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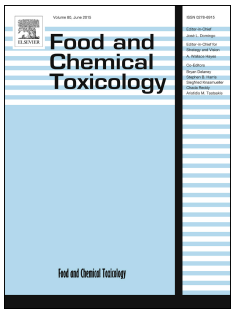

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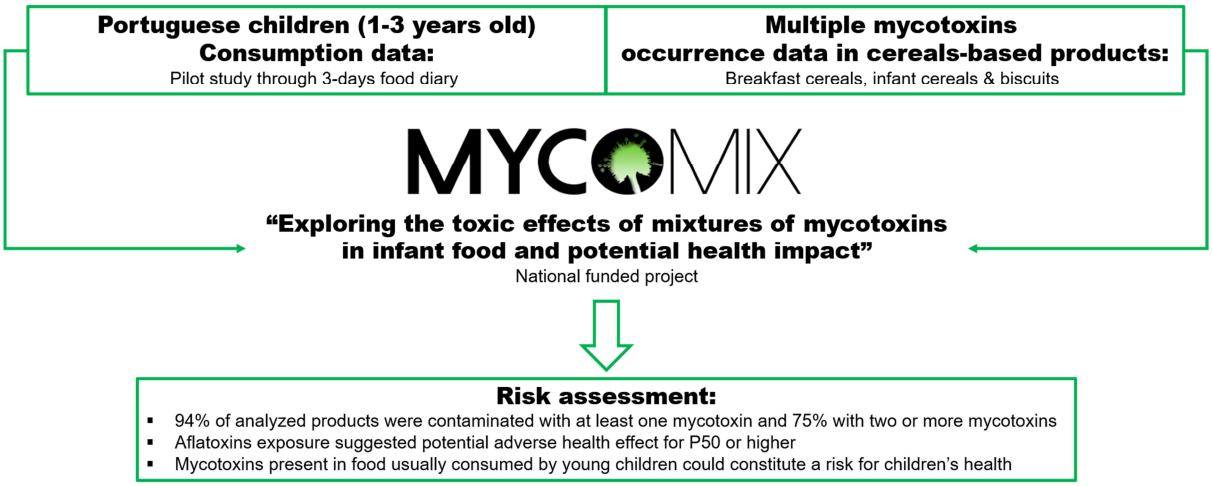
Portuguese children dietary exposure to multiple mycotoxins – an overview of risk assessment under MYCOMIX project

Ricardo Assunção[a,b,c,*], Carla Martins[a,c,d], Elsa Vasco[a], Alessandra Jager[e], Carlos Oliveira[f], Sara Cunha[g], José Fernandes[g], Baltazar Nunes[h,i], Susana Loureiro[j,c] and Paula Alvito[a,c]

[a]*Food and Nutrition Department, National Institute of Health Dr. Ricardo Jorge, Portugal;* [b]*IIFA, Universidade de Évora, Portugal;* [c]*CESAM, Centre for Environmental and Marine Studies, University of Aveiro, Portugal;* [d]*National School of Public Health, NOVA University of Lisbon, Portugal;* [e]*Department of Physics and Chemistry, School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Brazil;* [f]*Department of Food Engineering, School of Animal Science and Food Engineering, University of São Paulo, Brazil;* [g]*LaQV-REQUIMTE, Laboratory of Bromatology and Hydrology, Faculty of Pharmacy, University of Porto, Portugal;* [h]*Departamento de Epidemiologia, Instituto Nacional de Saúde Dr. Ricardo Jorge, Portugal;* [i]*Centro de Investigação em Saúde Pública, Escola Nacional de Saúde Pública, Universidade NOVA de Lisboa, Portugal,* [j]*Departamento de Biologia, Universidade de Aveiro, Portugal.*

*Food and Nutrition Department, National Institute of Health Dr. Ricardo Jorge, Av. Padre Cruz, 1649-016 Lisboa, Portugal. Telephone: (+351) 217 519 340. E-mail address: ricardo.assuncao@insa.min-saude.pt



Portuguese children dietary exposure to multiple mycotoxins – an overview of risk assessment under MYCOMIX project

Abstract: Mycotoxins are secondary fungi metabolites that induce acute and chronic toxic effects in humans and animals. Simultaneous contamination of cereal-based products by multiple mycotoxins has been increasingly reported, including in food products usually consumed by children. Although some previous authors assessed the health risk of children exposed to multiple mycotoxins, scarce data are available on the risk associated with the ingestion of multiple mycotoxins through different food products. MYCOMIX, a national funded project, intended to contribute to fill this gap. The present study aimed to overview the risk assessment of multiple mycotoxins performed under MYCOMIX, assessing for the first time, the risk associated with the exposure to 13 mycotoxins in breakfast cereals, infant cereals and biscuits consumed by children from Lisbon region, Portugal. Results on mycotoxins occurrence showed that 94% of samples were contaminated with at least one mycotoxin, although at levels below the legislated limits. Co-contamination was observed in 75% of the analysed samples. Estimated aflatoxins exposure suggested a potential adverse health effect for percentiles of intake above or equal to P50. The obtained results suggested that future research actions should be set in motion in order to protect children health.

Keywords: cumulative risk assessment, children, multiple mycotoxins, cereal-based foods, MYCOMIX project

1. Introduction

Mycotoxins are naturally occurring secondary metabolites produced by certain fungi species and these toxins have become increasingly important worldwide, considering their impact on human and animal health (Borchers et al., 2010; Marin et al., 2013; Wu et al., 2014). These contaminants may be acutely toxic but currently, the main human and animal health burdens of mycotoxin exposure are related to chronic toxicity, such as carcinogenic, teratogenic, immunotoxic, nephrotoxic, and estrogenic effects. Human exposure to mycotoxins occurs directly through the intake of contaminated agricultural

products (e.g. cereal-based products, fruits) or indirectly through the consumption of products of animal origin (e.g. meat, milk, eggs) prepared or obtained from animals that were fed with contaminated material (Capriotti et al., 2012; Flores-Flores et al., 2015). Some international institutions developed significant efforts reflecting a specific concern about the potential impact of mycotoxins on human health. To ensure a high level of public health protection, the International Agency for Research on Cancer (IARC) evaluated several food-contaminating mycotoxins relatively to their impact on human carcinogenicity and classified them from human carcinogens (group 1) to not classifiable as to its carcinogenicity to humans (group 3) (IARC 2016). In addition, the European Union (EU) established maximum levels for the major mycotoxins present in food products (EC 2006a).

