academicJournals

Vol. 9(22), pp. 1487-1491, 3 June, 2015
DOI: 10.5897/AJMR2014.7492
Article Number: 9C8393553428
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Microbiological and mycotoxicological evaluation of rice products used in human food in northeastern Brazil

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Received 23 March, 2015; Accepted 18 May, 2015

Rice consumption is part of Brazilian food culture, the per capita consumption, considering different forms is approximately of 14.9 kg of rice. The storage of rice grains in inappropriate conditions favor fungal growth and mycotoxin production. A survey was carried out to determine presence of coliforms, *Salmonella, Bacillus cereus,* fungal and mycotoxin contamination (aflatoxins, ochratoxin A and zearalenone) in 40 rice products (rice flakes and rice dough) samples traded in Terezina. Also, the ability to produce mycotoxins by *Aspergillus* and *Fusarium* isolates was shown. Regarding the microbiological standards, the results were within the established pattern. Several fungal species, especially *Aspergillus flavus* and *Penicillium citrinum*, were isolates, but the strains were not able to produce aflatoxins and citrinin, respectively. The samples commercialized in Terezina had satisfactory hygienic and sanitary conditions, and free of mycotoxins analyzed.

Key words: Rice flakes, rice dough, mycotoxins, fungi, bacteria.

INTRODUCTION

Rice production is of fundamental importance in the world, because it is considered a staple food in many countries. Brazil is among the top ten world producers with 11 million tons produced per year. Santos et al. (1994) points

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out that the Asian countries, where a considerable amount of rice is consumed daily, the problem of fungal contamination and mycotoxin in this cereal is relevant. Rice is part of the food habits of the brazilian people, which is confirmed by high consumption, considering its different forms. The average consumption of rice in Brazil is approximately 12 million tonnes (Conab, 2012).

For the great importance of their consumption, studies have reported the involvement of this cereal in outbreaks of microbiological contamination, both by fungi, such as bacteria. *Bacillus cereus* is a natural soil bacterium and can also contaminate the rice planting, remain viable in the form of spores and subsequent treatment to withstand the processing of rice (Ghelard, 2002). Other bacteria such as *Salmonella*, an important causative agent of infections have been described as contaminants in feed ingredients, such as corn, sorghum, rice bran and cottonseed meal (Jones and Richardson, 2004). The occurrence of *Salmonella* in these grains may be related to the stages of growth, harvest, storage or transport, as well as to contamination by effluents, sewage, fecal waste, where this pathogen can be incorporated into crops by irrigation system (Freitas et al., 2003).

The monitoring of fungal contamination of rice is indispensable to ensure the quality and safety of this cereal (Guimarães et al., 2010). The fungi are widely distributed in the environment, being frequently contaminants of food, especially of plant origin. