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Incidence of toxigenic fungi and zearalenone in rice grains from Brazil

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ABSTRACT

Rice (*Oryza sativa* L.) is one of the most important food crops worldwide. In Brazil, the southern region is the area with the highest production of rice in the country and also has a high average daily intake of rice by the population. The mycoflora, mainly toxigenic *Aspergillus* and *Fusarium* species, the presence of AFB_1, DON and ZEA in rice grains, as well as daily intake estimates for the Southern Brazilian population were evaluated. The rice grain samples were collected during the 2017 crop from different harvest periods. According to the mycological tests, the samples presented a high count of fungal colonies in the pre and post-harvest, where the incidence of the *F. graminearum* species complex (52%) was significantly predominant. This group can be responsible for ZEA production, as found in this study in parboiled rice, mainly because most of the isolated strains were producers of high ZEA levels in the pre-harvest (77%) and post-harvest after natural (79%) and artificial (75%) drying of the rice. Only ZEA showed significant results in the rice grain analyzed (60%) at levels of 90.56 to 126.31 μg/kg, where 36% of the samples were significantly higher than the current maximum limit stipulated in Brazilian regulations and by the European Commission. Despite this, the dietary exposure of ZEA estimated for the southern Brazilian population was below the provisional maximum tolerable daily intake level of 0.5 μg/kg body weight/day set at international regulations.

1. Introduction

Rice (*Oryza sativa* L.) is one of the most important food crops that feed approximately 75% of the world's population. In Brazil, rice is widely cultivated in subtropical environments which are characteristically warm and humid, such as the southern region of Brazil, which is the area with the highest production of rice in the country. Southern Brazil contains 64% of the rice fields in the country and 81% of the Brazilian production. The irrigated rice cultivated in Southern Brazil accounts for an average of 90% of the Brazilian rice production, then the cultivation practiced in this region is almost totally irrigated (CONAB, 2017). After harvesting, it is one of the food crops that is most prone to contamination and under inappropriate storage conditions is considered an excellent substrate for fungal growth (Al-Zoreky and Saleh, 2017). According to FAO, 15% of the rice harvest is lost every year due to inappropriate storage conditions, resulting in fungal growth and other deleterious agents (FAO, 2006).

In order to avoid the reduction in quality of rice during production, the period of harvest is performed when the moisture content is around 22%. During post-harvest, the rice is dried up to 14% by way of natural or artificial drying. Natural drying is characterized by the use of the sun as the primary source of heat, while artificial drying through driers may reach the temperature of 70 °C. The rice is stored in metal silos until further processing. Different types of rice can be obtained from processing after de-husking: whole, polished and parboiled rice (EPAGRI, 2017). The parboiling process is used as a way to minimize grain loss during processing and to avoid the excessive removal of compounds that are nutritiously imperative (Dors et al., 2011). However, parboiled rice has been one of the most frequently contaminated products, mainly because the parboiling process allows mycotoxin migration from the outside to the inner layer of the rice grain (Dors et al., 2009; Dors et al., 2011; Nunes et al., 2003).

The compositions of fungal communities established during pre-harvest greatly influence the post-harvest quality (Paterson and Lima,

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2010). Both periods promote mycotoxin production resulting in annual losses of useful food bio-resources, in addition to greatly affecting human and animal health. Although different mycotoxins have been described in rice by some countries (Aydin et al., 2011; Bansal et al., 2011; Ferre, 2016; Fredlund et al., 2009; Makun et al., 2011), few studies have been performed in the Brazilian context. Only the aflatoxins (AFLs) are frequently studied, mainly the AFLs B_1, B_2, G_1 and G_2 (da Silva et al., 2008; Ferre, 2016). AFB_1 produced by *Aspergillus flavus* and *A. parasiticus* is considered the most potentially hepatotoxic, teratogenic and with high genotoxic activity (IARC, 1993).

On the other hand, the presence of *Fusarium* genera has also been associated with mycotoxin production, such as deoxynivalenol (DON) and zearalenone (ZEA) in cereals. DON, also known as vomitoxin, is a type B trichothecene, a sesquiterpenoid metabolite, produced mainly by the *Fusarium graminearum* species complex (Pestka, 2007). This group also produces ZEA that has high affinity for estrogen receptors and can cause significant changes in reproductive organs and even fertility loss in animals and humans (Zinedine et al., 2007). Previously, a study performed by Almeida et al. (2012) showed the occurrence of AFLs, DON and ZEA in 58.7%, 8.3% and 45.2% of the rice samples collected from different regions of Brazil in the period of 2007–2009. The co-occurrence was observed for AFLs and ZEA in 17% of the samples. The co-occurrence of DON was not observed since the great majority of the samples showed no contamination by this toxin.

According to our knowledge, no current study has assessed the presence of these toxins in rice grains from Southern Brazil, although Brazilian regulations have established a maximum limit for AFLs and DON levels since 2011 and for ZEA levels in this year of 2017.The current ML is of 5 μg/kg, 750 μg/kg and 100 μg/kg for AFLs, DON and ZEA in rice for human consumption (ANVISA, 2011, 2017).

