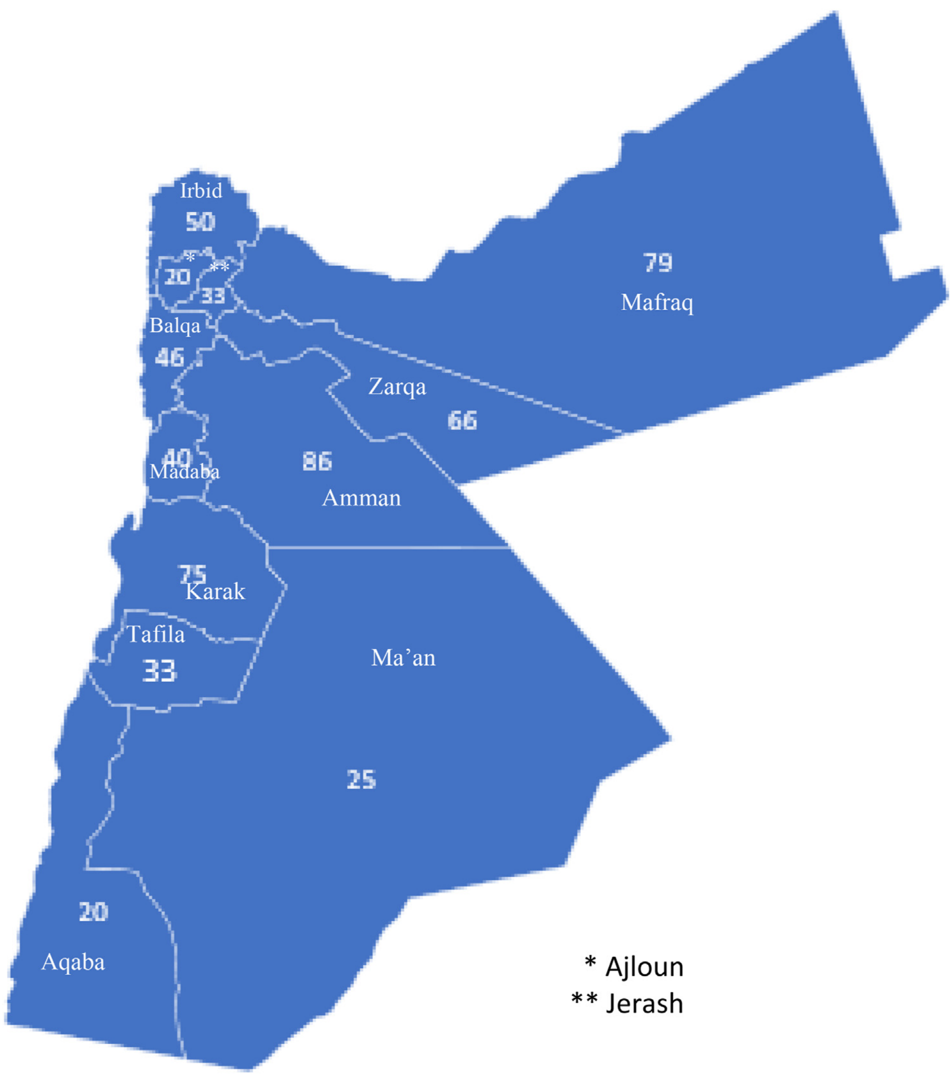


Figure 1. Sample collection of hen eggs over a period of six months in the year 2017 in Jordan (n = 250). Numbers represent number of samples collected from each governorate.

## 2.3. Preparation of standards

To prepare standard stock solutions, each of DON, and ZEA standards was dissolved in 4 ml methanol, $AFB_1$ standard was dissolved in 5 ml acetonitrile, and $FB_1$ standard was dissolved by 10 ml acetonitrile/water (5:5; v:v).

Working solutions of DON and ZEA standards were prepared by dissolving 100μl of the stock solution in 50 ml of methanol. $AFB_1$ and $FB_1$ stock solutions were dissolved in acetonitrile, and diluted acetonitrile (5:5; v:v), respectively. A mixture of 1000 ng/ml final concentration was prepared from all the individual working solutions. Stock solutions and first working solutions were stored at −40 °C. Standard curves (Supplementary file) were appropriately determined using Liquid Chromatography–Mass Spectrophotometry (LC–MS/MS).

