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Assessment of mycotoxin exposure and risk characterization using occurrence data in foods and urinary biomarkers in Brazil

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ABSTRACT

This study aimed to assess the exposure of Brazilian residents ($N = 86$) from rural areas to multiple mycotoxins and characterize the associated risk in two sampling periods (SP) (April–May and December/2016). Mycotoxins in food and urine samples were determined by liquid chromatography coupled to tandem mass spectrometry. Mean probable daily intake (PDI) values based on occurrence data in foods in both SP varied from 0.007 to 0.013, 0.069 to 1.002, 0.119 to 0.321 and 0.013–0.156 μg kg^{-1} body weight (bw) day^{-1} for aflatoxins (AFs), deoxynivalenol (DON), fumonisins (FBs) and zearalenone (ZEN), respectively. Mean PDI values based on urinary biomarkers were 0.001, 84.914, 0.031, 0.377 and 0.002 μg kg^{-1} bw day^{-1} for AFB_1, DON, ochratoxin A (OTA), FB_1 and ZEN, respectively. Hazard quotient (HQ) calculated using food data revealed a potential health concern for ZEN in 2nd SP. HQ > 1 based on urinary biomarkers were observed for DON in the two SP. Although OTA was not detected in any food sample, the HQ based on urinary OTA levels was > 1 in the 1st SP. Margin of exposure values for AF from food and urine data in the 1st SP were below 10,000, indicating potential health risks.

1. Introduction

Mycotoxins are organic compounds of low molecular weight produced as secondary metabolites by various species of fungi during growth on foodstuffs. They present different chemical structures and occur in several agricultural products, especially cereals (Abbas, 2005). The most well-know and studied mycotoxins are the aflatoxins (AF), deoxynivalenol (DON), ochratoxin A (OTA), fumonisins (FB), and zearalenone (ZEN). *Aspergillus* species (mainly *A. flavus*, *A. parasiticus* and *A. nomius*) are the main producers of the most important, highest toxic AF compounds (AFB_1, AFB_2, AFG_1 and AFG_2) showing teratogenic, mutagenic and carcinogenic effects in several animal species and humans (Jager et al., 2013). DON, also called vomitoxin, is a type B trichothecene mainly produced by *Fusarium graminearum*, which causes nausea, diarrhea, reduced nutritional efficiency, gastrointestinal tract injuries, and weight loss in animals (Oliveira et al., 2014). OTA, which is produced by *Aspergillus* and *Penicillium* (Abbas, 2005), interferes with the synthesis of macromolecules in the cells of the renal parenchyma, including DNA, RNA, and proteins. *F. verticillioides* produces FB_1, as the most toxic compound, FB_2 and FB_3 predominantly found in natural conditions (Oliveira et al., 2014). ZEN is an estrogenic substance derived from resorcylic acid produced by several *Fusarium* species, such as *F. graminearum, F. culmorum,* and *F. equisetum* (Magan and Olsen, 2006).

Due to the risks posed to human health, several countries, including Brazil, have set maximum permitted levels (MPL) for mycotoxins in different food products. Brazilian regulations for mycotoxins determine MPL for total AF (sum of AFB_1, AFB_2, AFG_1 and AFG_2), DON, OTA, total FB (sum of FB_1 and FB_2), and ZEN in the most susceptible foods for contamination, including cereals and cereal-based products, such as rice, bean, wheat flour, corn flour, and corn meal (Anvisa, 2014). Brazilian MPLs for OTA and DON in rice, beans, wheat flour, corn flour and corn meal are 10 and 750 μg kg^{-1}, respectively. For ZEN, the MPL

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is 100 μg kg^{-1} in rice and wheat flour, and 150 μg kg^{-1} in corn products. MPLs established for total AF (B_1 + B_2 + G_1 + G_2) and FB (B_1 + B_2) in corn products are 20 and 1500 μg kg^{-1}, respectively. However, a stringent MPL for total AF of 5 μg kg^{-1} was adopted for rice, beans and wheat flour (Anvisa, 2014). Despite regulations, the occurrence of mycotoxins in Brazilian food products has been highlighted by several studies, indicating high frequencies and concentrations of total AF, total FB and, more recently, DON, especially in corn, peanuts, wheat, and products made with these cereals (Almeida-Ferreira et al., 2013; Del Ponte et al., 2012; Machinski et al., 2001; Martins et al., 2012a; Moreno et al., 2009; Oliveira et al., 2009; Piacentini et al., 2015; Scaff and Scussel, 2004; Simas et al., 2007). Although the published data indicate a high potential for consumption of food products contaminated with more than one mycotoxin in Brazil, there is no published study on the exposure assessment or risk characterization, the two main steps of risk assessment, regarding the presence of multiple mycotoxins in the diet. However, previous studies have shown high occurrences of other regulated mycotoxins in food, indicating a potential high exposure in the Brazilian population, especially in rural areas (Bordin et al., 2015; Van Der Westhuizen et al., 2003; Franco et al., 2018).

Exposure assessment can be determined by 2 different approaches, one indirect by combining food consumption and occurrence data, and another, direct approach based on urine biomarkers. In both approaches, exposure assessment is expressed as probable daily intake (PDI). For the risk characterization, the outputs of exposure, namely the daily intake values, are compared with the reference dose (Assunção et al., 2015). Exposure assessments based on food consumption and occurrence data have important limitations due to the heterogeneous distribution of mycotoxins in foods and to the limited accuracy of food consumption data (Heyndrickx et al., 2015). These caveats may be overcome with the measurement of specific urinary biomarkers to assess exposure to mycotoxins, since biomarker excretion correlates well with the intake of some mycotoxins (Qian et al., 1994; Groopman and Kensler, 1999; Rodríguez-Carrasco et al. 2014, 2015). Urinary biomarkers suitable for AFB_1 and ZEN are AFM_1, AFP_1 and AFQ_1 (Groopman and Kensler, 1999), and non-metabolized ZEN + α-zearalenol (α-ZEL) + β-zearalenol (β-ZEL) (Solfrizzo et al., 2014), respectively. Non-metabolized FB_1, OTA and DON + de-epoxideoxynivalenol 1 (DOM-1) + 15-acetyl-DON (15-Ac-DON) are urinary biomarkers for FB_1, OTA and DON, respectively (Solfrizzo et al., 2014). Because these biomarkers are excreted in urine as free and conjugated forms, urine samples are generally digested with β-glucuronidase/sulfatase to deconjugate the conjugated forms, to increase the concentration and detectability of the free analytes (Solfrizzo et al., 2011). In this context, human biomonitoring of urinary biomarkers of mycotoxins may provide adequate exposure data for a more accurate risk characterization of mycotoxins in foods.

Human biomonitoring studies using urinary biomarkers for mycotoxins in Brazil have only been carried out for aflatoxins, and were based on the assessment of AFM_1 (Romero et al., 2010; Jager et al., 2014, 2016) and AFB_1-N^7-guanine in urine (Jager et al., 2016), which is a biomarker of early carcinogenic effects of AFB_1 (Qian et al., 1994). Thus, the present study aimed to assess the multiple mycotoxin exposure of consumers from São Paulo (SP) and Santa Catarina (SC) (Brazil). So that mycotoxin analysis in food samples and urine biomarkers were done. A dietary recall questionnaire (RQ24h) was used to report the consumption.

2. Materials and methods

2.1. Subject background information

Participants were recruited among residents in small-scale dairy and poultry farms located in the surroundings of Descalvado and Pirassununga cities, Northeast region of the state of São Paulo, and of Pinhalzinho and Erval Velho, Western region of the state of Santa Catarina. The states of São Paulo and Santa Catarina were chosen because they have large number of family-operated farms with a centralized system of regulation and control. The climates in both regions are classified as humid subtropical, with slight differences in their annual mean temperatures and rainfalls: 18-20 °C and 1300–1600 mm without dry season in São Paulo, and 16-18 °C and 1600–1900 mm with dry winter in Santa Catarina (Alvares et al., 2013). The farms from the state of São Paulo contained silos for grain drying. Storage of cereals, in most cases, was carried out in sealed sheds protected from the rain. In the state of Santa Catarina, farms were simpler, with grains stored in old sheds that showed cracks and had no protection against the entry of water, insects, and animals.

The study was submitted to and approved by the Research Ethics Committee (REC) of the School of Animal Sciences and Food Engineering, University of São Paulo (Opinion No. 1.500.317). All volunteers ($N = 86$) were older than 18 years of age (mean of 46.6 ± 17.0 years old), and included 44 women (51.2%) and 42 men (48.8%), with average weight 73.4 ± 15.8 kg. Before starting the experiment, they were invited to sign a Free and Informed Consent Form that was approved by the aforementioned REC, and to answer general questions about their health status. People with signs and/or symptoms of liver or kidney illness or any chronic disease were not included in the study, due to potential interferences with the metabolism of mycotoxins and creatinine.

2.2. Sampling design

Two sampling procedures were carried out, one during April and May (Brazilian Fall), and the other 7–8 months after that, in December (Summer). The total number of small-scale farms visited was 32, and the number of volunteers in the first sampling was 86 (30 in São Paulo and 53 in Santa Catarina). In the second sampling, 76 volunteers agreed to participate in the study (24 in São Paulo and 52 in Santa Catarina). Samples of rice ($N = 66$), bean ($N = 59$), wheat flour ($N = 39$), corn flour ($N = 21$) and corn meal ($N = 18$) were collected if available and stored in the farm households, and were immediately sent to the laboratory for analysis. The total number of food products analyzed was 203. Most of the food products collected were industrialized products that were previously purchased by volunteers in supermarkets in nearby cities, and were available in their original packages (0.5–1.0 kg) in the households at the time of sampling. Because not all types of food products were available in each farm at the time of sampling, the number of samples of each type of food varied according to their availability in the farms visited. Upon arrival at the laboratory, all food samples were homogenized, finely milled and kept frozen at −20 °C until the moment of mycotoxin determination. During the visit to each farm, volunteers were instructed to collect their first morning urine (min. 10 mL) in the following day after food sample collection. After collection, urine samples were transported to the laboratory in a cooler with dry ice, and kept frozen at −20 °C until analysis. The total number of urine samples analyzed in the two sampling periods was 162. Fig. 1 presents an overview of the experimental protocol including sampling procedures adopted in the study.