Due to the scarcity of data on the mycobiota and toxin content of rice grains, particularly ZEA, the objective of the present study was to investigate the mycoflora, mainly toxigenic *Aspergillus* and *Fusarium* species, and the presence of AFB_1, DON and ZEA in rice grains in the most important productive regions in Brazil. In addition, whether or not the quality of rice produced in Brazil is in accordance with the Brazilian regulation was evaluated, including the daily intake estimates for the population of southern Brazil.

2. Materials and methods

2.1. Chemicals and mycotoxin standards

The reagents used were of analytical grade and the solvents for chromatography were of HPLC and LC/MS grade, obtained from Sigma Aldrich (St. Louis, MO, USA). The culture medium was purchased from Himedia (West Chester, Pennsylvania, USA). Water was purified using a Milli-Q system on 18.2 MΩ/cm (Millipore, Bedford, MA, USA).

AFB_1, DON and ZEA standards were supplied by Sigma Aldrich. The stock solutions standards were prepared in methanol at a concentration of 1 mg/mL. Working standard solutions, ranging from 0.005 to 0.5 μg/mL for AFB_1 and 0.025 to 0.5 μg/mL for DON and ZEA were prepared from suitable dilutions of the stock solution in methanol and were stored at 4 °C.

2.2. Sample characterization

A total of 100 rice grain samples of different varieties recommended for cultivation in southern Brazil were collected during the 2017 crop. The samples were randomly collected from various periods and obtained from local industries during different processing treatments: (1) pre-harvest (freshly harvested) (n = 25); post-harvest after (2) natural (n = 25) and (3) artificial drying (n = 25) and (4) parboiled rice (commercialization) (n = 25) and equally divided for analyses. After rice processing, the parboiled rice was chosen to be analyzed due to high consumption by the population of southern Brazil and also for the possible contamination found by other authors that affirm that the parboiling process allows mycotoxin migration from the outside to the inner layer of the rice grain (Dors et al., 2009; Dors et al., 2011) which doesn't appear to occur with the polished rice (Almeida et al., 2012). Collection was performed using a grain auger from different points of the bulk batches. Each sample was homogenized and reduced separately and aseptically in portions varying around 1.0 kg. Then, the rice samples were collected in portions of 25 g, which were stored into sterile polyethylene bags at 4 °C for analysis of the mycoflora. At the same time, the rice grain samples used for mycotoxin analysis were finely ground using a mill with automatic quartering, thoroughly mixed, and a representative rice subsample of 100 g was collected. Samples were sent to the laboratory as soon as they were collected and were tested upon arrival.

Moisture content (mc) was determined in triplicate by drying 2 g of samples in an oven (105 °C) until constant weight was reached, according to the gravimetric method (AOAC, 2005), and water activity (a_w) was performed in triplicate with 2 g of samples using the equipment model Aw43 (Etec, Sao Paulo, SP, Brazil).

2.3. Characterization of isolated fungi

2.3.1. Morphological characterization

The total fungal count was carried out (Silva et al., 2010) by applying a series of dilutions (10^{-1} to 10^{-4}) onto the surface of potato dextrose agar (PDA) medium with chloramphenicol (100 mg/L). The results were presented taking into account the colony forming units per gram (CFU/g).

In addition, sixteen rice grains were taken from each sample at random, their surfaces were disinfected and they were plated on *Aspergillus flavus* and *parasiticus* agar (AFPA) and dichloran chloramphenicol peptone agar (DCPA). The medium AFPA was incubated at 30 °C for 48 h in order to identify *Aspergillus* sp., it is recommended for the detection and enumeration of potentially aflatoxigenic fungi (Nelson et al., 1983; Pitt and Hocking, 2009). The colonies of *A. flavus*, *A. parasiticus* and *A. nomius* were distinguished by bright orange-yellow reverse colors. Then, the medium DCPA was incubated at 25 °C for 7 days for the identification of *Fusarium* species (Hocking and Andrews, 1987; Samson et al., 2006).

The isolated strains were also sub-cultured on malt extract agar (MEA), glycerol nitrate agar (GN25) and czapek yeast extract agar (CYA) media for macro-morphological observations according to Pitt and Hocking (2009), the PDA medium was used to aid the characterization of the strains isolated from DCPA. The medium coconut agar (COCO) was only inoculated with the strains of *Aspergillus* in order to detect aflatoxin production of *A. flavus* and *A. parasiticus* at 30 °C for 7 days. After the incubation, the strains were examined under long wavelength ultra violet light. The colonies producing aflatoxins presented fluorescence bluish white or white and were identified as toxigenic strains (Dyer and McCammon, 1994).

Species identification was performed through microcultivation in czapek-dox (Samson et al., 2006; Weber and Pitt, 2000). The isolates were examined under a light microscope (100× and 400× magnifications) and species identification was carried out according to available taxonomic keys and guides (Nelson et al., 1983; Pitt and Hocking, 2009; Raper and Fennell, 1965).

2.3.2. Molecular characterization of the Aspergillus and Fusarium species

In order to confirm morphological identification, one *Aspergillus* and ten *Fusarium* strains, each one of the eleven isolates representing different groups based on macro and micromorphology, were selected for sequencing analysis.

2.3.2.1. Aspergillus identification. Beta tubulin locus (*BenA*) (Samson et al., 2014) was used for identification of the *Aspergillus* section *Flavi* isolates. All of the *Aspergillus* isolates were morphologically identical;