## 2.4. Method optimization

Egg (specific-pathogen and mycotoxin free) white and yolk were separated. A volume of 0.5 ml of serially diluted mixed standard was added to 2.0 ± 0.1 g of separated egg yolk and white samples, respectively. A volume of 2 ml of deionized water was then added and the sample was mixed thoroughly. Multiple extraction solutions were tested (acetonitrile: water (90:10, v:v), acetonitrile: water (80:20, v:v), acetonitrile: water: acetic acid (79:20:1, v:v:v), methanol: water: acetic acid (79:20:1, v:v:v), water: acetone (1:1, v:v), acetonitrile: water: methanol: acetic acid (39:30:30:1, v:v:v:v). The optimum extraction solution was determined to be acetonitrile: water: methanol, acetic acid (39: 30: 30: 1; v:v:v:v). The extracted sample was further purified using the solid phase extraction technique. Multiple eluents were investigated to identify the optimum eluent (acetonitrile: water, acetic acid (49:50:1, v:v:v), methanol: water, acetonitrile - methanol (40%), methanol 100%, acetonitrile 100%). The optimum eluent was identified to be 1.5 ml of acetonitrile-methanol (40%). The spiked mycotoxins were quantitatively determined by evaluating the retention time and peak areas of the chromatogram. A control sample was evaluated in every cycle for quality assurance purposes. The LOD and LOQ were determined (Supplementary file).

## 2.5. Mycotoxin extraction

Separated egg yolks and whites (2.0 ± 0.1 g) were weighed. A volume of 15 ml of acetonitrile: water: methanol: acetic acid (40:30:30:1; v:v:v:v), was added and the sample was kept in an ultrasonic water bath for 20 min at room temperature. The tubes were then centrifuged at 4000 rpm for 10 min. After discarding the supernatant, to ensure efficient extraction of mycotoxins, a hexane solution containing $MgSO_4$ (2 g) and NaCl (0.5 g) was added. The sample was re–centrifuged (4000 rpm; 10 min) after which the hexane layer was discarded. The sample was washed with chloroform thrice to complete the extraction. The collected chloroform was then rotary evaporated (35 °C) and the mycotoxins were re–dissolved in 6 ml chloroform for further analysis.

## 2.6. Sample clean up

The Clean–up of the extract was performed using a solid phase extraction (C18) column (ISOLUTE/$C_{18}$ (100 mg/10 ml–1 ml XL), Biotage, Uppsala, Sweden). The column was conditioned using 1 ml of MeOH (10 %). A volume of 6 ml of the extract was added to the column using a vacuum manifold. Elution was performed twice using 1.5 ml of acetonitrile–methanol (40%) (optimized) and soaked for 2 min. The eluted sample was dried by evaporation and re–dissolved in 500 μL of the isocratic mobile phase (A:B, 1:1). Prior to loading the sample in LC-MS/MS, the samples were filtered through a syringe filter (0.45μm).

## 2.7. Liquid chromatography–mass spectrophotometry (LC–MS/MS) test conditions

The LC-MS/MS parameters followed were as per previously published protocol (Alaboudi et al., 2022). A LC $C_{18}$ column (Zorbax, 4.6 * 150 mm, 3.5 μm) was used with a binary pump autosampler (Agilent 1200). A volume of 100 μl was injected. The flow rate was adjusted to 0.5 ml/min with run time of 15 min. A maximum pressure of 5801 psi was used. A gradient elution was used in the mobile phase. The first solution was of ammonium acetate dissolved in water containing 0.1% formic acid (eluent A) while the second solution was of 5 mM of ammonium acetate dissolved in methanol containing 0.1% formic acid (eluent B). Analysis was initiated with 25% eluent A.

## 2.8. Risk analysis

The Probable Daily Intake (PDI) was calculated using Eq. (2). An average body weight of 70 kg was considered (Milićević et al., 2021). Risk assessment was performed for raw egg consumption.

$$PDI = \frac{\text{Concentration of a mycotoxin } (\mu g\ kg^{-1}) * \text{ consumption of the food } (kg\ day^{-1})}{\text{Body weight } (kg)} \quad (2)$$

## 2.9. Refrigeration

Eggs positive (3 samples) for mycotoxins were refrigerated at 2–5 °C for four weeks. Samples were analyzed on a weekly basis to investigate any changes in mycotoxin concentrations.

