Research Article

Fungi and Mycotoxins from Pre- and Poststorage Brewer’s Grain Intended for Bovine Intensive Rearing

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The aim of the study was to determine the mycobiota and natural levels of mycotoxins such as aflatoxin B1 (AFB1), ochratoxin A (OTA), fumonisin B1 (FB1), and deoxynivalenol (DON) present in brewers grains pre- and poststored intended for bovine intensive rearing. Poststored (80%) samples had counts higher than $1 \times 10^4$ colony-forming units (CFU/g). Cladosporium spp. and Aspergillus spp. were isolated at high frequencies. Aspergillus flavus was the prevalent isolated species. Prestored (70%) and poststored (100%) samples showed AFB1 levels over the recommended limits (20 µg/Kg), and OTA levels were below the recommended limits (50 µg/Kg) while pre- and poststored samples did not show FB1 and DON natural contamination levels. The presence of mycotoxins in this substrate indicates the existence of contamination. Regular monitoring of feeds is required in order to prevent chronic and acute toxic syndromes related to this kind of contamination.

1. Introduction

The use of agroindustrial residues as a food supplement for animal production plays a significant economic role due to the availability and versatility of these materials. Brewer’s grains (beer industry residue) are an interesting alternative option as feeding for animal production, being a rich source of protein and fibber at a low price [1, 2]. Inadequate management of raw materials during storage can result in excessive moisture or dryness, condensation, heating, leakage of rainwater, and insect infestation, leading to undesirable growth of fungi [3]. Worldwide, the contamination of animal feed and the potential contamination of animal meat by mycotoxins represent a serious hazard to humans and animals. Mycotoxins are toxic, chemically diverse secondary substances or metabolites produced by a wide range of fungi. They are mainly produced by Aspergillus, Penicillium, and Fusarium genera [4]. Due to the diversity of their toxic effects and their synergetic properties, mycotoxins are considered as risky to the consumers of contaminated foods and feeds [5]. Aflatoxins (AFs), the fungal metabolites produced by some strains of A. flavus and A. parasiticus, are of great concern because of their detrimental effects on the health of humans and animals, including carcinogenic, mutagenic, teratogenic, and immunosuppressive effects [6, 7]. Ochratoxin A (OTA) is one of the most common and dangerous mycotoxin in food and feed, naturally produced by A. ochraceus, A. carbonarius and A. niger aggregate mainly in tropical regions, and P. verrucosum in temperate areas [8–10]. This toxin has a potent toxicity, and the nephrotoxic, hepatotoxic, teratogenic, carcinogenic, and immunosuppressive effects have been demonstrated in all mammalian species [11]. Fumonisins (FBs), produced by Fusarium verticillioides and F. proliferatum, occur worldwide and are predominantly found in maize and in maize-based animal feeds. Fumonisin B1 (FB1) is the most common and
the most thoroughly studied, causing toxicities in animals such as equine leukoencephalomalacia (ELEM) and porcine pulmonary edema (PPE), diseases long associated with the consumption of mouldy feed by horses and pigs, respectively [12]. Deoxynivalenol (DON) or vomitoxin is a commonly occurring mycotoxin produced primarily by *F. graminearum* and *F. culmorum* [13]. This toxin can cause vomiting, feed refusal, gastrointestinal irritation, and immunosuppression [14].

Previous studies performed in Brazil determined the fungal biota as well as the presence of different mycotoxins in brewer’s grain and barley rootlets intended for cattle and pigs [15–18]. There are no data about the contamination with fungi and mycotoxins in breweer’s residue stored in farms in a similar manner of trench type silos during 3 months. Therefore, the aims of this work were to determine mycobiota occurrence and to evaluate AFs, OTA, FBs, and DON incidence on pre- and poststorage brewer’s grains.

### 2. Materials and Methods

#### 2.1. Characteristic of Storage

Brewer’s grains were transported from brewery industry to farms in trucks, deposited in five (5) structures similar to a trench type and stored in large pits dug into ground (1 m) and covered with a black plastic sheet. Storage and compaction was performed during 3 months, and brewer’s grains were kept closed until to be used. The removal of material for animal feeding was made by shovelling.