Cereal-based products constitute one of the main contributors to human exposure to mycotoxins. In fact, the Food and Agriculture Organization of the United Nations (FAO) estimated that approximately 25% of the cereals produced in the world are contaminated by mycotoxins, but perhaps this value is closer to 50%, if one takes into account emerging mycotoxins of which so far limited data are available (Fraeyman et al., 2017; Rice and Ross, 1994). This issue assumes particular importance specially for specific population groups as children, considering their vulnerability due to their physiology, a fairly restricted diet and a higher consumption relative to their body (Alvito et al., 2010). Additionally, cereals, namely cereal-based products as breakfast cereals, infant cereals and biscuits, are among their first solid foods (Amezdroz et al., 2015; Rodrigues et al., 2007). Therefore, the significance and potential health risk associated with mycotoxins present in foods usually consumed by children requires meticulous attention.

Nowadays, the simultaneous contamination of food products by numerous known or unknown mycotoxins constitutes a rising concern, especially because health effects resulting from multiple mycotoxins exposure could lead to different output toxicity and carcinogenicity than exposure to single mycotoxins (Bouaziz et al., 2008).

However, most studies have focused on the risk assessment of single mycotoxins (Assunção et al., 2015; De Ruyck et al., 2015; Smith et al., 2016; Stoev, 2015). Diverse approaches have been used to predict the toxicity and risk of mixtures based on their chemical composition and knowledge of the toxicities of the mixture components [e.g. Hazard Index (HI) and the Combined Margin of Exposure Index (MoET)]. Most of these methods are based on the concepts of Concentration Addition (CA) and Independent Action (IA) (Borg et al., 2013). The HI, the mostly used for non-genotoxic and non-carcinogenic mycotoxins, provides a measure of the total risk based on the individual risk of each component. The MoE (margin of exposure) is proposed for the risk assessment of substances that have both genotoxic and carcinogenic properties and the MoET is usually used for the cumulative risk assessment (EFSA, 2013).

There are scarce published reports concerning the risk associated with human exposure to multiple mycotoxins in foods, especially for those intended for children consumption (Raiola et al., 2015; Sherif et al., 2009). The inexistence of Portuguese data concerning the risk associated to children dietary exposure to multiple mycotoxins led to the development of MYCOMIX, a national funded project (https://www.youtube.com/watch?v=CsKaz3mt2J4) that was entitled “Exploring the toxic effects of mixtures of mycotoxins in infant food and potential health impact”. Between other tasks, MYCOMIX included the characterization of Portuguese children consumption pattern (through a pilot study), the assessment of their mycotoxin exposure through the cereal-based foods and the associated risk estimate. The present work aimed to overview the main results obtained under MYCOMIX concerning the Portuguese children dietary exposure to multiple mycotoxins. For the first time, the present study assessed the cereal-based food products consumption by children aged from one year up to and including three years of age and estimated the risk associated with the exposure to multiple mycotoxins through the simultaneous consumption of different cereal-based products (including breakfast cereals, infant cereals and biscuits) consumed by the studied population group.

2. Materials and methods

2.1. Occurrence data

2.1.1. Samples

A total of 52 different cereal-based products primarily marketed for children [26 breakfast cereals (BC), 20 infant cereals (IC) and six biscuits (BIS)] were purchased from supermarkets in Lisbon region, in 2014 and 2015. The minimum amount of each sample was 1 kg, in accordance with Commission Regulation (EC) 401/2006 of 23 February 2006 (EC 2006b). The samples were homogenized in a food homogenizer, saved in plastic bags and stored at 4 °C until further analysis. To avoid cross contamination during the homogenization process, good laboratory practices were applied.

2.1.2. *Analytical determination and method performance*

The determination of 13 mycotoxins [aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2, AFM_1), OTA, fumonisins (FB_1, FB_2), trichothecenes (deoxynivalenol, DON; nivalenol, NIV; T-2 toxin and HT-2 toxin) and zearalenone (ZEA)] in cereal-based products was performed using a set of extraction and analytical methods, as previously described (Martins et al., 2018). Following a procedure based on solvent and salted extraction and a purification step using immunoaffinity columns (IAC), according to EN ISO (2010), aflatoxins and ochratoxin A were determined by high performance liquid chromatography with fluorescence detection (HPLC-FLD). HPLC analysis was performed using a Waters® Alliance 2695 equipped with fluorescence detector Waters 2475 (Waters®, USA) and Empower Chromatography Software®. Following an extraction based on QuEChERS and derivatization procedure developed by Pereira et al. (2014), trichothecenes were determined by gas chromatography coupled to mass spectrometry (GC-MS) analysis

performed on an Agilent® (Little Falls, DE, USA) gas chromatograph 6890 equipped with an electronically controlled split/splitless injection port and an inert 5973N mass selective detector with electron impact (EI) ionization chamber. The analytical separation was conducted in DB-5 MS (30 m x 0.25 µm x 0.25 mm film thickness; J&W Scientific, Folsom, CA, EUA) capillary column in the same conditions described in Pereira et al. (2014). Following an extraction procedure based on liquid-liquid extraction with methanol/water as referred by Ndube et al. (2011), fumonisins and zearalenone were determined by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The UPLC-MS/MS system consisted of a Waters® Acquity I-Class UPLC coupled to a Waters® Xevo TQ-S mass spectrometer (Milford, MA, USA) and equipped with a BEH C_{18} column (2.1 × 50 mm, 1.7 µm). Electrospray interface in positive mode was used for detection in multiple reactions monitoring (MRM) mode. ZEA and fumonisins determination was accomplished using a labelled standard ($^{13}C_{18}$-ZEA and $^{13}C_{34}$-FB_1) for spiking experiments. Mycotoxin contents were expressed as mean, median and maximum, using Microsoft® Excel 2013.

All the analytical methods showed a good linear response over the working range, a coefficient of determination (r^2) > 0.995, with mean recoveries ranging between 44% (for T-2 toxin) and 93% (for DON), limit of detection (LOD) values ranging between 0.001 (for AFB_2) and 6.8 µg/kg (for T-2), and limit of quantification (LOQ) values ranging between 0.004 (for AFB_2) and 22.3 µg/kg (for T-2) (Assunção et al., 2015). ZEA presented an r^2 > 0.995, with a LOD and LOQ of 0.12 and 0.40 µg/kg, respectively. The linearity range used to ZEA quantification purposes was 0.24 – 10.0 µg/kg (Martins et al., 2018). All the presented results were in agreement with the criteria mentioned in the Commission Regulation (EC) No. 401/2006 (EC 2006b).

2.2. Consumption data

In order to obtain children food consumption data, a pilot study was developed in a

Primary Health Care Unit in Lisbon region (Cidadela, Cascais), between February and June 2014. Participants enrolled in the study were attended in the Health Care Unit during the survey period, parents signed an informed consent and filled a brief health and socio-demographic questionnaire and a three days food diary (Assunção et al., 2015). This study was conducted according to the guidelines laid down in the declaration of Helsinki (World Medical Association, 2013) and was approved by the Ethical Committee of the National Institute of Health Dr. Ricardo Jorge and by the Portuguese Data Protection Authority. Considering that children may begin to eat cereal-based products mainly after one year old, a subsample of 75 children aged from one year up to and including three years of age was considered in the present study.