## 2.10. Statistical analysis

Data was analyzed using the Statistical Package for the Social Sciences (SPSS, IBM cooperation, NY, USA). Data were presented in the form of mean ± standard deviation. The effect of refrigeration was analyzed using a one-way ANOVA. All analysis were conducted in triplicate unless stated.

# 3. Results and discussion

The Middle Eastern weather conditions provide optimal conditions for mycotoxin production in food/feeds. The ability of mycotoxins to dangerously impact human and animal health is extensively reported. Decreasing Aflatoxins in food products to non-detectable levels is estimated to decrease hepatocellular cancer prevalence by 23% (Liu et al., 2012). Fumonisins has been associated with esophageal cancer and neural tube defects (Shephard, 2011), while ZEA has been associated with cervical cancer (Reddy et al., 2010). Symptoms associated with DON ingestion include nausea, vomiting, abdominal pain, diarrhea, dizziness and headache (Reddy et al., 2010). It is estimated that Aflatoxins cause up to 25,200 to 155,000 liver cancer cases per year (Wu et al., 2014). About a quarter of Hepatocellular carcinoma cases detected globally have been attributed to Aflatoxins. Thereby, determining the prevalence of mycotoxins in food products is essential.

Studies indicated that feeding hens a mycotoxin contaminated feed resulted in the deposition of these toxins in the hens' kidneys, liver and muscle. Moreover, transmission to the egg has also been observed (Aly and Anwer, 2009). A sixty-day hen exposure to 100 μg Aflatoxin per kilogram of feed resulted in Aflatoxin transfer to the egg in a ratio of 1428:1 (Aly and Anwer, 2009).

In the current study, 67.2% of the egg samples tested positive for mycotoxins. The highest prevalence of mycotoxins was observed in Amman (85.7%) followed by Mafraq (78.6%), Karak (75.0%) and Zarqa'a (66.6%). In all other governates, a prevalence of less than 50.0% was observed (Figure 2). From the positive samples, 42.0% contained at least one mycotoxin while 16.8 and 8.4% of the samples contained two or three types of mycotoxins, respectively. The overall prevalence of $AFB_1$, ZEA, $FB_1$ was 56.8, 16.0 and 7.6%, respectively. DON was not found in any of the samples (Figure 3); however, it is possible that the DON may have been transformed into its metabolites. The mycotoxin, $AFB_1$ concentration in the tested egg samples ranged from 0.19 to 1.06 μg/kg with a mean concentration of 0.54 ± 0.43 μg/kg, respectively (Figure 4). $FB_1$ concentration ranged from 0.39 to 0.60 μg/kg with mean of 0.5 ± 0.2 μg/kg. Lastly, ZEA concentration ranged from 1.86 to 4.67 μg/kg with a mean of 3.2 ± 1.5 μg/kg. Previous studies indicated the prevalence of Aflatoxins, ZEA and DON in chicken eggs to be 28% (1.39 μg/kg), 32% (1.58 μg/kg) and 85% (2.3–4.5 ng/kg), respectively (Iqbal et al., 2014; Tangni et al., 2009). The variation could be explained by the different feeds, feed manufacturing plants, feed storage conditions and storage periods, origin and quality of the feed, sanitary conditions and season (Milani, 2013). International limits for $AFB_1$, DON, ZEA and Fumonisin range from 1 to 20 μg/kg, 300–2000 μg/kg, 50–1000 μg/kg, 1000–3000 μg/kg, respectively. The Jordanian maximum tolerated level for $AFB_1$ is 15 μg/kg (FAO, 2003). The concentrations of the mycotoxins ($AFB_1$, ZEA, $FB_1$ and DON) in all investigated samples in this study were well below both the International and Jordanian acceptable limits.