2.3. Food consumption questionnaires

At the time of food and urine sample collection, participants were instructed to complete a dietary recall questionnaire (RQ24h) describing the consumption of foods in the 24 h before sample collection, including those that are usually considered of higher risk for mycotoxin contamination and regulated in Brazil (Anvisa, 2014). In addition, volunteers were instructed to complete a Food Consumption Frequency Questionnaire. This questionnaire estimates the portion of high-risk foods (susceptible to mycotoxins occurrence) eaten by volunteers per unit of time. Consumption frequencies were estimated on the basis of

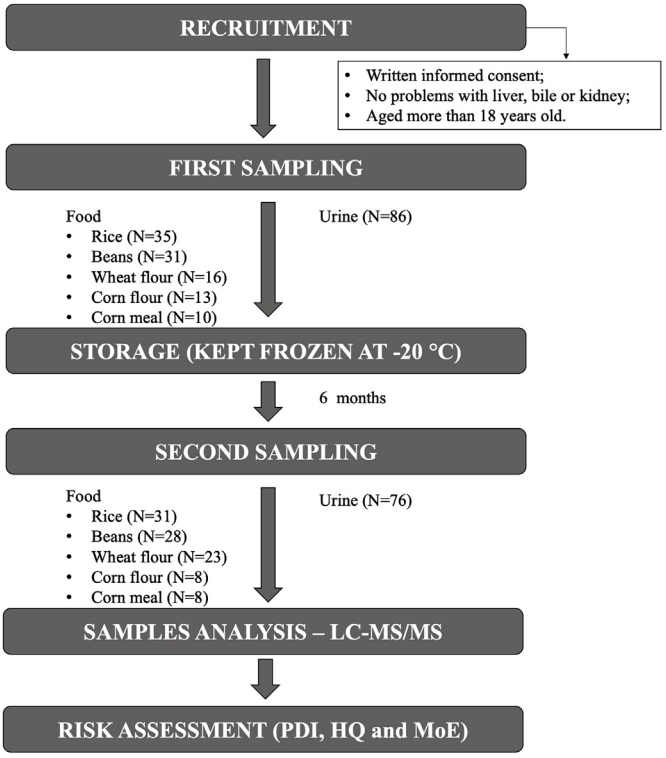

Fig. 1. Flow chart describing the experimental procedures adopted in the study. N: number of collected samples.

the following terms: "Never", "Less than once a month", "1–2 times a month", "3–4 times a week", "Every day" and "2 times a day". Portions were estimated in grams, based on home measures that were common to the participants, such as cups, spoons, etc. (Jager et al., 2013). RQ24h values were used to estimate the dietary intake of mycotoxins.

2.4. Reagents and solutions

Analytical grade reagent and Milli-Q (Millipore, Bedford, MA, USA) water, HPLC-grade acetonitrile and methanol (JT Baker, Xalostoc, Mexico) were used in all laboratory procedures. Mycotoxin standards (AFM_1, AFB_1, AFB_2, AFG_1, AFG_2, AFP_1, AFQ_1, OTA, FB_1, FB_2, ZEN, α-ZEL, β- ZEL, DON, DOM-1, 15-Ac-DON, T-2 and HT-2) were purchased from Sigma (Sigma, St Louis, MO, USA). Isotopically labeled standards (IS) of [$^{13}C_{17}$]-AFB_1 and [$^{13}C_{17}$]-AFM_1 (Sigma, St. Louis, MO, USA), [$^{13}C_{20}$]-OTA, [$^{13}C_{34}$]−FB_1, [$^{13}C_{18}$]-ZEN and [$^{13}C_{15}$]-DON (Biopure, Romer Labs, Tulln, Austria) were also used. Individual stock solutions were prepared in water/acetonitrile.

2.5. Preparation of food samples

Extraction of mycotoxins from food products was performed strictly following the procedures as described by Sulyok et al. (2007), including minor modifications as proposed by Franco et al. (2018). Briefly, food samples were finely grinded and weighed (1.0 g) in duplicate 15-mL Falcon tubes. Then, 4 mL of the acetonitrile/water/acetic acid extraction solvent (80:20:0.1%, v/v/v) were added to the tubes, which were vortexed for 1 min, and homogenized in shaker (Tecnal) for 60 min. After that, they were vortexed again and kept under stirring for other 30 min. Samples were then centrifuged at 3000 rpm (Centrifugal Quimis, Brazil) for 5 min. Supernatants were removed and filtered through 0.22 μm membrane filters (Millex, Millipore Corp.). An aliquot of 80 μL of the solution was transferred to a glass insert placed in a vial, and mixed with 20 μL of a mixture of IS previously prepared in water/acetonitrile (1:1, v/v) at the concentrations of 5 ng mL^{-1} for [$^{13}C_{17}$]-AFB_1, [$^{13}C_{20}$]-OTA, [$^{13}C_{24}$]-T-2 and [$^{13}C_{34}$]−FB_1; 250 ng mL^{-1} for [$^{13}C_{18}$]-ZEN and 500 ng mL^{-1} for [$^{13}C_{15}$]-DON. Final concentrations in each sample extract were 1 ng mL^{-1} for [$^{13}C_{17}$]-AFB_1, [$^{13}C_{20}$]-OTA, [$^{13}C_{24}$]-T-2, [$^{13}C_{34}$]−FB_1, 50 ng mL^{-1} for [$^{13}C_{18}$]-ZEN and 100 ng mL^{-1} for [$^{13}C_{15}$]-DON. Calibration curves were prepared with working solutions in water/acetonitrile (1:1, v/v) at levels ranging from 0.05 to 4.0 ng mL^{-1} of AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, ZEN and T-2, 0.15–12.0 ng mL^{-1} of HT-2, and 0.37–30 ng mL^{-1} of DON.

2.6. Preparation of urine samples

The analysis of multiple mycotoxin residues and biomarkers in human urine was performed as described by Solfrizzo et al. (2011), with minor modifications. Prior to the extraction of mycotoxins in urine, samples were centrifuged at 3000 × *g* for 5 min, to remove particulate matter and supernatants. After this procedure, 300 μL of β-glucuronidase/sulfatase was added to 6 mL of sample for the enzymatic deconjugation of mycotoxins. After this procedure, samples were incubated under static conditions at 37 °C overnight. Samples were then diluted in ultra-pure water (1:1, v/v), and 20 μL of an IS solution containing 3 μg mL^{-1} of [$^{13}C_{17}$]-AFM_1, [$^{13}C_{20}$]-OTA, [$^{13}C_{34}$]−FB_1, [$^{13}C_{24}$]-T-2 and [$^{13}C_{18}$]-ZEN and 6 μg mL^{-1} of [$^{13}C_{15}$]-DON was added to each sample. Immuno-affinity columns (Oasis HLB) and (Mycosep6in1) (Vicam, Watertown, MA, USA) were used for sample clean-up. The Oasis HLB column was attached under the Mycosep6in1 column (empty). For the Oasis HLB column conditioning, 2 mL of methanol was added, followed by 2 mL of ultra-pure water. The diluted urine sample previously prepared was passed through the two stacked columns, and then the two columns were separated and treated differently. The mycotoxins in the Mycosep6in1 column were eluted with methanol strictly following the procedures of Solfrizzo et al. (2011). The Oasis HLB column was washed with 1 mL of methanol:water (2:8, v/v), then vacuum-dried for 15 s. The mycotoxins were eluted from the column with 1 mL methanol:water (4:6, v/v) by gravity, and collected in the same vial containing the eluate from the Mycosep6in1 columns. The combined, final eluates were dried under N_2, re-suspended in methanol-water solution (1:9, v/v), filtered through 0.22 μm membrane filters (Millex, Millipore Corp.), and reserved for analysis of by liquid chromatography coupled to mass spectrometry (LC-MS/MS). For the calibration curves, working solutions were prepared in water/acetonitrile (9:1, v/v) at concentrations ranging from 0.01 to 0.3 ng mL^{-1} of AFM_1, AFP_1, AFQ_1, FB_1, FB_2, OTA, T-2, HT-2, ZEN, α-ZEL and β- ZEL, and 0.1–5.0 ng mL^{-1} for DON, DOM-1 and 15-Ac-DON.

2.7. Liquid chromatographic and mass spectrometry conditions

Final extracts from food and urine samples were analyzed in a Waters Acquity I-Class UPLC system (Waters, Milford, MA, USA) equipped with a BEH C_{18} column (2.1 × 50 mm, 1.7 μm) and coupled to a Xevo TQ-S mass spectrometer (Waters, Milford, MA, USA). For chromatographic separation, extracted samples and standards, 10 μL of food samples or 5 μL of urine samples were injected into the LC-MS/MS system. The column was kept at 40 °C during the analyses, and samples were maintained at 15 °C. The mobile phase was composed by water (eluent A) and acetonitrile (eluent B), both containing 0.1% of formic acid. For elution of injected samples, percentage of eluent A was kept at 95% for 0.5 min. After this period, percentage of eluent B was linearly raised to 25% over 4.5 min (5.0 min). Then, eluent B was increased to 90% over 0.5 min, followed by a hold time of 0.25 min (5.5 min). After that, percentage of eluent B was reduced to 5% over 0.5 min (6.0 min), and the column re-equilibrated to the initial conditions for 0.5 min. Total chromatographic run time was 6.5 min, and the mobile phase flow rate was maintained at 0.5 mL min^{-1}. The mass spectrometer was operated in MRM mode using electrospray ionization in either positive or negative ion mode. Mass spectrometry parameters were as follows: capillary voltage: 0.75 kV; source temperature: 150 °C; desolvation

temperature: 500 °C, desolvation gas flow: 800 L h^{-1}; cone gas flow: 150 L h^{-1}. Cone voltage, collision energy, and MRM transitions (major precursor ion > fragment ion) were manually optimized for individual mycotoxins. Data collection and processing was performed using software MassLynx version 4.1.