Two groups were considered concerning consumption of cereal-based products ("consumers and non-consumers" including all individuals, and "only consumers"). The consumption pattern (g of intake per kg of body weight per day) of the studied population for each considered cereal-based product was characterized through different percentiles (P5 and P95), minimum, maximum and mean values, using Microsoft® Excel 2013.

2.3. Exposure assessment

The exposure assessment of each detected mycotoxin was performed considering two mathematical approaches: deterministic, with a point evaluation, and probabilistic, with a Monte Carlo simulation. For data treatment in relation to non-detects (<LOD), three scenarios were considered as recommended by EFSA [LOD=LOD (H1), LOD= ½ LOD (H2), LOD=0 (H3)] (EFSA, 2010). In the deterministic approach, two different calculations were performed, according to two different consumption settings: 1) the consumption of cereal-based products by all studied population ("consumers and non-consumers"); and 2) "only consumers" of cereal-based products. In the probabilistic approach, a fourth scenario for non-detects was considered replacing the censored

data by random values from a uniform distribution with zero as minimum and LOD as maximum (H4) (Assunção et al., 2015). An estimation of the exposure was evaluated, computing the intake of each mycotoxin from different food products. Calculations for the deterministic and the probabilistic approach were executed using Microsoft® Excel 2013 and the software @Risk® for Microsoft Excel version 6 (Palisade Corporation, USA), respectively.

2.4. Risk characterization

For the risk characterization, the outputs of exposure (daily intake values) were compared with the reference dose values. Two different approaches were performed considering mycotoxins carcinogenicity. The MoE was applied for individual aflatoxins and was calculated as a ratio of benchmark dose lower confidence limit ($BMDL_{10}$) and aflatoxin exposure. $BMDL_{10}$ value was derived from the study of Benford et al. (2010) (0.00025 mg/kg bw/day). For the remaining individual mycotoxins, the output of exposure was compared to the dose reference value [provisional maximum tolerable daily intake (PMTDI) or provisional tolerable weekly intake (PTWI)] in order to calculate the hazard quotient (HQ, ratio between exposure and a reference dose). The generated outputs were evaluated according to different criteria. The magnitude of the MoE gives an indication of the risk level and the Scientific Committee of EFSA and WHO have concluded that a MoE of 10000 or more was of low concern for public health (EFSA 2013). For the HQ, a tolerable or a non-tolerable exposure level was considered if HQ was below or above one, respectively (Borg et al. 2013; EFSA 2013). For the cumulative risk assessment of multiple mycotoxins, and considering the insufficient information about the mode of action of the identified combinations of mycotoxins, the CA concept was applied. CA is usually preferred over the independent action approach for those cases when no mode of action information is available. This approach is considered the most conservative on the analysis of combined exposure to

multiple compounds, contributing to a higher health assurance (EFSA, 2013; Meek et al., 2011). Mycotoxins were grouped by families as the cumulative assessment group (aflatoxins, ochratoxin A, fumonisins, trichothecenes and zearalenone) (EFSA 2013). The MoET was determined for aflatoxins and the HI for fumonisins and trichothecenes. The MoET was calculated as the reciprocal of the sum of the reciprocals of the individual margins of exposure (MoEs) and the HI, as the sum of the respective Hazard Quotients (HQs) for the individual mixture components of the same family (EFSA 2013).

3. Results and discussion

3.1. Assessment of children exposure to mycotoxins

3.1.1. Mycotoxins occurrence data

Table 1 shows the percentage of positive samples (mycotoxin contents above LOD) and mean (µg/kg), median (µg/kg) and maximum (µg/kg) contents of each mycotoxin in each food category.

Table 1. Occurrence (%) of mycotoxins in cereal-based products primarily marketed for children in Portugal.

Forty nine out of 52 samples (94%) evidenced at least one of the analysed mycotoxins (with values above the detection limit). All the studied mycotoxins were detected in the analysed samples, except AFG_2, T-2 and HT-2 toxins. Overall, OTA, ZEA and DON were the most commonly detected mycotoxins with 65%, 48% and 44% of analysed samples revealing values above the LOD, respectively (Table 1). All analysed samples presented mycotoxin contents bellow the legislated limits, when available (EC 2006a). Mycotoxin mean contents attained a maximum of 95.9 µg/kg of DON in a breakfast cereals sample and a maximum contamination value of 207.8 µg/kg for the same toxin (Table 1). This value exceeded the maximum limits

established for DON in processed cereal-based foods and baby foods (200 µg/kg) (EC 2006a), which is lower than the limit adopted for food intended for adult population, considering the specific vulnerability of infant and children.

In Portugal, several previous studies evaluated the occurrence of aflatoxins and OTA (Alvito et al., 2010; Assunção et al., 2016a), fumonisins (Lino et al., 2007; Martins et al., 2008; Silva et al., 2007), trichothecenes (Cunha and Fernandes, 2010; Martins and Martins, 2001) and ZEA (Aldana et al., 2014; Cunha and Fernandes, 2010) in foods usually consumed by children. These studies presented some similarities and dissimilarities with the results obtained in the present study. All these works revealed a considerable degree of heterogeneity in the contamination of the cereal-based products intended to be consumed by children. This could be explained by the fact that different fungi can grow and produce mycotoxins in the same environmental conditions. Cereals production, processing and storage conditions could favour the production of several mycotoxins and consequently, different mycotoxins contamination, in type and contents, could be detected (Ibáñez-Vea et al., 2011).

Co-occurrence of mycotoxins

The detected co-occurring mycotoxins, the number of mycotoxins included in the mixtures as well as their frequency of occurrence in the three cereal-based products considered are presented in Table S1 (Supplementary material). The analysis revealed a co-occurrence of mycotoxins in 75% of the analysed samples, with two or more mycotoxins occurring simultaneously. The highest number of mycotoxins detected simultaneously was seven and the combinations of two (OTA and DON; OTA and fumonisins) and four (aflatoxins, OTA and ZEA) mycotoxins were the most commonly detected, with a percentage of occurrence of 6% for each combination. The present results contribute to the growing evidence related to the more frequent human

exposure to multiple than to single mycotoxins (Alassane-Kpembi et al., 2017; De Ruyck et al., 2015; Grenier and Oswald, 2011; Stoev, 2015). As a consequence, there has been an increasing concern about the health hazard from exposure to multiple mycotoxins in human and animals. In a recent review, Smith et al. (2016) verified that several surveys reported the natural co-occurrence of mycotoxins from all over the world, and most of them concerned the aflatoxins, OTA, ZEA, fumonisins and trichothecenes, especially DON. The same authors referred that among the 116 mycotoxin combinations found in cereal and derived cereal product samples, aflatoxins and fumonisins, DON and ZEA, aflatoxins and OTA, and fumonisins and ZEA were the most present. These results agree well with those obtained in the present study and point out the importance to develop more toxicity studies that consider the co-exposure to multiple mycotoxins simultaneously considering the potential impact for public health, especially in those cases of possible synergism and additive effects. The present data were crucial to perform a more accurate Portuguese children risk assessment through consumption of cereal-based products.