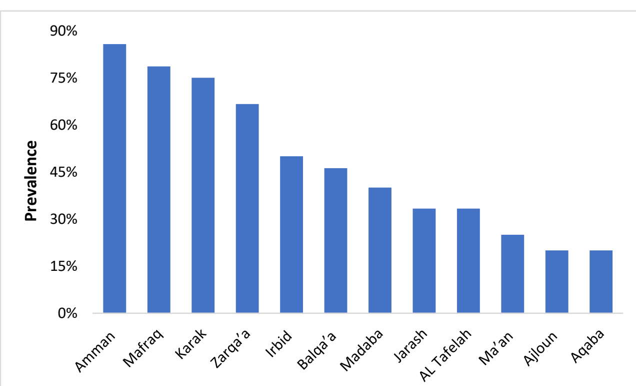


**Figure 2.** The prevalence of mycotoxins in the different governorates of Jordan (n = 250).

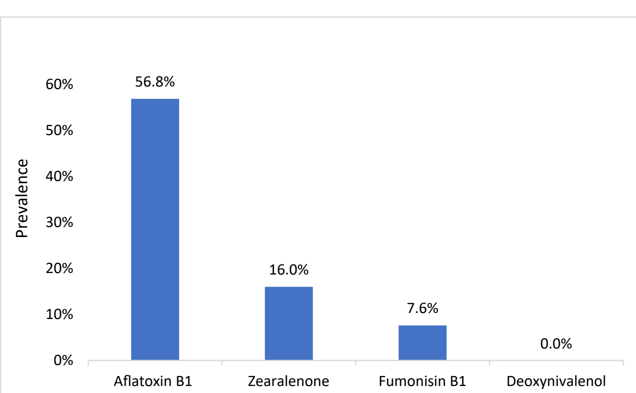


**Figure 3.** The prevalence of different types of mycotoxins in eggs from Jordan (n = 250).

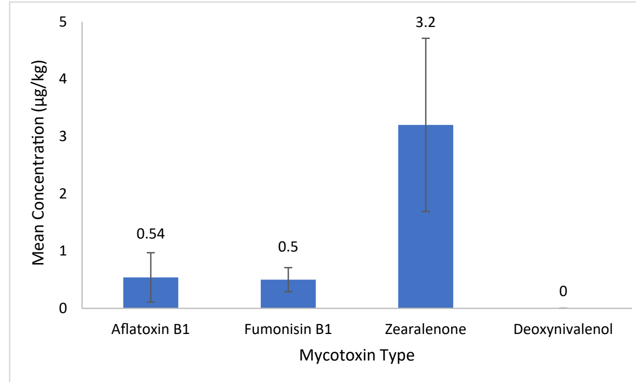


**Figure 4.** The mean concentration (μg/kg) of mycotoxins in eggs in Jordan (n = 250).

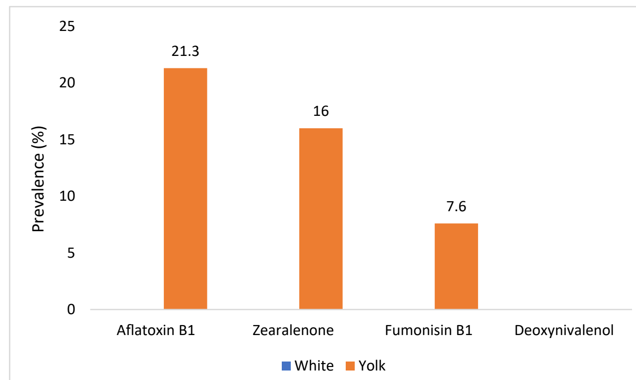


**Figure 5.** Prevalence of mycotoxins in egg whites and yolk in Jordan (n = 250).

Comparing the prevalence of mycotoxins in egg yolk and egg white, no residues of $AFB_1$, ZEA, $FB_1$ and DON were detected in egg whites. Their prevalence in egg yolk was 21.3, 16, 7.6, and 0%, respectively (Figure 5).

$AFB_1$ residues have been observed in eggs only when the hen consumes a feed with a minimum $AFB_1$ concentration of 500 g/kg feed (Oliveira et al., 2000). Another study indicated hen feed inoculated with DON and ZEA up to 2228, 820 μg/kg did not contaminate the eggs (Emmanuel et al., 2020). The detection of $AFB_1$, $FB_1$ and ZEA in egg samples in the current study gives an indirect indication that the feeds given to the hens may be contaminated with high levels of these toxins. Although a study on feed mycotoxin concentration conducted in Jordan does not exist (to the best of our knowledge), this hypothesis may hold true as a recent study conducted in Jordan on chicken indicated that the $AFB_1$, $FB_1$, ZEA and DON concentrations ranged from 0.03 – 2.84 μg/kg, 6.19–1170 μg/kg, 0.60–676 μg/kg and 12.20–2920 μg/kg, respectively (Alaboudi et al., 2022). The carry-over of mycotoxins in the egg is expected to vary based on the toxin type, toxin concentration in feed, period of exposure and ability of the hen to detoxify the mycotoxin.