2.8. Performance of the analytical methods

The analytical method for foods was previously validated for corn products (Franco et al., 2018). Thus, the performances of the analytical methods were evaluated using blank samples of rice, bean and wheat flour, as well as urine. Parameters evaluated included limits of detection (LOD) and quantification (LOQ) (calculated based on signal-to-noise ratios of 3:1 and 10:1, respectively, of peaks of confirmatory MRM transitions); apparent recovery (RA); linearity; signal suppression/enhancement (SSE) due to matrix effects; and extraction recovery (RE). All these parameters were determined based on calibration curves constructed from the analytical data obtained in samples spiked before extraction, spiked after extraction, and standards diluted in solvent, with the addition of the IS working solution in all prepared samples.

Spiked food samples were prepared exactly as described by Franco et al. (2018) in triplicate tubes containing blank samples (1.0 g) at concentrations ranging from 1.25 to 20 μg kg^{-1} for AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, T-2, HT-2 and ZEN, and 18.7–150 μg kg^{-1} for DON. The spiked samples were submitted to the same extraction procedures as described for samples collected in the farms. For the matrix-matched calibration curves, spiked extracts were prepared in triplicate with blank samples (1.0 g) of each type of product at concentrations ranging from 0.25 to 4.0 ng mL^{-1} for AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, T-2, HT-2 and ZEN, and 3.7–30 ng mL^{-1} for DON. The same levels were used to prepare standard solutions in water/acetonitrile (9:1, v/v). Eighty μL of each level of final extracts from spiked samples, spiked extracts and liquid standards were combined with 20 μL of the IS working solution in glass inserts inside amber vials and stored at −20 °C until analysis by LC-MS/MS.

Urine fortified samples were prepared by adding appropriate volumes of standard working solutions to triplicate tubes containing blank samples (urine:ultrapure water, 1:1, v/v) before the elution, to achieve concentrations ranging from 0.01 to 0.3 ng mL^{-1} for AFM_1, AFP_1, AFQ_1, FB_1, FB_2, OTA, T-2, HT-2, ZEN, α-ZEL and β- ZEL, and 0.1–5.0 ng mL^{-1} for DOM-1, DON, 15-Ac-DON. Clean-up and elution procedures were carried out as described in section 2.6. Standard solutions in water/acetonitrile (9:1, v/v) and matrix-matched calibration curves were prepared by spiking triplicate blank extracts with appropriate volumes of the mycotoxin working solutions to reach the same concentrations as described for spiked samples. For all urine samples, 20 μL of the IS solution was added before elution to 80 μL of each level from fortified samples, spiked and liquid standards. Values for RA (Eq. (1)), SSE (Eq. (2)) and RE (Eq. (3)) for the analytical methods for foods and urine were calculated as proposed by Sulyok et al. (2006) and Varga et al. (2012).

$$\text{RA (\%)} = 100^{*} \text{ slope spiked sample / slope standard diluted in solvent} \quad (1)$$

$$\text{SSE (\%)} = 100^{*} \text{ slope spiked extract / slope standard diluted in solvent} \quad (2)$$

$$\text{RE (\%)} = 100^{*}\text{RA / SSE} \quad (3)$$

2.9. Creatinine analysis in human urine

Creatinine analysis was performed in urine samples using a commercial kit (Bioplus - Bio 200), with two-point kinetic assay based on the Jaffe reaction principle (Vasiliades, 1976). The concentration of creatinine in each urine sample was used to correct differences in dilution between individuals and excretion rates, and the results were expressed in ng mycotoxin/mg creatinine.

2.10. Exposure assessment (probable daily intakes estimates) and risk characterization

In the present study, exposure assessment was determined based on estimated intake through food (indirect approach) and through urine biomarkers (direct approach). Eq. (4) shows the calculation of PDI through food, according to Assunção et al. (2015). Occurrence (analyzed samples) and consumption (reported by the participants in the RQ24h) data were used to determine PDI. For the PDI calculations and risk characterization purposes, the results of samples presenting < LOQ were replaced with zero.

$$\text{PDI} = \frac{\text{Occurrence}^{*}\ \text{Consumption}}{\text{bw}^{*}\ 1000} \quad (4)$$

- PDI = Probably Daily Intake (μg kg^{-1} bw day^{-1});
- Occurrence = mycotoxin content (μg.kg^{-1}) determined in food analysis;
- bw = body weight (kg) reported by volunteers;
- Consumption = reported consumption (g) of food on the previous day.

The methodology described by Turner et al. (2010) was used to calculate the PDI through urinary biomarkers data (Eq. (5)).

$$\text{PDI} = \frac{\text{Occurrence} * \text{V}}{\text{ER} * \text{bw} * 1000} \quad (5)$$

- PDI = Probably Daily Intake (μg kg^{-1} bw day^{-1});
- Occurrence = mycotoxin content (ng mL^{-1}) determined in urine analysis;
- V = daily urine production of adults, assumed to be 1500 mL (Turner et al., 2010);
- bw = body weight (kg) reported by volunteers;
- ER = urinary excretion ratio of AFM_1 for women: 1.5% (Zhu et al., 1987);
  = urinary excretion ratio of AFM_1 for men: 1.7% (Zhu et al., 1987);
  = urinary excretion ratio of DON for women: 72% (Vidal et al., 2018);
  = urinary excretion ratio of DON for men: 50% (Vidal et al., 2018);
  = urinary excretion ratio of OTA: 50% (Schlatter et al., 1996);
  = urinary excretion ratio of FB_1: 0.5% (Riley et al., 2012);
  = urinary excretion ratio of ZEN: 36.8% (Gambacorta et al., 2013).

Risk characterization was performed comparing PDI values with dose reference values of tolerable daily intake (TDI) for OTA (0.016 μg kg^{-1} bw.day^{-1}) (FAO/WHO, 2007), FBs (2 μg kg^{-1} bw day^{-1}) (FAO/WHO, 2011a) DON (1.0 μg kg^{-1} bw day^{-1}) (FAO/WHO, 2011b) and ZEN (0.25 μg kg^{-1} bw day^{-1}) (EFSA, 2016). Comparisons were performed using hazard coefficients (HQ) ratio between exposure and a reference dose) as referred at Eq. (6). HQ < 1 indicated tolerable exposure and a HQ > 1 ratio indicated a non-tolerable exposure level (Borg et al., 2013; EFSA, 2013).

$$\text{HQ} = \frac{\text{PDI}}{\text{TDI}} \quad (6)$$

- HQ = Hazard Quotient;
- PDI = Probable Daily Intake (μg kg^{-1} bw day^{-1});
- TDI = Tolerable Daily Intake (μg kg^{-1} bw day^{-1}).

Considering the carcinogenic potential of aflatoxins, the Margin of Exposure (MoE) was calculated for exposure to this toxin (Eq. (7)) as a ratio of the Benchmark Dose Lower Confidence Limit ($BMDL_{10}$) and the level of exposure (PDI). MoE indicates the risk level, with MoE ≥10,000 being of low public health concern, and MoE < 10,000 being of high public health concern (EFSA, 2013). For aflatoxins, the $BMDL_{10}$ value was in accordance with Benford et al. (2010).

$$MoE = \frac{BMDL_{10}}{PDI} \quad (7)$$

- MoE = Margin of Exposure;
- $BMDL_{10}$ = Benchmark Dose Lower Confidence Limit (0.25 μg kg^{-1} bw day^{-1});
- PDI = Probably Daily Intake (μg kg^{-1} bw day^{-1}).

2.11. Statistical analysis

Data were statistically analyzed using an IBM SPSS Statistics 23 software. The differences in the PDI of each mycotoxin evaluated in the two sampling periods and places of sampling were evaluated using a non-parametric Mann-Whitney test, considering a 95% confidence interval and $P < 0.05$. For the purpose of data analysis, only positives samples (mycotoxin concentration above the LOQ) were considered.

3. Results

3.1. Performance of the analytical methods

Table 1 presents the performance parameters of the analytical method for determination of mycotoxins in rice, bean, and wheat flour samples. The LOD and LOQ values for individual mycotoxins ranged from 0.12 to 6.1 μg kg^{-1} and 0.3–18.8 μg kg^{-1}, respectively. R_A, SSE and R_E values for mycotoxins ranged from 59 to 127%, 63–113% and 76–143%, respectively. The same parameters were determined for the analytical method used for determination of mycotoxin biomarkers in urine samples, and the results are presented in Table 2. LODs and LOQs values ranged from 0.001 to 0.633 ng mL^{-1} and 0.003–2.000 ng mL^{-1}, respectively. The determined R_A, SSE and R_E values ranged from 56 to 93%, 62–103% and 80–113%, respectively.

3.2. Occurrence of mycotoxins in food products

Table 3 presents the mycotoxin levels in food samples collected in households of small-scale farms from the states of São Paulo and Santa Catarina. All types of foods showed positive samples containing at least one type of mycotoxin above LOQ, comprising 38% of rice ($N = 66$), 12% of bean ($N = 59$), 97% of wheat flour ($N = 39$), 100% of corn flour ($N = 21$), and 94% of corn meal ($N = 18$) samples. AFs were found in two samples of rice (3%), one sample of bean (2%), and two samples of corn flour (10%), with the highest median of total AF observed in rice samples (2.9 μg kg^{-1}). DON was found in all types of foods, with median values of 13.2 μg kg^{-1} (rice), 51.3 μg kg^{-1} (bean), 408.2 μg kg^{-1} (wheat flour), 56.7 μg kg^{-1} (corn flour), and 51.8 μg kg^{-1} (corn meal). OTA was only found in three samples (8%) of wheat flour with a median value of 0.9 μg kg^{-1}. T-2 toxin was found in only one sample of rice (1.5%) containing 1.0 μg kg^{-1}, which is close to the LOQ values (Table 1). However, HT-2 was not detected in any sample evaluated. FBs were also detected in all types of food, except for bean samples, with median levels (FB_1 + FB_2) of 1.5, 14.6, 131.6 and 131.9 μg kg^{-1} in rice, wheat flour, corn flour, and corn meal, respectively. ZEN was detected in 18 (27%) samples of rice, 4 (19%) of corn flour and a single sample of corn meal (6%), at median values of 4.9, 98.6 and 10.7 μg kg^{-1}, respectively.