3.1.2. Consumption data

The participants enrolled in this study consisted of 75 children aged from one year up to and including three years of age with the following distribution by gender and age: 18 male and 20 female (13-24 months), 9 male and 9 female (25-36 months), 7 male and 12 female (36-47 months). Food consumption data analysis revealed that approximately 92% of the considered children population consumed one or more cereal-based products, at least one time in three days, as reported in food diary (40%, 65% and 65% consumed breakfast cereals, infant cereals and biscuits, respectively) which represents a considerable consumption of cereal-based products by Portuguese children. Table 2 shows the cereal-based products consumption values by age of the two considered groups “consumers and non-consumers” and “only consumers”. As

would be expected the average consumption is higher when considering the group "only consumers". Infant cereals are the group of foods most consumed by children (3.3 g/kg bw/day), especially at one year old (3.9 g/kg bw/day). The mean daily consumption of these food groups, for all children, were 5.6 g, 25.3 g and 8.7 g for breakfast cereals, infant cereals and biscuits, respectively (data not shown). For the group "only consumers", the values of the mean daily consumption were higher (15.4g, 38.7g and 13.4g, for the same food groups, respectively) (data not shown). Guerra et al. (2012), who reviewed the knowledge and practices on infant feeding, suggested that for nutritional requirements and inherent neurosensory, motor and social infant development, foods other than milk and with less homogeneous texture should be progressively introduced, leading to entry in the family diet, which should occur around 12 months of age. Hence, the present results corroborate these practices, revealing that Portuguese children from early ages usually eat other foods than milk, including cereal-based products.

Table 2. Distribution of Portuguese children cereal-based products' consumption by age (from one year up to and including three years of age).

3.1.3. Exposure assessment

In the present study, the exposure assessment of children to multiple mycotoxins in cereal-based products was assessed through the estimation of the mycotoxins daily intake. Table 3 presents the estimated daily intake (ng/kg bw/day) by Portuguese children for the different cereal-based products, considering three different scenarios (according to the data treatment of the non-detects). Two different set of calculations were performed, one considering all the studied population ("consumers and non-consumers" of cereal-based products) and another only considering the cereal-based consumers ("only consumers"). For the H1 scenario (< LOD = LOD, worst

case), the sum of daily intake through consumption of cereal-based products presented the highest value for DON (57.22 ng/kg bw/day), followed by FB_1 (6.4 ng/kg bw/day), NIV (2.68 ng/kg bw/day), FB_2 (1.0 ng/kg bw/day), ZEA (0.86 ng/kg bw/day), OTA (0.131 ng/kg bw/day) and AFM_1 (0.069 ng/kg bw/day). The remaining scenarios followed approximately the same pattern. As expected, the estimates for the daily intake for consumers presented higher values, although quite comparable with the obtained results for the all studied population.

Considering the daily intake through the consumption of each cereal-based product, the highest mycotoxin intake was verified through the consumption of breakfast cereals (for AFB_1, fumonisins, DON and NIV) and through the consumption of infant cereals (for AFM_1, AFB_2, AFG_1, OTA and ZEA).

Table 3. Children's daily intake of mycotoxins present in cereal-based products (ng/kg bw/day) considering three different scenarios for non-detects (<LOD), through a deterministic approach.

The results of the estimated daily intake through the probabilistic approach (mean and percentiles 50, 75, 90, 95, 99) for individual toxins in each cereal-based product and its sum in the three cereal-based products are presented at Table 4.

Table 4. Children's daily intake of mycotoxins present in cereal-based products (ng/kg bw/day) considering four different scenarios for non-detects (<LOD), through a probabilistic approach.

Considering all the studied scenarios in probabilistic analysis (H1, H2, H3, H4), the worst case was reflected in H1 scenario, and the results reinforced the outcomes obtained with the deterministic approach. Considering the mean sum of daily intake through the consumption of the three groups of cereal-based products, the highest

estimated value was presented for DON (53.93 ng/kg bw/day), followed by FB_1 (6.7 ng/kg bw/day), NIV (2.74 ng/kg bw/day), ZEA (0.89 ng/kg bw/day) and OTA (0.165 ng/kg bw/day). Within aflatoxins, AFM_1 presented the highest value for the mean sum of daily intake (0.058 ng/kg bw/day). Considering each analysed food group, the breakfast cereals was the highest contributor for the estimated daily intake of mycotoxins by Portuguese children under three years old, revealing the highest values for fumonisins, trichothecenes, ZEA and AFB_1. On the other hand, processed cereal-based foods (flours) presented the highest contribution for the estimated daily intakes of AFM_1, AFB_2, AFG_1 and OTA.

3.2. Risk characterization

Relatively to aflatoxins, Figure 1A presents the risk characterization results using MoE (individual aflatoxins) or MoET (combined aflatoxins). The results were obtained considering the probabilistic estimates of aflatoxins exposure through the consumption of the considered three food categories (breakfast cereals, infant cereals and biscuits). The H1 scenario, as the worst case, was presented. The remaining scenarios followed the same pattern (data not shown).

Figure 1. Characterization of risk associated with the exposure to mycotoxins (A, aflatoxins; B, remaining mycotoxins) through consumption of the three considered food products (breakfast cereals, infant cereals and biscuits). MoE and MoET were derived from estimates obtained by the probabilistic approach. Data for H1 scenario (H1: < LOD = LOD), as the worst case scenario, is presented.