#### 2.2. Samples Source

Brewer’s grain samples were collected from bovine intensive rearing (feedlot) 2 farms in São Paulo State, Brazil. These samples were collected at different times: day zero (0) (immediately deposited) and after 90 days of storage (before feeding animals). To guarantee a correct sampling, each structure similar to trench type was imaginary divided along its length into three equal parts with four sections each: upper, lower, border, and middle sections. Six subsamples (500 g) were collected from each section to obtain a total of three kilograms sample. A total of 100 samples (3 kg each) of brewer’s grains were taken at different times: 50 were taken at day 0, and 50 samples were taken at day 90. Samples were properly packed in bags and immediately sent to the laboratory. Samples were immediately processed for physical and mycological analyses and kept at −4°C until mycotoxins analyses.

#### 2.3. Physical Properties of Samples

The pH and dry matter percentage for 100 g of each sample were determined according to Ohyama et al. [19].

#### 2.4. Mycological Analysis

The quantitative enumeration of fungi as colony-forming units per gram of food (CFU/g) was performed using the surface-spread method described by Pitt and Hocking [20]. Ten grams of each sample were homogenized in 90 mL distilled water solution for 30 min in an orbital shaker. Serial dilutions (from 10−2 to 10−5) were made, and 0.1 mL aliquots were inoculated in duplicates onto the media dichloran that rose bengal chloranphenicol agar (DRBC) for estimating total culturable fungi [21] and dichloran 18% glycerol agar (DG18) that favors xerophilic fungi development. The plates were incubated at 25°C for 5–7 days. All samples were also inoculated onto Nash and Snyder agar (NSA) to enumerate *Fusarium* species [22]. Nash-Snyder plates were incubated at 24°C for 7 days under a 12 h cold white/12 h black fluorescent light photoperiod. Only plates containing 10–100 CFU were used for counting, with results expressed as CFU per gram of sample. On the last day of incubation, individual CFU/g counts for each colony type considered to be different were recorded. Colonies representative of *Aspergillus* and *Penicillium* were transferred for subculturing to tubes containing malt extract agar (MEA) whereas *Fusarium* spp. were transferred for subculturing to plates containing carnation leaf agar (CLA). Fungal species were identified according to Klich [23], Nelson et al. [22], and Samson et al. [24]. The results were expressed as isolation frequency (% of samples in which each genus was present) and relative density (% of isolation of each species among the same genera).

#### 2.5. Mycotoxins Detection and Quantification

##### 2.5.1. Aflatoxin B1 and OTA Determination

The extraction of AFB1 was determined according to Soares and Rodriguez-Amaya [25]. Quantitative evaluation was made using high-performance liquid chromatography (HPLC). The detection limit of the technique for AFB1 was 1.0 µg/kg.

##### 2.5.2. Fumonisin B1 Determination

A commercially available enzyme-linked immunosorbent assay (ELISA) plate kit (Beacon Analytical Systems Inc., Portland, USA) was applied for the extraction and quantification of FB1. Mycotoxin extraction and testing were performed according to manufacturer’s introductions. A 20 g portion of each sample was extracted with 100 mL methanol:water (70:30, v/v) during 3 min into a blend jar. The mixture was filtered through filter paper Whatman N° 4 (Whatman, Inc., Clifton, NY, USA) and an aliquot taken and placed into a culture plate. Detection limit of the technique was 0.3 µg/g.

##### 2.5.3. Deoxynivalenol Determination

An ELISA tube kit (Beacon Analytical Systems Inc., Portland, USA) was also applied for the extraction and quantification of DON. Mycotoxin extraction and testing (ELISA) were performed according to manufacturer’s introductions. A 20 g portion of each sample was extracted with 100 mL distilled water during 3 min into a blend jar. The mixture was filtered through filter paper Whatman N° 4 (Whatman, Inc., Clifton, NY, USA) and an aliquot taken and placed into a culture tube. Detection limit of the technique was 0.5 µg/g.

##### 2.5.4. Statistical Analyses

Statistical analysis of data was by the general linear models model (MLGM). Fungal counts per 100 g mycotoxin analyses were compared using Fisher’s protected LSD test.
3. Results

3.1. Chemical and Physical Properties of Samples. Table 1 shows the physical properties of the sorghum samples. The pH mean levels ranged from 5.7 to 6.0 in prestored brewer’s grain while the values of pH, from poststored were from 4.5 to 5. In both types of samples, dry matter values were from 39.7 to 41%.