3.3. Estimation of mycotoxin exposure through food data

Total PDI estimates through food are presented in Table 4. PDI mean values were calculated for mycotoxins with reference values available for AF, DON, OTA, FB and ZEN, and based on the intake of foods as reported in the RQ24h, the body weight of the volunteers (Supplementary material A) and the mycotoxin occurrence data. For total AF, only rice and bean contributed for the PDI at mean values of 0.013 ± 0.007 and 0.007 μg kg^{-1} bw day^{-1}, respectively. All types of foods analyzed contributed for a PDI of DON at mean values ranging

Table 1
Method performance parameters for determination of mycotoxins in samples of rice, bean and wheat flour.

Mycotoxin	RT (min)	Mass (g/mol)	Molecular ion	Transition (*m/z*)	Concentration range (μg kg^{-1})	R_A range (%)	SSE range (%)	R_E range (%)	LOD (μg kg^{-1})	LOQ (μg kg^{-1})
AFB_1	4.80	312.3	$[M+H]^+$	312.7 > 284.9[a] 312.7 > 241.1[b]	1.25–20	105–110	79–99	109–140	0.4–0.5	0.8–1.0
AFB_2	4.50	314.3	$[M+H]^+$	314.7 > 259.0[a] 314.7 > 287.0[b]	1.25–20	64–106	68–103	94–102	0.4–0.5	0.8–1.0
AFG_1	4.46	328.3	$[M+H]^+$	328.9 > 243.0[a] 328.9 > 199.5[b]	1.25–20	89–107	95–113	94–98	0.4–0.5	0.8–1.0
AFG_2	4.18	330.3	$[M+H]^+$	330.9 > 245.0[a] 330.9 > 188.9[b]	1.25–25	64–103	84–97	76–108	0.4–0.6	0.9–1.0
DON	1.98	296.3	$[M+H]^+$	397.3 > 249.1[a] 397.3 > 231.1[b]	18.7–150	101–103	63–109	93–105	5.8–6.1	18.5–18.8
OTA	5.99	403.1	$[M+H]^+$	404.0 > 238.9[a] 404.0 > 357.9[b]	1.25–20	84–106	92–99	92–109	05.-0.7	1.0–1.1
HT-2	5.50	424.2	$[M + NH_4]^+$	442.2 > 263.3[a] 442.2 > 215.4[b]	15–60	97–105	99	98–106	6.0–6.5	15.0–16.0
T-2	5.91	466.2	$[M + NH_4]^+$	484.2 > 305.2[a] 484.2 > 185.0[b]	1.25–20	105–115	97–98	105–108	0.3–0.6	1.0–1.4
FB_1	5.40	721.8	$[M+H]^+$	722.5 > 334.0[a] 722.5 > 352.1[b]	2.5–25	59–109	98–102	97–111	0.9–1.0	2.0–2.8
FB_2	3.74	705.8	$[M+H]^+$	706.5 > 336.2[a] 706.5 > 318.3[b]	2.5–25	91–101	90–99	101–102	07–0.9	2.0–2.5
ZEN	5.98	318.1	$[M-H]^-$	317.1 > 175.1[a] 317.1 > 130.9[b]	0.25–20	97–127	89–98	95–143	0.12–0.23	0.3–0.6

RT: retention time; R_A: apparent recovery; SSE: signal suppression/enhancement; R_E: extraction recovery; LOD: limit of detection; LOQ: limit of quantification; AF: aflatoxin; DON: deoxynivalenol; OTA: ochratoxin A; FB: fumonisin; ZEN: zearalenone.

[a] Transitions used for quantification.

[b] Transitions used for confirmation.

Table 2
Method performance parameters for determination of mycotoxins' biomarkers in urine samples.

Mycotoxin	RT (min)	Mass (g/mol)	Molecular ion	Transition (*m/z*)	Concentration range (ng mL^{-1})	R_A (%)	SSE (%)	R_E (%)	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
AFM_1	4.03	328.3	$[M+H]^+$	329.0 > 273.1[a] 329.0 > 243.0[b]	0.01–0.27	81	101	80	0.001	0.004
AFP_1	4.46	328.3	$[M+H]^+$	328.9 > 243.0[a] 328.9 > 199.5[b]	0.05–0.8	67	82	82	0.013	0.050
AFQ_1	4.50	314.3	$[M+H]^+$	314.7 > 259.0[a] 314.7 > 287.0[b]	0.05–0.8	51	62	81	0.020	0.067
DON	1.98	296.3	$[M+H]^+$	397.3 > 249.1[a] 397.3 > 231.1[b]	0.10–5.0	75	90	84	0.333	1.223
DOM-1	2.58	282.1	[M+Ac]-	339.2 > 249.1[a] 339.2 > 59.1[b]	0.10–5.0	90	80	113	0.200	0.310
15-Ac-DON	3.38	338.3	[M+Ac]-	397.2 > 337.2[a] 397.2 > 307.2[b]	0.10–5.0	70	87	81	0.633	2.000
OTA	5.99	403.1	$[M+H]^+$	404.0 > 238.9[a] 404.0 > 357.9[b]	0.01–0.27	57	71	80	0.005	0.017
T-2	5.91	466.2	$[M + NH_4]^+$	484.2 > 305.2[a] 484.2 > 185.0[b]	0.01–0.27	59	75	80	0.013	0.040
HT-2	5.50	424.2	$[M + NH_4]^+$	442.2 > 263.3[a] 442.2 > 215.4[b]	0.05–0.8	56	72	80	0.024	0.075
FB_1	5.40	721.8	$[M+H]^+$	722.5 > 334.0[a] 722.5 > 352.1[b]	0.01–0.27	81	100	81	0.003	0.007
FB_2	3.74	705.8	$[M+H]^+$	706.5 > 336.2[a] 706.5 > 318.3[b]	0.01–0.27	82	103	80	0.001	0.013
ZEN	5.98	318.1	$[M-H]^-$	317.1 > 175.1[a] 317.1 > 130.9[b]	0.01–0.27	93	100	93	0.001	0.003
α-ZEL	5.53	320.2	$[M-H]^-$	319.1 > 275.2[a] 319.1 > 160.2[b]	0.05–0.8	92	90	103	0.053	0.183
β-ZEL	5.76	320.2	$[M-H]^-$	319.1 > 275.2[a] 319.1 > 160.2[b]	0.05–0.8	90	91	99	0.060	0.200

RT: retention time; R_A: apparent recovery; SSE: signal suppression/enhancement; R_E: extraction recovery; LOD: limit of detection; LOQ: limit of quantification; AF: aflatoxin; DON: deoxynivalenol; DOM-1: de-epoxdeoxynivalenol; 15-Ac-DON: 15- acetyl-DON; OTA: ochratoxin A; FB: fumonisin. ZEN: zearalenone; α-ZEL: α-zearalenol; β-ZEL: β-zearalenol.
[a] Transitions used for quantification.
[b] Transitions used for confirmation.

Table 3
Occurrence of mycotoxins in food samples collected in small-scale farms from the states of São Paulo and Santa Catarina, Brazil.

	n (%)	Rice (*N* = 66)	*n* (%)	Bean (*N* = 59)	*n* (%)	Wheat flour (*N* = 39)	*n* (%)	Corn flour (*N* = 21)	*n* (%)	Corn meal (*N* = 18)
		Median (Range) (μg kg^{-1})		Median (Range) (μg kg^{-1})		Median (Range) (μg kg^{-1})		Median (Range) (μg kg^{-1})		Median (Range) (μg kg^{-1})
AFB_1	1 (1.5)	3.9	0	< LOQ	0	< LOQ	1 (5)	3.6	0	< LOQ
AFB_2	0	< LOQ	0	< LOQ	0	< LOQ	0	< LOQ	0	< LOQ
AFG_1	0	< LOQ	0	< LOQ	0	< LOQ	1 (5)	1.3	0	< LOQ
AFG_2	1 (1.5)	2.0	1 (2)	2.0	0	< LOQ	0	< LOQ	0	< LOQ
ΣAF	2 (3)	2.9 (2.0–3.9)	1 (2)	2.0	0	< LOQ	2 (10)	2.48 (1.3–3.6)	0	< LOQ
DON	8 (12)	13.2 (7.0–171.7)	6 (10)	51.3 (46.5–60.2)	38 (97)	408.2 (27.8–2203.8[2])	9 (43)	56.7 (43.9–78.6)	2 (11)	51.8 (43.0–60.6)
OTA	0	< LOQ	0	< LOQ	3 (8)	0.9 (0.6–1.4)	0	< LOQ	0	< LOQ
HT-2	0	< LOQ	0	< LOQ	0	< LOQ	0	< LOQ	0	< LOQ
T-2	1 (1.5)	1.0	0	< LOQ	0	< LOQ	0	< LOQ	0	< LOQ
FB_1	0	< LOQ	0	< LOQ	3 (8)	25 (3.2–43.6)	21 (100)	115.6 (6.8–883.0)	16 (89)	122.2 (2.9–796.2)
FB_2	1 (1.5)	1.5	0	< LOQ	4 (10)	101.5 (11.5–272.4)	9 (43)	235.0 (89.8–630.5)	9 (50)	87.0 (5.9–496.5)
ΣFB	1 (1.5)	1.5	0	< LOQ	5 (13)	14.6 (3.2–316.0)	21 (100)	131.6 (6.8–1513.5[3])	17 (94)	131.9 (2.9–1169.2)
ZEN	18 (27)	4.9 (1.7–230.0[1])	0	< LOQ	0	< LOQ	4 (19)	98.6 (8.7–508.9[4])	1 (6)	10.7

[1] One sample with concentration above the maximum permitted level (MPL) for ZEN in rice (100 μg kg^{-1}) in Brazil (Agência Nacional de Vigilância Sanitária, 2014).
[2] Five samples with concentration above the MPL for DON in wheat flour (750 μg kg^{-1}) in Brazil (Agência Nacional de Vigilância Sanitária, 2014).
[3,4] One sample with concentration above the MPL for total FB and two samples above the MPL for ZEN in corn flour (1500 and 150 μg kg^{-1}, respectively) in Brazil (Agência Nacional de Vigilância Sanitária, 2014).
n: Number of samples with concentrations above the limit of quantification (LOQ), see Table 1 for LOQ of each mycotoxin.
AF: aflatoxin; DON: deoxynivalenol; OTA: ochratoxin A; FB: fumonisin; ZEN: zearalenone; NA: Not applicable (no regulations in Brazil).

from 0.069 ± 0.032 to 1.002 ± 0.772 μg kg^{-1} bw day^{-1}. The PDI of total FB contributed by wheat flour, corn flour and corn meal ranged from 0.119 ± 0.133 to 0.321 ± 0.312 μg kg^{-1} bw day^{-1}. Regarding ZEN, only rice and corn flour contributed for mean PDI values of 0.156 ± 0.310 and 0.013 ± 0.001 μg kg^{-1} bw day^{-1}.