As presented in Figure 1A, AFB_2 was the aflatoxin that revealed a MoE above 10000 for all percentiles of exposure, which represent low risk for children. However, for the remaining aflatoxins and for some percentiles of intake (AFB_1 and AFG_1, for percentiles 90, 95 and 99), the MoE values were below 10000, suggesting a potential

health concern. When considered percentiles P50 or higher of AFM_1 intake, the MoE values were below 10000, suggesting also a potential health concern. When considered the simultaneous exposure to aflatoxins, MoET for percentiles P50 or higher revealed a potential health concern. Values of MoE or MoET below 10000 signifies that continuous exposure to such cereal-based products could pose serious adverse health effect to such susceptible groups of individuals, as young children. Assunção et al. (2015) had already referred that a potential health concern could arise from the consumption of breakfast cereals, especially for high consumers (percentiles 90, 95 and 99), being AFB_1 the main contributor (87.3%) for the risk (MoET < 10000). As suggested by Serrano et al. (2012), one possible explanation for the higher risk associated with infants and children is the fact that they have an exceptionally high intake in relation to their body weight. Sherif et al. (2009) refereed that there is evidence that suggest increased susceptibility to cancer from early-life exposures, particularly for chemicals acting through a mutagenic mode of action, as aflatoxins. Additionally, Raiola et al. (2015) pointed out other potential effects of children exposure to aflatoxins, namely i) reduction of the efficiency of immunization in children with the consequent increase of susceptibility to infections; ii) children vulnerability to the risk of cancer from aflatoxin-contaminated milk since milk is an important constituent of their diet (the same could be proposed to aflatoxins present in cereal-based products); iii) young animals have been found to be more susceptible to AFB_1 and AFM_1 toxicity than adults and repeated exposures to aflatoxins *in-utero* and through childhood might predispose to liver cancer later in life.

Relatively to the remaining mycotoxins, Figure 1B presents the results concerning the risk characterization for OTA, fumonisins, and trichothecenes using HQ (individual mycotoxins) and HI (combined mycotoxins). The outputs were derived from estimate of these mycotoxins exposure performed by the probabilistic approach and through the consumption of the three food categories considered. The H1 scenario, as

the worst case, was presented. The remaining scenarios followed the same pattern (data not shown).

These results showed that all HQs were below one, *i.e.*, indicating no cause for concern for individuals exposed to mycotoxins through consumption of cereal-based products. The HQ for DON and HI for the simultaneous exposure to trichothecenes showed the highest values, however well below one.

The exposure assessment results were compared to other reports available in the literature. In Spain, several authors studied children (including different age groups) exposure to mycotoxins (Cano-Sancho et al., 2013, 2012a, 2012b, 2011; Coronel et al., 2012; Hernández-Martínez and Navarro-Blasco, 2010; Rodríguez-Carrasco et al., 2013). Generally, Spanish results agreed well with those obtained in the present study. From all the Spanish studies, it is important to highlight the results obtained by Cano-Sancho et al. (2013). These authors assessed the exposure to aflatoxins by Catalonian (Spain) population. The study included children aged above 4 years old. The authors reported a children daily intake of aflatoxins of 0.105 ng/kg bw/day with results for MoE of 8208 (mean) and of 2582 (P95), representing similarly to the present study a significant health risk. The authors concluded that the MoEs achieved in the study were in the line with those estimations reported for other European countries. In France, Sirot et al. (2013) assessed the dietary exposure of the French population to mycotoxins through a total diet study. The authors reported that the highest mean concentrations were found in wheat and cereal-based products and only the exposure to DON and its acetylated derivatives was found to significantly exceed the reference values in adults and children. Similarly to the results obtained in the present study, DON revealed the highest values of daily intake.

The present study was conducted in a children population from one year up to and including three years of age, a particular vulnerable population group considering their high intake relatively to body weight. This study included, for the first time, the exposure to multiple mycotoxins through consumption of various cereal-based

products, thus reinforcing the importance of cumulative analysis of the risk of exposure to mycotoxins through food. The analysis of the present results should take into consideration some sources of uncertainties, namely i) the number of analysed samples, ii) the children sample size and iii) the toxicological data. These facts may have influenced the obtained intake values estimates and the characterization of the risk. Relatively to the toxicological data, it should be highlighted that reference doses are only defined for the adult population and this renders difficulty in the children and infants risk assessment for which the available reference doses are not suitable (Assunção et al., 2016b). The $BMDL_{10}$ described by Benford et al. (2010) was determined for AFB_1. However, and assuming a worst case perspective, in the current study, it was also applied to the remaining aflatoxins. Additionally, the present study estimated the children exposure through an indirect approach, combining data of mycotoxin occurrence in food and food consumption. This common approach has some limitations for the mycotoxins exposure assessment, including the heterogeneous distribution of mycotoxins in food, the possible exposure through other exposure routes than ingestion, the presence of masked mycotoxins, the influence of food processing, inter-individual variation in absorption, distribution, metabolism and excretion (ADME), and the under- and overestimation in food consumption data (Arcella and Leclercq, 2004; Heyndrickx et al., 2014). These limitations could lead to an under- and/or overestimation of the exposure, and biomarkers have been proposed as a suitable alternative (Assunção et al., 2016b).

3.3. Strategies to reduce risk associated with aflatoxins exposure

The risk associated with the children consumption of foods contaminated by mycotoxins is dependent of the magnitude, frequency of exposure as well as the hazard from each mycotoxin (Kuiper-Goodman, 2004). A simulation considering a quarter (1/4) of the aflatoxins daily intake previously estimated was performed, in order

to evaluate the potential measures that could be suggested to reduce the aflatoxins MoET values. The results showed an improvement of the aflatoxins MoET, revealing that just percentiles of intake above P90 could be under health concern (data not shown). This reduction of aflatoxins daily intake could be attained considering some measures as i) improvement on the variety and diversity of cereal-based products consumed by children, ii) reduction on the amount of daily ingestion of these products and iii) reduction on mycotoxins contaminations of cereal-based raw materials used for food products primarily marketed for children. Consequently, industry is strongly encouraged to have a more rigorous monitoring and quality control of the ingredients used in these products.

4. Conclusions

The present work overviewed the main results concerning Portuguese children exposure to multiple mycotoxins through cereal-based products consumption, obtained under MYCOMIX project. The combination of data from mycotoxins occurrence in different cereal-based products and their consumption by Portuguese children included in the present study allowed to conduct a first exposure assessment to multiple mycotoxins, and to characterize the associated risk. Bringing together all the generated results, the present study also highlights three major areas that should be considered in future research activities, including i) biomonitoring of children exposure to mycotoxins, ii) attainment of detailed toxicological data, including health consequences resulting from early-life exposures to multiple mycotoxins and iii) reduction and decontamination of foods, especially those that will be destined for children consumption. The acquisition of better information on children's exposure patterns, sources, and the health consequences are of crucial importance. Additionally, and because mycotoxins are natural contaminants and cannot be completely prevented, significant efforts should be set in motion, from farm to fork, to improve the safety and

quality of foods, reducing mycotoxin exposure. Risk-benefit should be systematically assessed in order to obtain all the information needed to implement strategies to reduce and prevent health consequences of mycotoxins exposure. Special attention should be dedicated to aflatoxins contamination, as presented, considering their potential toxicity as carcinogenic compounds. Altogether, these aspects will contribute to reduce the children exposure to mycotoxins through food consumption and consequently, improve the protection of children health.