Table 1: Physical properties from pre- and poststored brewer’s grains in several section of silo.

| Section of silo | pH | Dry matter (%) |
|-----------------|----|----------------|
|                 | Pre | Post | Pre | Post |
| Upper           | 5.7 ± 1.0 | 4.6 ± 0.8 | 46 ± 0.07 | 40 ± 0.13 |
| Middle          | 6.0 ± 0.3 | 4.5 ± 0.4 | 37 ± 0.11 | 41 ± 0.13 |
| Lower           | 5.9 ± 0.4 | 5.0 ± 0.8 | 44 ± 0.09 | 41 ± 0.12 |
| Border          | 6.0 ± 0.35 | 4.6 ± 0.7 | 37 ± 0.12 | 37 ± 0.11 |

SD: standard deviation.

3.2. Mycological Survey. Table 2 shows fungal counts from pre- and poststored brewer’s grains in different culture media. Total fungal count analyses from prestored shown values with means ranging from $1.7 \times 10^3$ to $2.9 \times 10^3$ CFU/g and $1.5 \times 10^3$ to $1.8 \times 10^3$ CFU/g in DRBC and DG18, respectively. Eighty percent of poststored samples had counts higher than $1 \times 10^4$ CFU/g. Means varied from $2.5 \times 10^4$ to $2.3 \times 10^5$ CFU/g in DRBC and from $6.2 \times 10^3$ to $1.5 \times 10^5$ CFU/g in DG18. There were significant differences between pre- and poststored brewer’s grain samples. No statistically significant differences were found between different layer of the silo prestored brewer’s grain in DRBC and DG18 while there were significant differences between fungal counts from poststored samples ($P < 0.05$).

Figure 1 shows the isolation frequency of fungal genera (%) from pre- and poststored brewer’s grains samples. *Cladosporium* spp., *Aspergillus* spp., *Mucor* spp., and yeasts were isolated at high frequencies. *Eurotium* spp., *Penicillium* spp. and *Alternaria* spp. were isolated at low frequencies. *Fusarium* spp. were isolated only from poststored brewer’s grain samples.

Figure 2 shows the relative density of isolated *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp. from pre- and poststored brewer’s grain samples. Three *Aspergillus* spp. were isolated. *Aspergillus flavus* was the prevalent isolated species, followed by *A. fumigatus* and *A. terreus*. *Aspergillus flavus* was isolated at levels that ranged from 50 to 78% for pre- and poststored samples, respectively. While *A. fumigatus* and *A. terreus* were isolated from pre- and poststored samples. *Penicillium citrinum* was the only species isolated within this genus. *Fusarium verticilloides* was only present in prestored brewer’s grain samples.

3.3. Determination of Mycotoxins. Table 3 shows the AFB$_1$ and OTA levels found in pre- and poststored brewer’s grain samples. Pre- and poststored samples did not show FB$_1$ and DON natural contamination levels. Four percent of pre- and poststored samples were contaminated with AFB$_1$ at levels that varied from 10 to 35 µg/Kg and 24 to 47 µg/Kg, respectively. Seventy percent of prestored and all poststored samples (100%) showed AFB$_1$ levels over the recommended limits (20 µg/Kg). None of the analyzed of prestored samples showed OTA levels. While 5% of poststored samples were contaminated with average levels of 9.8 µg/Kg. However, none of these samples were contaminated with OTA levels over the recommended limits (50 µg/Kg). No statistically significant differences were found between pre- and poststored brewer’s grain for AFB$_1$ and OTA contamination ($P < 0.05$).
Table 2: Fungal counts (CFU/g) from pre- and poststored brewer’s grains samples in DRBC and DG18 culture media.