Individual PDI estimates through contaminated food products in the two sampling periods are presented in Fig. 2. Statistical differences ($P < 0.05$) between the first and the second sampling were observed in the PDI values of FBs and ZEN, both being higher in the second collection. For FBs, PDI values varied from 0.20 ± 0.21 to 0.52 ± 0.49 μg kg^{-1} bw day^{-1}, in the first and second sampling, respectively. For ZEN, PDI varied from 0.04 ± 0.05 to 0.32 ± 0.43 μg kg^{-1} bw day^{-1} in the first and second sampling, respectively. PDI estimates through contaminated foods collected in small-scale farms from different states of Brazil are presented in Fig. 3. Overall the highest values were found in the state of Santa Catarina, although no significant differences were found except for total FB, which was significantly lower ($P < 0.05$) in São Paulo.

Table 4
Probable daily intake (PDI) of mycotoxins based on their levels in foods consumed by volunteers in small-scale farms from the states of São Paulo and Santa Catarina, Brazil[a].

Mycotoxin	PDI (μg kg^{-1} bw day^{-1})[b]				
	Rice	Bean	Wheat flour	Corn flour	Corn meal
AFB_1	0.018 ± 0.001	0	0	0	0
AFG_2	0.007 ± 0.001	0.007 ± 0.00	0	0	0
ΣAF	0.013 ± 0.007	0.007 ± 0.00	0	0	0
DON	0.069 ± 0.073	0.086 ± 0.044	1.002 ± 0.772	0.124 ± 0.070	0.069 ± 0.032
OTA	0	0	0	0	0
FB_1	0	0	0.032 ± 0.019	0.257 ± 0.234	0.311 ± 0.316
FB_2	0	0	0.177 ± 0.151	0.475 ± 0.153	0.351 ± 0.344
ΣFB	0	0	0.119 ± 0.133	0.305 ± 0.235	0.321 ± 0.312
ZEN	0.156 ± 0.310	0	0	0.013 ± 0.001	0

AF: aflatoxin; DON: deoxynivalenol; OTA: ochratoxin A; FB: fumonisin; ZEN: zearalenone.
[a] Results are expressed as mean ± standard deviation of individual PDI values, calculated considering food samples with concentrations above the limit of quantification (LOQ) and food consumption data from each volunteer in the RQ24h (Supplementary material A).
[b] Calculated as follows: PDI = (occurrence rate x consumption)/(body weight x 1000).

3.4. Occurrence of mycotoxin biomarkers in human urine

Table 5 presents the levels of mycotoxins biomarkers in positive samples (above LOQs) of urine ($N = 162$) collected in small-scale farms from the states of São Paulo and Santa Catarina. DON was the most frequent mycotoxin found in urine, with 88% of positive samples at levels ranging from 0.62 to 72,439 ng mg^{-1} creatinine (median: 12.0 ng mg^{-1} creatinine). However, DOM-1 was detected in only one sample (0.6%) at 7.3 ng mg^{-1} creatinine. Concerning AFs, only AFM_1 and AFP_1 were detected in 17% of urine samples, at median levels of 0.02 ng mg^{-1} of creatinine. OTA and ZEN were detected in 27% and 7% of samples, respectively, with the same median value for both mycotoxins (0.02 ng mg^{-1} creatinine). FB_1 was present in 23% of the samples at 0.06 ng mg^{-1} creatinine. AFQ_1, 15-Ac-DON, T-2, HT-2, FB_2, α-ZEL and β- ZEL were not detected in any urine sample.

3.5. Estimation of mycotoxin exposure through urinary levels

PDI estimates through urinary levels of mycotoxins biomarker are shown in Table 6. The PDI values were calculated according to previously described urinary excretion rates for AFs, DON, OTA, FBs and ZEN. In our study, the mean PDI ranged from 0.001 ± 0.002 to 84.914 ± 469.333 μg g^{-1} bw day^{-1} for AFM_1 and DON, respectively. When comparing the differences between the first and second sampling (Fig. 4), urinary PDI for DON and OTA were greatly reduced from 172.31 ± 661.92 to 1.11 ± 1.35 μg kg^{-1} bw day^{-1}, and from 0.0538 ± 0.1443 to 0.0081 ± 0.290 μg kg^{-1} bw day^{-1}, respectively. However, the mean PDI for FB_1 did not vary ($P > 0.05$) between the two sampling periods. Mean PDI values for DON, OTA, and FB_1 in the state of Santa Catarina were higher than the values found in the state of São Paulo, as presented in Fig. 5, although differences were significant ($P < 0.05$) only for DON.

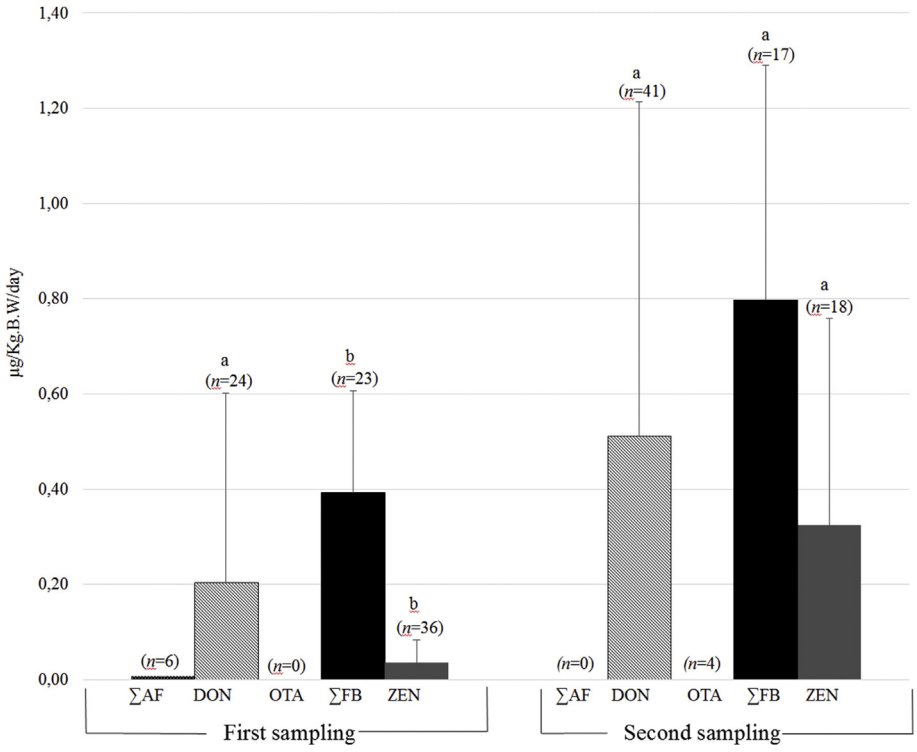

Fig. 2. Estimated dietary intake of mycotoxins in positive samples (*n*) based on consumed food levels in two sampling periods. Results are expressed as mean ± SD. For the same type of mycotoxin, bars with different superscript letters differ significantly ($P < 0.05$). Total AF: sum of aflatoxins B_1, B_2, G_1 and G_2; DON: deoxynivalenol (DON); OTA: ochratoxin A; Total FB: sum of fumonisins B_1 and B_2; ZEN: zearalenone.

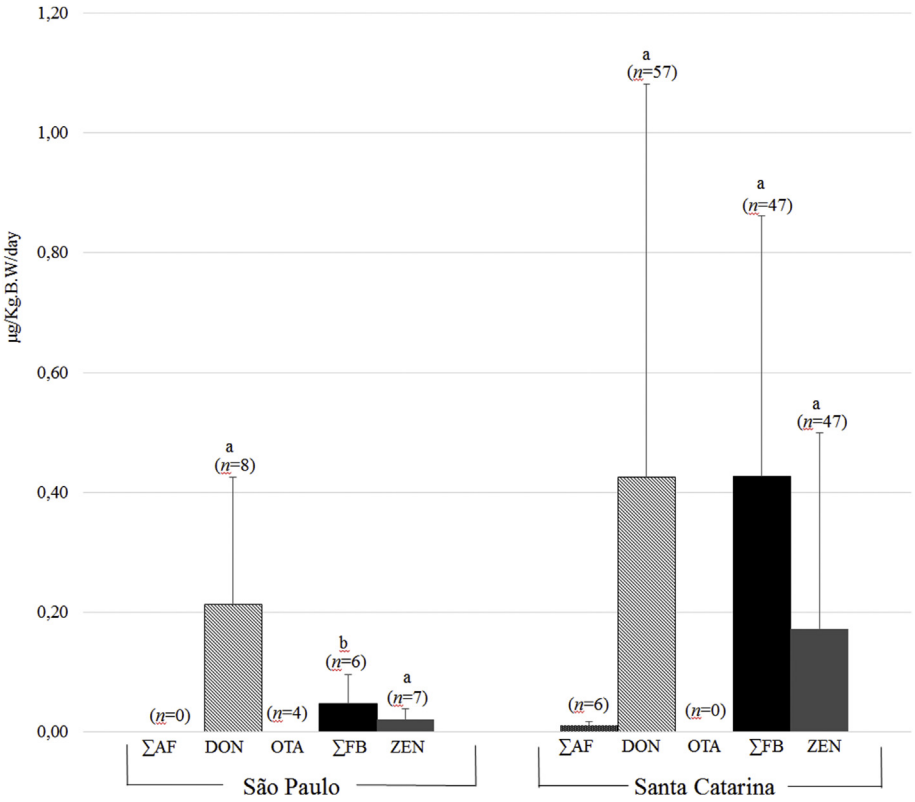

Fig. 3. Estimated dietary intake of mycotoxins in positive samples (n) based on consumed food levels in the states of Santa Catarina and São Paulo, Brazil. Results are expressed as mean ± SD. For the same type of mycotoxin, bars with different superscript letters differ significantly ($P < 0.05$). Total AF: sum of aflatoxins B_1, B_2, G_1 and G_2; DON: deoxynivalenol (DON); OTA: ochratoxin A; Total FB: sum of fumonisins B_1 and B_2; ZEN: zearalenone.