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Table 2. Distribution of Portuguese children cereal-based products' consumption by age (from one year up to and including three years of age).

Age (years)	Consumption of cereal-based products (g/kg bw/day)									
	"Consumers and non-consumers"			"Only consumers"						
	(n)	Mean[a]	P95	(n)	(%)	Min	P5	Mean[a]	P95	Max
Breakfast cereals										
1	38	0.4 ± 1.4	1.8	8	21	0.4	0.4	2.0 ± 2.6	6.5	8.0
2	18	0.3 ± 0.4	1.2	9	50	0.2	0.2	0.6 ± 0.5	1.3	1.4
3	19	0.7 ± 0.7	2.0	13	68	0.3	0.3	1.0 ± 0.7	2.3	2.8
1 - 3	75	0.5 ± 1.1	1.6	30	40	0.2	0.2	1.2 ± 1.6	3.3	8.0
Infant cereals										
1	38	3.0 ± 3.4	10.8	29	76	0.8	0.9	3.9 ± 3.4	10.9	12.0
2	18	1.8 ± 2.5	6.7	10	56	0.7	0.9	3.2 ± 2.5	7.7	8.9
3	19	0.8 ± 0.9	2.2	10	53	0.3	0.3	1.5 ± 0.7	2.2	2.3
1 - 3	75	2.1 ± 2.9	8.4	49	65	0.3	0.8	3.3 ± 3.0	10.1	12.0
Biscuits										
1	38	0.6 ± 0.6	1.6	26	68	0.2	0.3	0.9 ± 0.5	1.7	2.5
2	18	0.9 ± 1.2	2.5	13	72	0.2	0.2	1.2 ± 1.2	3.1	4.7
3	19	0.5 ± 0.6	1.4	10	53	0.5	0.5	1.0 ± 0.4	1.6	1.9
1 - 3	75	0.7 ± 0.8	1.9	49	65	0.2	0.3	1.0 ± 0.7	2.1	4.7

[a] Mean ± Standard deviation; bw: body weight

Table 3. Children's daily intake of mycotoxins present in cereal-based products (ng/kg bw/day) considering three different scenarios for non-detects (<LOD), through a deterministic approach.

Estimated daily intake (ng/kg bw/day) - **consumers and non-consumers**

Toxins	Breakfast cereals			Infant cereals			Biscuits			**Sum of daily intake (ng/kg bw/day)**		
	H1	H2	H3	H1	H2	H3	H1	H2	H3	H1	H2	H3
AFM_1	0.005	0.003	0.001	0.064	0.057	0.051	0.000	0.000	0.000	0.069	0.060	0.052
AFB_1	0.012	0.011	0.011	0.000	0.000	0.000	0.000	0.000	0.000	0.012	0.011	0.011
AFB_2	0.001	0.001	0.001	0.002	0.002	0.000	0.000	0.000	0.000	0.003	0.003	0.001
AFG_1	0.003	0.001	0.000	0.013	0.008	0.002	0.000	0.000	0.000	0.016	0.009	0.002
OTA	0.011	0.010	0.010	0.064	0.060	0.059	0.056	0.056	0.056	0.131	0.126	0.124
FB_1	6.0	5.4	5.3	0.4	0.3	0.3	0.0	0.0	0.0	6.4	5.7	5.6
FB_2	1.0	1.0	0.8	0.0	0.0	0.0	0.0	0.0	0.0	1.0	1.0	0.8
DON	24.83	24.80	24.77	16.34	16.06	15.78	16.05	15.15	14.24	57.22	56.01	54.79
NIV	2.68	1.56	0.44	0.00	0.00	0.00	0.00	0.00	0.00	2.68	1.56	0.44
ZEA	0.42	0.41	0.41	0.43	0.36	0.28	0.00	0.00	0.00	0.86	0.77	0.69

H1: < LOD = LOD; H2: < LOD = 1/2 LOD; H3: < LOD = 0.
Mean consumption data: Breakfast cereals 5.6 g/day; Infant cereals 25.3 g/day; Biscuits 8.7 g/day.
Mean weight data (consumers and non-consumers): 13.4 kg.

Estimated daily intake (ng/kg bw/day) - **only consumers**

Toxins	Breakfast cereals			Infant cereals			Biscuits			**Sum of daily intake (ng/kg bw/day)**		
	H1	H2	H3	H1	H2	H3	H1	H2	H3	H1	H2	H3
AFM_1	0.012	0.007	0.002	0.104	0.091	0.082	0.000	0.000	0.000	0.116	0.099	0.084
AFB_1	0.028	0.028	0.027	0.000	0.000	0.000	0.000	0.000	0.000	0.028	0.028	0.027

AFB_2	0.003	0.003	0.002	0.003	0.003	0.000	0.000	0.000	0.000	0.006	0.006	0.002
AFG_1	0.006	0.004	0.001	0.021	0.012	0.003	0.000	0.000	0.000	0.028	0.016	0.004
OTA	0.035	0.035	0.034	0.104	0.098	0.094	0.088	0.088	0.088	0.227	0.221	0.217
FB_1	13.4	13.2	13.1	0.6	0.5	0.5	0.0	0.0	0.0	14.0	13.8	13.6
FB_2	2.6	2.27	2.07	0.0	0.0	0.0	0.0	0.0	0.0	2.6	2.3	2.1
DON	61.16	61.08	61.01	26.36	25.90	25.44	25.27	23.84	22.42	112.78	110.83	108.88
NIV	6.60	3.84	1.07	0.00	0.00	0.00	0.00	0.00	0.00	6.60	3.84	1.07
ZEA	0.94	0.92	0.91	0.70	0.58	0.46	0.00	0.00	0.00	1.64	1.50	1.37

H1: < LOD = LOD; H2: < LOD = 1/2 LOD; H3: < LOD = 0.
Mean consumption data: Breakfast cereals 15.4 g/day; Infant cereals 38.7 g/day; Biscuits 13.4 g/day.
Mean weight data: Breakfast cereals consumers 14.9 kg; Infant cereals consumers 12.7 kg; Biscuits consumers 13.1 kg.

Table 4. Children's daily intake of mycotoxins present in cereal-based products (ng/kg bw/day) considering four different scenarios for non-detects (<LOD), through a probabilistic approach.