### 3.1. Risk assessment

The average hen egg consumption in Jordan is about 153 eggs/person/year (News, 2019). Considering an average egg weight of around 60 g (Travel et al., 2011), the egg consumption would be around 25.1 g/day (Table 1). This would translate to less than 0.002 μg $kg^{-1}$ bw $day^{-1}$ of consumption for all the studied mycotoxins (Table 1). The provisional maximum tolerable daily intake (PMTDI) for Fumonisins and DON are 2 and 1 μg $kg^{-1}$ bw $day^{-1}$, respectively (EFSA, 2014). The Tolerable Daily Intake (TDI) for ZEA is established at 0.25 μg $kg^{-1}$ bw $day^{-1}$ (EFSA, 2014). The levels of Aflatoxins in food are recommended to be as low as

**Table 1.** Probable Daily Intake (PDI) of mycotoxins from raw hen eggs in Jordan in comparison to International Standards.

| Mycotoxin | Mean | Maximum value | Minimum value | International (μg $kg^{-1}$ bw $day^{-1}$) |
|---|---|---|---|---|
| Aflatoxin $_{B1}$ | 0.00019 | 0.0004 | 0.0000 | As low as possible* |
| Fumonisin $_{B1}$ | 0.00018 | 0.00021 | 0.0001 | 2** |
| Zearalenone | 0.00115 | 0.00170 | 0.0007 | 0.25*** |
| Deoxynivalenol | 0 | 0 | 0 | 1** |

*As determined by Codex Alimentarius (2019).
**Provisional maximum tolerable daily intake (PMTDI) as determined by EFSA (2014).
***Tolerable Daily Intake (TDI) as determined by EFSA (2014).

reasonably possible (Codex Alimentarius, 2019). The concentration of mycotoxins in raw hen eggs in our study thereby meet International Recommendations by a good margin and can thereby be considered safe for consumption purposes. In a French total diet study, DON and its derivative exposure amongst the population was observed to exceed the recommended health based guidance values (Sirot et al., 2013). In one study, the estimated total intake for DON and $AFB_1$ in Mediterranean countries ranged from 0.000186 – 0.1888 and 0.000033–0.0489 μg $kg^{-1}$ bw $day^{-1}$, respectively (Serrano et al., 2012). Meanwhile, in Europe and Africa, the estimated total intake by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) of DON was estimated to be 1.4 and 0.77 μg $kg^{-1}$ bw $day^{-1}$, respectively (Serrano et al., 2012). The sum of $FB_1$ and $FB_2$ exposure in the two regions was estimated to be 0.2 and 2.4 μg $kg^{-1}$ bw $day^{-1}$, respectively. To reduce exposure, countries may introduce tighter regulations. However, this may result in massive export losses which could be up to 5 folds for certain countries/regions (Wu, 2004). Decisions which balance the health of consumers (with regards to mycotoxin concentration) without causing massive disruption in food related economy may need to be made on a regular basis by different countries/regions.

### *3.2. Effect of refrigeration*

The concentration of mycotoxins did not change significantly ($P \geq 0.5$) during storage at refrigeration temperatures up to four weeks. To the best of our knowledge, no study which evaluates the impact of refrigeration on mycotoxin concentrations has been done previously. The elimination of mycotoxins is fairly difficult due to their high stability (Kabak, 2009). The degradation has been observed to vary based on different factors such as moisture content of the food, presence of any other additives as well as the matrix of the food product (Temba et al., 2016).

## 4. Conclusion

The mycotoxin content in eggs from Jordan meets international standards. However, regular and high consumption may lead to bioaccumulation in the body. Refrigeration had no impact on reducing mycotoxin contaminations. Food safety authorities should eliminate the possibility of contamination at the root level during production and storage. Future studies should concentrate on the seasonal variation of mycotoxins in the feed and the metabolites of these mycotoxins.

## Declarations

### *Author contribution statement*

Tareq M. Osaili: Analyzed and interpreted the data; Wrote the paper.
Akram R. Al-Abboodi, Mofleh AL Awawdeh: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.
Samah Aref M. AL Jbour: Performed the experiments; Analyzed and interpreted the data.

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### *Data availability statement*

Data will be made available on request.

### *Declaration of interest's statement*

The authors declare no conflict of interest.

### *Additional information*

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2022.e11017.

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