Table 5
Mycotoxin levels in urine samples ($N = 162$) from volunteers in small-scale farms from the states of São Paulo and Santa Catarina, Brazil.

Mycotoxin	*n*	%	Range (ng mg^{-1} creatinine)	Median (ng mg^{-1} creatinine)
AFM_1	20	12	0.0005–0.64	0.02
AFP_1	9	6	0.01–0.08	0.02
AFQ_1	0	0	< LOQ	< LOQ
ΣAF	27	17	0.0005–0.72	0.02
DON	143	88	0.62–72,439	12.0
DOM-1	1	0.6	7.30	–
15-Ac-DON	0	0	< LOQ	< LOQ
OTA	44	27	0.01–11.71	0.02
T-2	0	0	< LOQ	< LOQ
HT-2	0	0	< LOQ	< LOQ
FB_1	37	23	0.01–0.29	0.04
FB_2	0	0	< LOQ	< LOQ
ΣFB	37	23	0.01–0.29	0.04
ZEN	12	7	0.01–0.77	0.02
α-ZEL	0	0	< LOQ	< LOQ
β-ZEL	0	0	< LOQ	< LOQ

n: Number of samples showing concentrations above the limit of quantification (LOQ), see Table 2 for LOQ of each mycotoxin.
AF: aflatoxin; DON: deoxynivalenol; DOM-1: de-epoxdeoxynivalenol; 15-Ac-DON: 15- acetyl-DON; OTA: ochratoxin A; FB: fumonisin. ZEN: zearalenone; α-ZEL: α-zearalenol; β-ZEL: β-zearalenol.

3.6. Risk characterization

Table 7 presents the HQ values derived from the PDI calculated through food data (indirect approach) and urinary biomarkers of mycotoxins (direct approach). For the indirect approach, HQ values greater than 1 was observed only for ZEN in the second sampling (1.07). For the direct approach, HQ values greater than 1 were observed for DON in both sampling periods (172.31 and 1.13), and for OTA in the first sampling (3.36). The MoE and MoET values for AFs related to their respective PDIs obtained in food and urine samples are presents in

Table 6
Probable daily intake (PDI) of mycotoxins based on their urinary excretion ratios (UER) determined in previous studies, and respective urinary levels of volunteers in small-scale farms from the states of São Paulo and Santa Catarina, Brazil[a].

Mycotoxin	UER		PDI (µg kg^{-1} bw day^{-1}) [b]
	%	Reference	
AFM_1	Women: 1.5; Men: 1.7	Zhu et al. (1987)	0.001 ± 0.002
DON	Women: 72; Men: 50	Vidal et al. (2018)	84.914 ± 469.333
OTA	50	Schlatter et al. (1996)	0.031 ± 0.106
FB_1	0.5	Riley et al. (2012)	0.377 ± 0.951
ZEN	36.8	Gambacorta et al. (2013)	0.002 ± 0.004

AFM_1: aflatoxin M_1; DON: deoxynivalenol; OTA: ochratoxin A; FB_1: fumonisin B_1; ZEN: zearalenone.
[a] Results are expressed as mean ± standard deviation of individual PDI values, calculated considering urine samples with concentrations above the limit of quantification (LOQ).
[b] Calculated as follows: PDI = (occurrence rate x daily urine production)/(UER x body weight x 1000).

Table 8. Food samples had concentrations above LOQs for AFB_1 and AFG_2 only in the first sampling. For both types of AF, MoE values were much lower than the reference level (10,000) in the first sampling, although no calculation was possible in the second sampling because at that time no sample had concentrations above LOQs for AFB_1 and AFG_2. Regarding the urine samples, MoE values for AFM_1 were 258.29 and 458.71 in the first and second samplings, respectively.

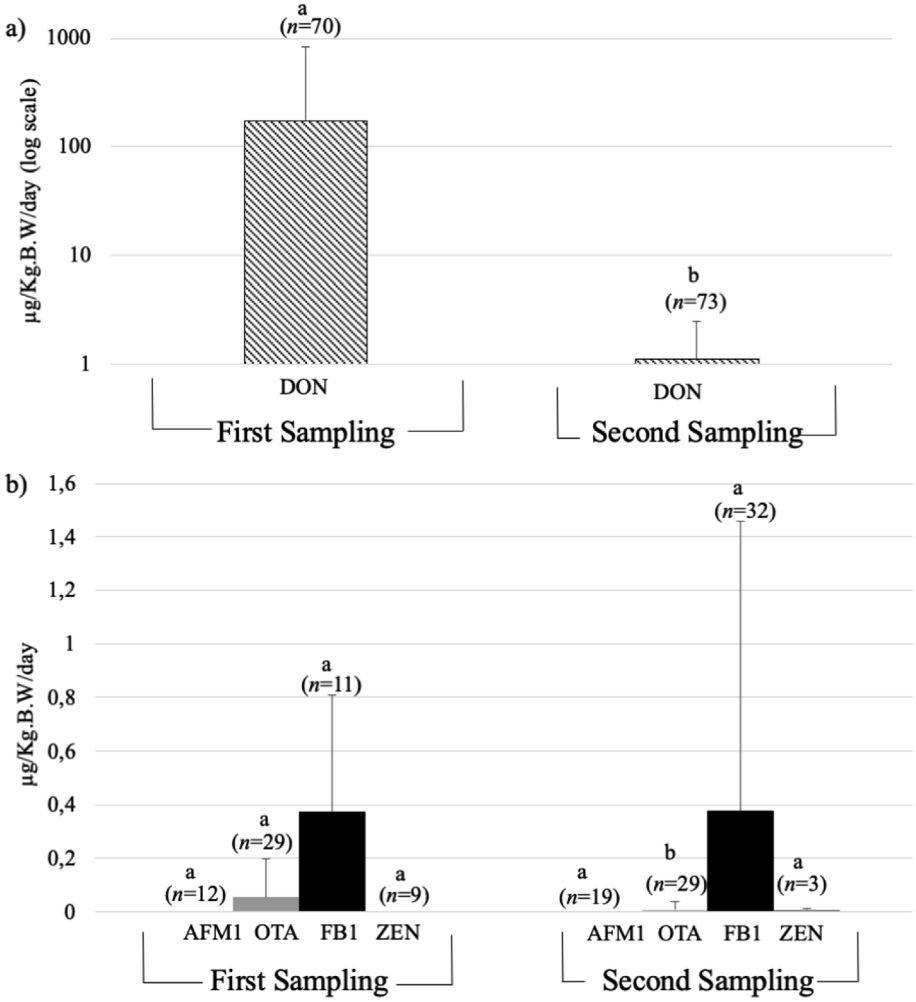

Fig. 4. Estimated dietary intakes based on urinary levels in positive samples (*n*), in two sampling periods, for: a) DON (log scale); b) AFM_1, OTA, FB_1 and ZEN. Results are expressed as mean ± SD. For the same type of mycotoxin, bars with different superscript letters differ significantly ($P < 0.05$). Total AF: sum of aflatoxins B_1, B_2, G_1 and G_2; DON: deoxynivalenol (DON); OTA: ochratoxin A; Total FB: sum of fumonisins B_1 and B_2; ZEN: zearalenone.

4. Discussion

4.1. Performance of the analytical methods

The LOQ values for individual mycotoxins in food products (Table 1) were much lower than the MPLs for mycotoxins in foods determined by the Brazilian regulations (Anvisa, 2014). In order to assess the capacity of the analytical method to compensate the matrix effects, R_A, SSE and R_E values for mycotoxins were determined in rice, bean, and wheat flour samples with the addition of IS to samples (Varga et al., 2012). R_E values were high for all mycotoxins evaluated (76–140%), which fulfil the requirements described by the European Commission (2006). The analytical method for determination of urinary biomarkers also showed high R_E values and suitable sensitivity for quantification of all mycotoxins evaluated, since LOQs ranged from 0.003 to 2.0 ng mL^{-1} (Table 2).

4.2. Mycotoxin contents in food products

All types of foods evaluated in the present study had samples containing quantifiable levels of at least one type of mycotoxin (Table 3). However, HT-2 was not detected in any sample analyzed, and T-2 was found in only one sample of rice at low concentration (1.0 µg kg^{-1}). The levels of AFB_1 and OTA found in rice were similar to those reported by Almeida et al. (2012), who observed mean concentrations of 2.49 and 0.64 µg kg^{-1} for total AF and OTA, respectively. AFs are the most frequently reported mycotoxins in rice, with AFB_1 values ranging from 0.1 to 308.0 µg kg^{-1} in India (Reddy et al., 2009), and mean levels of 4.6 µg kg^{-1} for total AF in Pakistan (Lutfullah and Hussain, 2012). Mean levels reported for AFB_1 and OTA in rice in Vietnam was 3.31 and 0.75 µg kg^{-1}, respectively (Nguyen et al., 2007). The median level of DON in rice in the present study was below the tolerable limit for this mycotoxin in rice (750 µg kg^{-1}) in Brazil (Anvisa, 2014), and much lower than the median level reported for DON (116 µg kg^{-1}) by Almeida et al. (2012). In our work, a high percentage of positive samples for ZEN were found in rice (27%), with one sample containing 230 µg kg^{-1}, which is above the Brazilian MPL for this toxin (100 µg kg^{-1}). However, the median level of ZEN in our study was similar to that reported by Almeida et al. (2012).