	Estimated daily intake (ng/kg bw/day)																							
Toxins	Breakfast cereals						Infant cereals						Biscuits						**Sum of estimated daily intake (ng/kg bw/day)**					
	Mean	P50	P75	P90	P95	P99	Mean	P50	P75	P90	P95	P99	Mean	P50	P75	P90	P95	P99	Mean	P50	P75	P90	P95	P99
AFM_1	0.005	0.004	0.007	0.013	0.017	0.027	0.053	0.022	0.054	0.117	0.186	0.458	0.000	0.000	0.000	0.000	0.000	0.000	0.058	0.026	0.062	0.130	0.203	0.485
AFB_1	0.013	0.003	0.011	0.030	0.055	0.160	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.013	0.003	0.011	0.030	0.055	0.160
AFB_2	0.001	0.000	0.001	0.002	0.004	0.010	0.002	0.001	0.003	0.005	0.006	0.011	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.002	0.004	0.007	0.010	0.020
AFG_1	0.003	0.002	0.004	0.007	0.010	0.016	0.012	0.008	0.017	0.029	0.038	0.062	0.000	0.000	0.000	0.000	0.000	0.000	0.015	0.010	0.021	0.036	0.048	0.078
OTA	0.019	0.009	0.023	0.047	0.069	0.126	0.091	0.018	0.05	0.131	0.246	0.951	0.056	0.029	0.072	0.142	0.202	0.356	0.165	0.056	0.145	0.321	0.517	1.433
FB_1	6.3	1.0	3.7	12.5	26.1	92.1	0.4	0.1	0.3	0.8	1.3	3.6	0.0	0.0	0.0	0.0	0.0	0.0	6.7	1.1	4.0	13.3	27.4	95.7
FB_2	1.2	0.5	1.2	2.6	4.3	11.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.2	0.5	1.2	2.6	4.3	11.7
DON	28.69	4.73	23.82	75.72	134.47	344.96	8.09	0.70	2.48	7.78	16.28	75.00	17.15	6.50	19.04	44.38	69.99	146.84	53.93	11.93	45.34	127.88	220.74	566.80
NIV	2.74	1.82	3.72	6.38	8.51	13.51	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.74	1.82	3.72	6.38	8.51	13.51
ZEA	0.44	0.14	0.41	1.05	1.82	4.65	0.44	0.23	0.52	1.04	1.53	3.15	0.00	0.00	0.00	0.00	0.00	0.00	0.89	0.37	0.94	2.09	3.34	7.80

AFM_1, aflatoxin M_1; AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; OTA, ochratoxin A; FB_1, fumonisin B_1; FB_2, fumonisin B_2; DON, deoxynivalenol; NIV, nivalenol; ZEA, zearalenone.

Table 1. Occurrence (%) of mycotoxins in cereal-based products primarily marketed for children in Portugal.

Toxins	Breakfast cereals (n = 26)[a]	Infant cereals (flours) (n = 20)	Biscuits (n = 6)	Total Samples (n = 52)

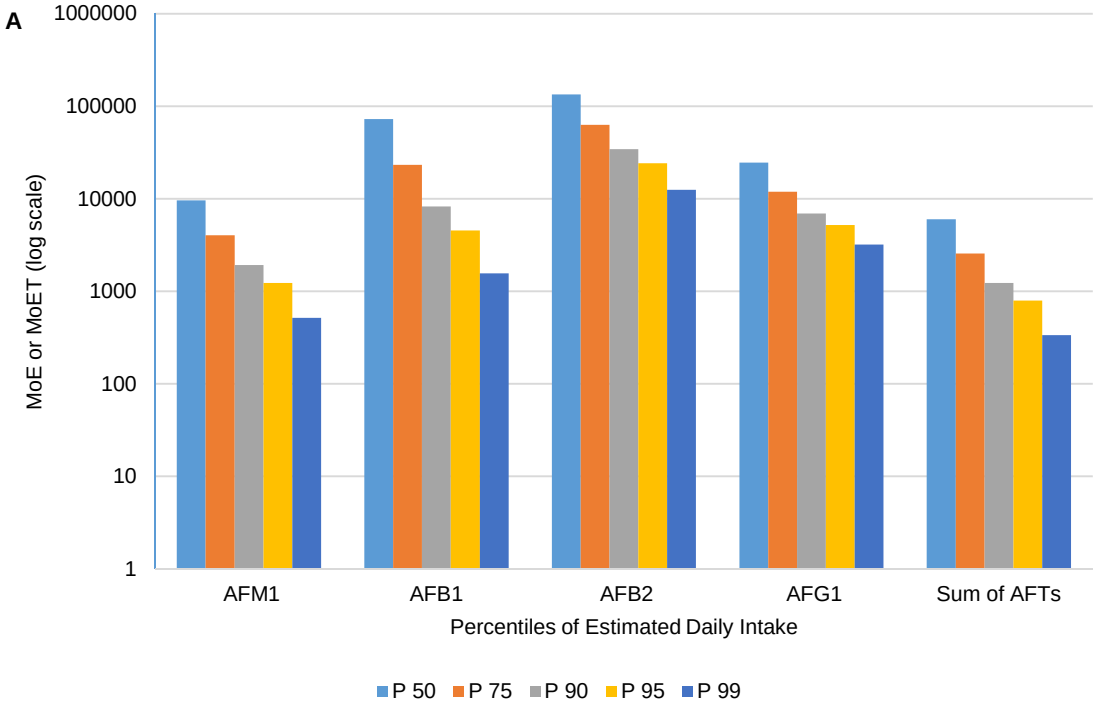
	Positive samples (%)	Mean (µg/kg)	Median (µg/kg)	Maximum (µg/kg)	EU maximum content (µg/kg)	Positive samples (%)	Mean (µg/kg)	Median (µg/kg)	Maximum (µg/kg)	EU maximum content (µg/kg)	Positive samples (%)	Mean (µg/kg)	Median (µg/kg)	Maximum (µg/kg)	EU maximum content (µg/kg)	Positive samples (%)
AFB_1	19 (73%)	0.036	0.013	0.130	2.0	0	ND	ND	ND	0.10	0	ND	ND	ND	2.0	19 (37%)
AFB_2	12 (46%)	0.007	0,004	0.011	-	1 (5%)	NA	NA	0.002	-	0	ND	ND	ND	-	13 (25%)
AFG_1	1 (4%)	NA	NA	0.017	-	2 (10%)	0.014	0.014	0.016	-	0	ND	ND	ND	-	3 (6%)
AFG_2	0	ND	ND	ND	-	0	ND	ND	ND	-	0	ND	ND	ND	-	0
AFM_1	3 (12%)	0.017	0.013	0.024	-	8 (40%)	0.068	0.023	0.190	-	0	ND	ND	ND	-	11 (21%)
AFTs	19 (73%)				-	9 (45%)				-	0				-	28 (54%)
OTA	18 (69%)	0.047	0.043	0.100	3.0	10 (50%)	0.061[b]	0.040[b]	0.263[b]	0.50	6 (100%)	0.086	0.091	0.134	3.0	34 (65%)
FB_1	15 (58%)	22.00	12.50	67.00	-	7 (35%)	0.44	0.31	0.86	-	0	ND	ND	ND	-	22 (42%)
FB_2	10 (39%)	5.10	4.20	14.00	-	0	ND	ND	ND	-	0	ND	ND	ND	-	10 (19%)
FMs	15 (58%)				200	7 (35%)				-	0				-	22 (42%)
ZEA	19 (73%)	1.20	0.69	5.61	50	6 (30%)	0.48	0.41	0.98	20	0	ND	ND	ND	50	25 (48%)
DON	16 (62%)	95.9	91.5	207.8	500	4 (20%)	41.8	37.5	71.0	200	3 (50%)	43.8	32.3	73.3	500	23 (44%)
NIV	1 (4%)	NA	NA	27.1	-	0	ND	ND	ND	-	0	ND	ND	ND	-	1 (2%)
T-2	0	ND	ND	ND	-	0	ND	ND	ND	-	0	ND	ND	ND	-	0
HT-2	0	ND	ND	ND	-	0	ND	ND	ND	-	0	ND	ND	ND	-	0