| Samples     | Section of silo | DRBC                        | DG18                        | Contaminated samples (%) over GMP (2008) [26] limits |
|-------------|-----------------|-----------------------------|-----------------------------|-----------------------------------------------|
|             |                 | Mean-Range*                 |                             |                                               |
| Prestored   | Upper           | 2.9 × 10^3a                 | 1.6 × 10^3a                 |                                               |
|             |                 | (3.3 × 10^2–6.1 × 10^3)     | (1 × 10^2–4.8 × 10^3)       |                                               |
|             | Lower           | 2.2 × 10^3a                 | 1.8 × 10^3a                 |                                               |
|             |                 | (2.8 × 10^2–5.2 × 10^3)     | (3.1 × 10^2–4.4 × 10^3)     |                                               |
|             | Border          | 2.5 × 10^3a                 | 1.5 × 10^3a                 | 0%                                            |
|             |                 | (2.8 × 10^2–6.1 × 10^3)     | (1 × 10^2–4.8 × 10^3)       |                                               |
|             | Middle          | 1.7 × 10^3a                 | 1.8 × 10^3a                 |                                               |
|             |                 | (1 × 10^2–2.8 × 10^3)       | (3.1 × 10^2–4.4 × 10^3)     |                                               |
| Poststored  | Upper           | 1.3 × 10^3b,c              | 1.5 × 10^3b,c              |                                               |
|             |                 | (3.1 × 10^2–3 × 10^3)      | (1.6 × 10^3–4.3 × 10^3)    |                                               |
|             | Lower           | 2.5 × 10^4abc              | 2.3 × 10^4abc              |                                               |
|             |                 | (1.2 × 10^3–5 × 10^3)      | (3.4 × 10^3–6.7 × 10^4)    | 80%                                           |
|             | Border          | 1.8 × 10^4b,c              | 6.2 × 10^3a                 |                                               |
|             |                 | (1 × 10^3–1.4 × 10^4)      | (1.2 × 10^3–2.1 × 10^4)    |                                               |
|             | Middle          | 2.3 × 10^5c                 | 5.2 × 10^4abc              |                                               |
|             |                 | (1.3 × 10^4–5.2 × 10^5)   | (1.3 × 10^4–1.5 × 10^5)    |                                               |

*Mean values of counts. Minor and major values count. Detection limit: 1 × 10^2 CFU/g. Maximum recommended level: 1 × 10^4 CFU/g [26]. DRBC: dichloran rose bengal chloramphenicol. DG18: dichloran glycerol 18%. Letters in common are not significantly different according to Fisher’s protected LSD test (P < 0.05).

Table 3: Incidence of aflatoxin B1 and ochratoxin A in pre- and poststored brewers grains samples.

| Samples | Mean levels | AFB1 (µg kg^-1) | (%)* | Mean levels | OTA (µg kg^-1) | (%)* |
|---------|-------------|----------------|------|-------------|----------------|------|
| Pre     | 25.8*       | 10–35          | 8    | ND*         | —              | —    |
| Post    | 38*         | 24–47          | 10   | 9.8*        | 2–25           | 5    |

*Prestored brewers grains; Poststored brewers grains; *Percentage of samples contaminated with mycotoxin (%). ND: not detected. Values with letters in common are not statistically significant, according to test of LSD (P ≤ 0.05).

4. Discussion

Mycobiota and natural occurrence of AFB1, OTA, FB1, and DON in pre- and poststored brewer’s grain were studied.

Physical properties of brewer’s grain samples showed that there was no difference in dry matter comparing pre- and poststored brewer’s grains. The dry matter content is one of the main factor for well-preserved samples. The ideal values of this parameter are between 26 and 38% with pH around 4.0 [27]. The physical factor that assures the preservation is pH. The pH difference between pre- and poststored samples is due to the acidification of carbohydrates present in the raw material by microorganisms present in this ecosystem. In this work, this substrate was acidified through time, and the pH values in poststored brewers’ grain were between 4.5 to 5.0 after 90 days of storage.

In this study, the average of fungal colony counts from all prestored brewer’s grain samples had counts lower than the maximum proposed limit (1 × 10^3 CFU/g) [26]. However, poststored brewer’s grain samples had high values, which were over the maximum of the recommended limits. These results suggest a high fungal activity that could affect the palatability of feed and reduce the animal nutrients absorption, determining a low-quality substrate [28, 29]. Simas et al. [15] and Rosa et al. [17] studied the same substrate intended for dairy cattle feed. They found media counts of 1 × 10^3 CFU/g and 6 × 10^5 CFU/g in potato dextrose agar and DRBC media, respectively. Cavagliè et al. [18] obtained counts ranging between 1 × 10^3 and 1 × 10^6 CFU/g in DRBC; however, they studied other waste derived from processing of barley intended for pigs (barley rootlet). This substrate was storage between 8 and 15 days while in this study the period was 90 days.