Concerning bean samples, low frequencies and levels were observed only for AFs and DON, and no quantifiable levels of the other mycotoxins evaluated were found in any bean sample. Low AF frequencies has also been reported in bean samples from the Brazilian state of Goiás (Silva et al., 2002). However, a much higher frequency (75%) was reported in a previous study conducted in the Brazilian state of São Paulo (Jager et al., 2013), although the total AF mean level (0.10 ± 0.09 µg kg^{-1}) was lower than the value obtained in the present study (2.0 µg kg^{-1}). Results from studies conducted in other countries indicate higher occurrence of AFs in bean from Pakistan (Lutfullah and Hussain, 2012), with 20% of red kidney bean and

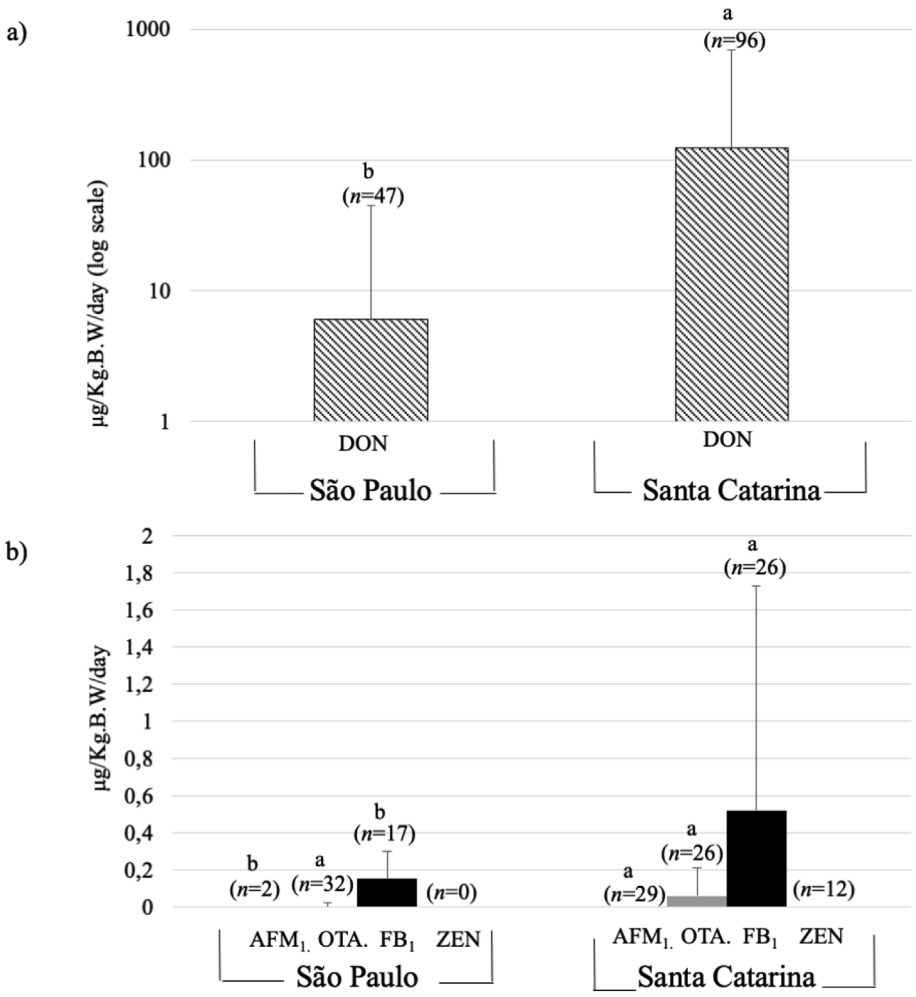

Fig. 5. Estimated dietary intakes based on urinary levels in positive samples (*n*) in the states of Santa Catarina and São Paulo, Brazil, for: a) DON (log scale); b) AFM_1, OTA, FB_1 and ZEN. Results are expressed as mean ± SD. For the same type of mycotoxin, bars with different superscript letters differ significantly ($P < 0.05$). Total AF: sum of aflatoxins B_1, B_2, G_1 and G_2; DON: deoxynivalenol (DON); OTA: ochratoxin A; Total FB: sum of fumonisins B_1 and B_2; ZEN: zearalenone.

Table 7
Risk characterization of mycotoxins through determination of Hazard Quotient (HQ) based on the occurrence data in foods and urinary biomarkers in small-scale farms from the states of São Paulo and Santa Catarina, Brazil.

Mycotoxin	HQ[b]	
	First sampling	Second sampling
Occurrence data in foods[c]:		
DON	0.22	0.58
OTA	0.00	0.04
ΣFB	0.11	0.29
ZEN	0.10	1.07[a]
Urinary biomarkers[c]:		
DON	172.31[a]	1.13[a]
OTA	3.36[a]	0.55
ΣFB	0.19	0.21
ZEN	0.00	0.04

DON: deoxynivalenol; OTA: ochratoxin A; FB: fumonisins; ZEN: zearalenone.
[a] Indicates a non-tolerable risk (HQ > 1).
[b] Calculated as follows: HQ = Probable daily intake/reference values.
[c] Relative to samples containing mycotoxin levels above the limit of quantification (LOQ), see Tables 1 and 2 for LOQ of each mycotoxin in food products and urine, respectively.

Table 8
Risk characterization of aflatoxins through the determination of Margin of Exposure (MoE) and Combined MoE (MoET) based on the occurrence data in foods and urinary biomarkers in the states of São Paulo and Santa Catarina, Brazil.

Mycotoxin	MoE[b]	
	First sampling	Second sampling
Occurrence data in foods[c]:		
AFB_1	0.019[a]	ND
AFG_2	0.007[a]	ND
ΣAF (MoET[d])	0.005[a]	ND
Urinary biomarkers[c]:		
AFM_1	258.29[a]	458.71[a]

ND: Not determined (no sample with concentrations above LOQ).
[a] Indicates high concern for public health (MoE < 10,000).
[b] Calculated as follows: MoE = Benchmark Dose Lower Confidence Limit ($0.00025\ mg\ kg^{-1}\ bw.day^{-1}$)/exposure data.
[c] Relative to samples containing mycotoxin levels above the limit of quantification (LOQ), see Tables 1 and 2 for LOQ of each mycotoxin in food products and urine, respectively.
[d] Calculated as follows: $MoET = 1/[(1/MoE_{AFB1}) + (1/MoE_{AFB2}) + (1/MoE_{AFG1}) + (1/MoE_{AFM1})]$.

cowpea samples containing 5.0 and 2.2 μg kg^{-1} AFB_1, respectively.

Wheat flour had positive samples only for DON, OTA and FBs. The fact that five samples of wheat flour had levels above the Brazilian MPL (750 μg kg^{-1}) (Anvisa, 2014) warrants concern about the incidence of this toxin in wheat flour in Brazil. This result confirms the data reported by Santos et al. (2011), who observed a mean level of 1385 μg kg^{-1} of DON in wheat flour from two states from Southern Brazil. Recently, Stanciu et al. (2017) observed lower levels of DON (mean: 190 μg kg^{-1}) in wheat flour samples from Romania. Concerning corn flour, four types of mycotoxins were found in samples analyzed (AFs, DON, FB and ZEN), with one sample above the Brazilian MPL for total FB and two samples above the MPL for ZEN (1500 and 150 μg kg^{-1}, respectively) (Anvisa, 2014). Corn meal also had positive samples for DON, FBs and ZEN, although no sample had levels above the respective MLPs for these toxins. These results indicate that corn products are important sources of exposure to multiple mycotoxins in small-scale farms in Brazil. FB levels above the Brazilian MPL in corn-based products in São Paulo were also observed by Bittencourt et al. (2005), who described mean concentrations of 6200 ± 4600 and 2800 ± 2000 μg kg^{-1} in corn meal and corn flour, respectively.

4.3. Estimates of mycotoxin exposure through food data

Risk assessment enables evaluation of the impact of the intake of food contaminated with mycotoxins on the health of the volunteers, leading to a different perspective on the occurrence of mycotoxin in foods. For total AF, the mean PDI values (Table 4) derived from rice and bean (0.013 ± 0.007 and 0.007 ± 0.007 μg kg^{-1} bw day^{-1}, respectively) were higher than the PDI through the consumption of peanut products (0.00023 μg kg^{-1} bw day^{-1}) in the Northeast region of São Paulo reported by Oliveira et al. (2009). Exposure of general population to AF from all foods was reported in Europe ranging from 0.00093 to 0.00245 μg kg^{-1} bw day^{-1}, in Africa from 0.0035 to 0.180 μg kg^{-1} bw day^{-1}, in Asia from 0.0003 to 0.053 μg kg^{-1} bw day^{-1}, and in the United States at 0.0027 μg kg^{-1} bw day^{-1} (EFSA, 2007). These values are not too different in Brazil, as described with this study (ranging from 0.006 to 0.019 μg kg^{-1} bw day^{-1}).

In the present study, the higher PDI values observed for FBs and ZEN in the second sampling (Fig. 2), compared with the first sample collection, indicate seasonal variations in the food contamination profile between the two sampling periods. Reasons for higher contamination levels of these *Fusarium* toxins in the food samples analyzed in the first sampling period are difficult to assess at this time. Although the majority of the food products collected were industrialized products that were previously purchased by volunteers and stored in the farm households, some of the corn products were from in-farm production. Additionally, some farms especially in Santa Catarina state had poor storage conditions of produced corn, such as silos with cracks that allowed the contact of the grains with water and insects. This scenario could increase the fungal growth and the contamination levels of mycotoxins in corn samples (Table 3), thus leading to the higher PDI values for FBs and ZEN based on food consumption data.