ND, not detected. NA, not applicable.

Positive samples, mycotoxin content ≥ LOD.

[a]Martins et al., 2018; [b]Assunção et al., 2016a

AFM_1, aflatoxin M_1, LOD = 0.011 µg/kg; AFB_1, aflatoxin B_1, LOD = 0.003 µg/kg; AFB_2, aflatoxin B_2, LOD = 0.001 µg/kg; AFG_1, aflatoxin G_1, LOD = 0.006 µg/kg; AFG_2, aflatoxin G_2, LOD = 0.010 µg/kg; AFTs, aflatoxins; OTA, ochratoxin A, LOD = 0.006 µg/kg; FB_1, fumonisin B_1, LOD = 0.08 µg/kg; FB_2, fumonisin B_2, LOD = 0.08 µg/kg; FMs, fumonisins; DON, deoxynivalenol, LOD = 0.37 µg/kg; NIV, nivalenol, LOD = 5.56 µg/kg; T-2, T-2 toxin, LOD = 6.8 µg/kg; HT-2, HT-2 toxin, LOD = 6.4 µg/kg; ZEA, zearalenone, LOD = 0.12 µg/kg.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10000
1000
100
10
1
MoE or MoET (log scale)
AFM1
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AFB2
AFG1
Sum of AFTs
Percentiles of Estimated Daily Intake
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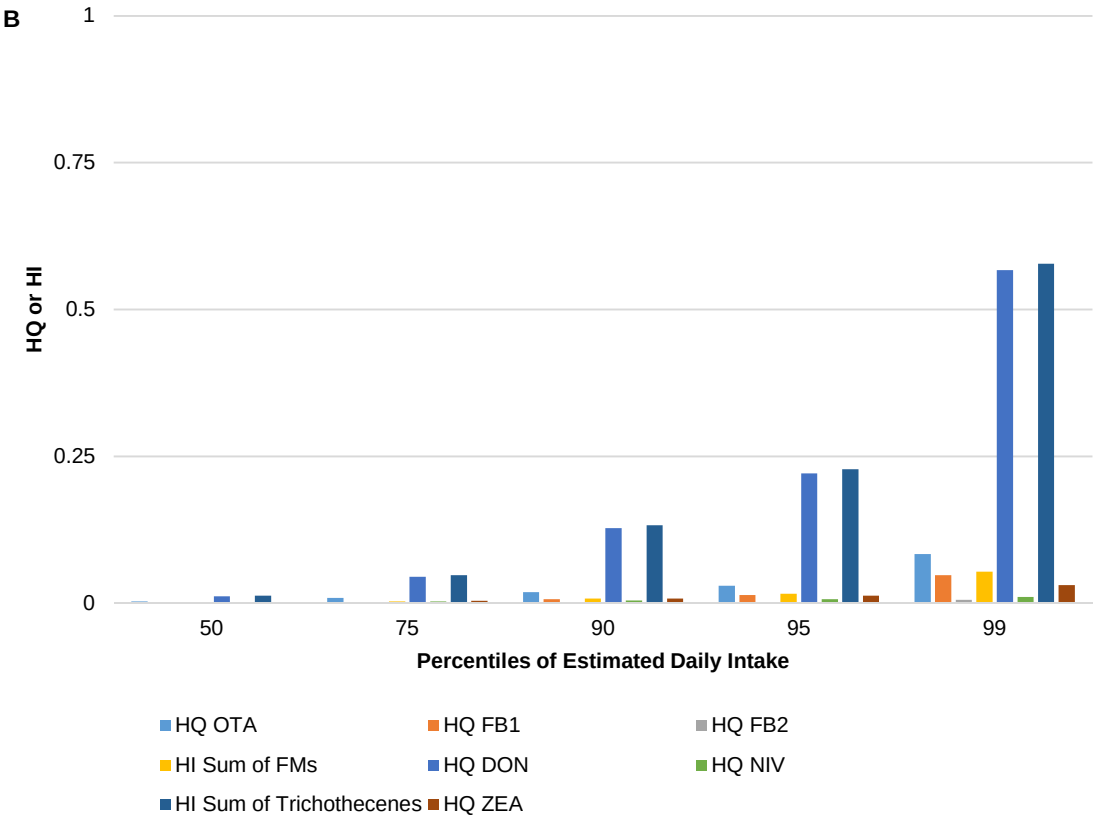
P 75
P 90
P 95
P 99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0.5
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0
HQ or HI
50
75
90
95
99
Percentiles of Estimated Daily Intake
HQ OTA
HQ FB1
HQ FB2
HI Sum of FMs
HQ DON
HQ NIV
HI Sum of Trichothecenes
HQ ZEA

Figure 1. Characterization of risk associated with the exposure to mycotoxins (A, aflatoxins; B, remaining mycotoxins) through consumption of the three considered food products (breakfast cereals, infant cereals and biscuits). Data for H1 scenario (H1: < LOD = LOD), as the worst case scenario, is presented. HQ: Hazard Quotient; HI: Hazard Index; MoE: Margin of Exposure; MoET: Combined Margin of Exposure Index.

Highlights

- Risk of Portuguese children exposed to multiple mycotoxins was evaluated under MYCOMIX project
- 94% of cereal-based products were contaminated with at least one mycotoxin
- 75% of analysed samples were contaminated with two or more mycotoxins
- Aflatoxins exposure suggested potential adverse health effect for P50 or higher
- Mycotoxins present in food usually consumed by young children could constitute a risk for children's health