In this work, Cladosporium spp. and Aspergillus spp. were the most prevalent genera isolated from pre- and poststored samples. Similar percentages of Aspergillus spp. were found by Cavagliè et al. [18] in barley rootlets; in addition they found Fusarium spp. as the prevalent genus. In this study,
the scarce presence of *Fusarium* sp. may be the result of brewer’s grain storage and processing conditions. These conditions may have favoured the development of storage and contaminant fungi instead of those known as field fungi, which include the genus *Fusarium*, more frequently found on recently harvested grain than on processed and stored ones [30]. Several studies have proved that *Aspergillus* and *Penicillium* genera were predominant in brewer’s such as Simas et al. [15], Rosa et al. [17], and Gerbaldo et al. [31]. A high frequency of yeasts was also found. The significance of yeasts, which were frequently isolated, is not known in this substrate.

In this study, *A. flavus* was the most prevalent species followed by *A. fumigatus* and *A. terreus*. These results agree with those of Gerbaldo et al. [31] who reported high percentages of *A. flavus* and *A. fumigatus* in brewer’s grains intended for pigs in Argentina. Rosa et al. [17] found *A. niger* aggregate as prevalent followed by *A. ochraceus*, *A. terreus* and *A. flavus* from dairy cattle feed. *Penicillium citrinum* was only species of *Penicillium* genus isolated. Previous studies in the some substrate have demonstrated high frequency of *P. citrinum* together with *P. funiculosum*, *P. janthinellum*, *P. rugulosum*, and *P. viridicatum* [17]. *Fusarium verticillioides* was isolated at low frequency in our study. Cavagliéri et al. [18] studied barley rootlets as feed for pigs. They found *F. verticillioides* as the only species within *Fusarium* genus, but at high frequency. Other researchers did not identify species of *Fusarium* sp. from the same substrate of this work [15, 17, 31].

Scientific reports on the contamination of brewer’s grain with mycotoxins in Brazil are scarce. Simas et al. [15] studied the presence of AFB1 and OTA in this substrate. In this study, levels of AFB1 found from prestored samples were higher than those obtained by Simas et al. [15]. Considering the vast territory of Brazil, this may be due to different climatic conditions between the two states.

Regulations on standard products in the animal feed sector established that the current maximum permitted level for AFB1 is 20 µg/Kg [26]. In this work, 75% and 100% of the samples contaminated at 0 and 90 days of storage, respectively, showed AFB1 levels higher than the recommended limits for feedstuffs. The OTA concentrations were observed in samples derived from poststored samples. Rosa et al. [17] found higher amounts of OTA in samples of brewer’s grains. In this work, OTA levels were below the recommend level which is 50 µg/Kg [26]. The presence of this mycotoxin in this substrate indicates the existence of contamination, a fact that would require periodic monitoring. Brewer’s grains samples did not show FB1 and DON contamination. Our results did not agree with Batatinha et al. [16] and Cavagliéri et al. [18] who found FB1 in brewer’s grains and barley rootlets, at levels that ranged from 198 to 295 µg/Kg and from 564 to 1383 µg/Kg, respectively. Preharvest contamination of the barley crop could be considered possible, barley could support *F. verticillioides/F. proliferatum* growth when grain is remoistened during the germination and malting process, and it might even continue during storage prior to use, providing that the water activity remained high. The malting process requires water to allow barley germination. If fumonisins were present, they could be diluted during the steeping process. No information is available about the study of DON in this substrate. While this report does not detect this toxin, this is the first study to investigate its presence.

The presence of mycotoxins in these substrates indicates the existence of contamination. Inadequate storage conditions promote the proliferation of mycotoxin-producing fungal species. Regular monitoring of feeds is required in order to prevent chronic and acute toxic syndromes related to this kind of contamination.

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