Regarding FBs, Bordin et al. (2015) analyzed samples of corn meal collected in two of the four cities of the present study (Pirassununga and Erval Velho), and reported lower PDI values for total FB (0.029 ± 0.037 μg kg^{-1} bw day^{-1}) than the mean value obtained for this type of food in the present study (0.321 ± 0.312 μg kg^{-1} bw day^{-1}). In another Brazilian southern state, PDI for total FB was similar to the values of the present study (0.121 μg kg^{-1} bw day^{-1}) (Martins et al., 2012b). Considering the established TDI value for FBs of 2.0 μg kg^{-1} bw day^{-1} determined by the FAO/WHO (2011a), consumption of food products evaluated in this study is considered safe for the population. However, the mean PDI for DON through wheat flour (1.002 ± 0.772 μg kg^{-1} bw day^{-1}) was higher than the TDI for this mycotoxin (1.0 μg kg^{-1} bw day^{-1}) (FAO/WHO, 2011b). The high occurrence of DON in wheat grains and flour in Brazil as described in previous studies (Santos et al. 2011, 2013; Tralamazza et al., 2016) is in agreement with the results obtained in this work for samples of wheat flour. Lower exposure levels of dietary DON (0.666 μg kg^{-1} bw day^{-1}) were described by Stanciou et al. (2018) for the Romanian population through the consumption of wheat-based products. PDI levels attributed to the consumption of wheat in Italy were 0.067 μg kg^{-1} bw day^{-1} for DON and 0.075 μg kg^{-1} bw day^{-1} for ZEN (Juan et al., 2017b). In Tunisia, estimated PDIs of DON (0.00033 μg kg^{-1} bw day^{-1}), OTA (0.00023 μg kg^{-1} bw day^{-1}), ZEN (0.001 μg kg^{-1} bw day^{-1}) and the sum of FBs (0.01971 μg kg^{-1} bw day^{-1}) through the consumption of barley and derived products were below the TDI values for the mentioned mycotoxins (Juan et al. (2017a).

4.4. Mycotoxin biomarkers in human urine

The analysis of urinary biomarkers revealed for the first time the presence of AFM_1, AFP_1, DON, DOM-1, OTA, FB_1 and ZEN in human urine in Brazil (Table 5). Importantly, urine samples were digested with β-glucuronidase to deconjugate the conjugated forms of DON. Hence, the urinary levels of DON indicate the sum of the parent compound and its conjugated forms in the urine samples (Solfrizzo et al., 2011). Risk assessment based on urinary mycotoxin levels have been performed in Brazil only for AF. Romero et al. (2010) confirmed the presence of AFM_1 in the urine of Brazilians (residents in a city of the state of São Paulo), with 78% of the analyzed samples presenting detectable levels of AFM_1. These results are higher than the present study which only showed 12%. In another study carried out in the state of São Paulo, Jager et al. (2014) also reported a higher frequency (61%) of samples with quantifiable levels (mean: 0.0012 ± 0.002 ng mg^{-1} creatinine). The percentage of positive samples reported in Northern Nigeria (14%) was more similar to the present study (Ezekiel et al., 2014), although another report in this same country described a lower incidence (5%) and higher level (mean: 0.31 ng mg^{-1} creatinine) of urinary AFM_1 (Warth et al., 2014).

4.5. Estimates of mycotoxin exposure through urinary levels

Urinary levels of AFM_1 indicated a lower PDI value (0.001 ± 0.002 μg kg^{-1} bw day^{-1}) for AFB_1 (Table 6), when compared with the PDI estimated through food data (0.018 ± 0.001 μg kg^{-1} bw day^{-1}). Jager et al. (2014) calculated the PDI for dietary AF in São Paulo state based on the AFM_1 levels in urine, obtaining values of 0.000034 and 0.000042 μg kg^{-1} bw day^{-1} for men and women, respectively, which are ten times lower than that of the present study in São Paulo (mean PDI: 0.0004 ± 0.0003 μg kg^{-1} bw day^{-1}).

As for DON and OTA, our results indicate that the mean PDIs based on urinary levels exceeded the established TDI values of 1.0 μg kg^{-1} bw day^{-1} (FAO/WHO, 2011b) and 0.016 μg kg^{-1} bw day^{-1} (FAO/WHO, 2007), respectively. Probable intakes greater than the TDI for DON based on urine samples has been reported in different parts of the world, such as Belgian (1.24 μg kg^{-1} bw day^{-1}) (Heyndrickx et al., 2015), Italy (1.03 μg kg^{-1} bw day^{-1}) (Solfrizzo et al., 2014) and South Africa (2.6 μg kg^{-1} bw day^{-1}); (Shephard et al., 2013). However, the PDI for DON presented in this study are much higher than the aforementioned estimates (84.914 μg kg^{-1} bw day^{-1}). This fact could be due to the cultural differences in the intake of food products. According to data from the Food Frequency Questionnaire (Supplementary material B), most of volunteers ate wheat-based bread every day. However, bread samples were not analyzed, and they may act as an additional source of exposure to DON. Heyndrickx et al. (2015) tested two formulas to calculate the DON PDI based on urine data and obtained different values: one below (0.62 μg kg^{-1} bw day^{-1}) the TDI, and one above it (1.24 μg kg^{-1} bw day^{-1}). Lower PDI estimates for DON were reported in Germany and the UK, with 0.52 μg kg^{-1} bw day^{-1} (Gerding et al., 2014), and 0.729 μg kg^{-1} bw day^{-1} (Turner et al., 2008),

respectively.

The lower PDI for DON estimated through urinary levels in the second sampling (Fig. 4) was surprising, since the PDI estimated from food data in this sampling period was numerically higher (hence indicating higher food contamination with DON) than the value obtained in the first sampling (Fig. 2). The differences in both PDI estimates reflects the uncertainties associated with exposure assessments based on food consumption and occurrence data, and based on urine biomarkers (De Boevre et al., 2013). The higher probable intakes of DON, FBs and ZEN in the state of Santa Catarina shown in Fig. 5 can be explained by some differences between daily food intakes and some climate differences. Food items that could possibly determine higher mycotoxin exposure in Santa Catarina according to the Food Consumption Frequency Questionnaires (Supplementary material B) included boiled corn (32 g in SP and 71 g in SC), bread (58 g in SP and 103 g in SC) and cooked corn meal (35 g in SP and 81 g in SC). Pirassununga and Descalvado (SP) have lower annual temperatures (18–20 °C) and rainfall (1300–1600 mm), when compared with Pinhalzinho and Erval Velho (SC) (16–18 °C, 1600–1900 mm, respectively) (Alvares et al., 2013), which could influence differently the fungi growth on food products in those areas.

4.6. Risk characterization

In our study, HQ values greater than 1 (indicative of health concern) in food samples was only observed for ZEN in the second sampling (Table 7), indicating tolerable levels of exposure for the other mycotoxins in both samplings. However, urinary biomarkers indicated that DON and OTA (first sampling) exceeded their tolerable levels of exposure since the HQ values were > 1. Although FB and ZEN are frequently found in corn products in Brazil, their biomarkers in urine indicated a tolerable level of exposure of the individuals evaluated in the present study. Another important observation was the absence of OTA in food samples and its percentage exceeding the TDI in urine samples.

Uncertainties associated with exposure assessments need to be considered for the interpretation of results (De Boevre et al., 2013). Moreover, PDI estimates from urinary biomarkers as obtained in the present study could have limited accuracy since the first morning urine was analyzed instead of the 24-h urine (Vidal et al., 2018). The major uncertainty in the present study should be associated with the difference between probable intake in food samples and the PDI in urine. These facts can be explained by possible additional daily exposure to mycotoxins by food sources not included in the sampling procedures, or by inhalation that may occur in several occupational settings of volunteers, such as handling animal feed (dairy cattle and poultry), or direct contact in grain production (Brera et al., 2002; Mayer et al., 2008; Viegas et al., 2018, 2019). However, two studies investigated the relationship of mycotoxin exposure between a control group with no occupational exposure, and a group of workers who were daily exposed at work. Both studies have concluded that mycotoxin levels and their biomarkers measured in urine mainly reflect dietary exposure (Degen, 2011; Föllmann et al., 2016). Therefore, it remains to be determined if the inconsistences found between levels of DON, OTA and ZEN in food and PDI based on urine data on mycotoxin urine biomarkers are attributable to underestimation of consumption by the volunteers in the self-assessment questionnaire, rather than occupational exposure.

MoE of 0.019 for AFB_1 exposure in this study (Table 8) was quite different from values reported in other regions, such as Asian countries (833) (Benford et al., 2010) and Malaysia (847) (Leong et al., 2011). However, MoE values in the present study and those mentioned were below 10,000, which indicate a high risk of toxic outcomes as a consequence of the exposure to AFs (Benford et al., 2010). Although AFs assume a significant importance as carcinogenic food contaminants, there are few studies reporting population exposure to this mycotoxin (Cano-Sancho et al., 2013). Previous studies identified peanuts as the main contributors to total intake of aflatoxins, worldwide (Qian et al., 1994; Oliveira et al., 2009; Njobeh et al., 2010).

In conclusion, the analysis of 203 food samples and 162 urine samples from adults living in rural areas revealed the presence of mycotoxins in 53% and 93% of the food and urine samples analyzed, respectively. Results demonstrated a clear exposure of this population to DON and high incidence of FBs in corn-based products. Although a low incidence of total AF was observed in food products, detectable concentrations indicated a potential health concern. However, uncertainties concerning the exposure assessment to AFs, ZEN and OTA highlight the need for future studies related to the exposure of the Brazilian population to multiple mycotoxins, in order to understand why occurrence and exposure levels in samples of food and urine samples showed inverted patterns for some mycotoxins, and to identify the main sources of exposure to mycotoxins in the Brazilian population. This is the first study describing the exposure to multiple mycotoxins and risk characterization in Brazil, which may help regulation agencies to better assess the mycotoxins in foods and exposure pattern in at least part of the population.

Authors' contributions

LF, GR and CO designed the study and analyzed the data. LF, KB and GG organized and conducted the sampling procedures. LF, TP and CO carried out the mycotoxin analyses. LF, PA and RA conducted the risk characterization. LF and RA drafted the manuscript. PA and GR made intellectual contributions for the manuscript. CO performed a final, critical review of the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare that there are no conflicts of interest relevant to this study.

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Transparency document

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Appendix A. Supplementary data

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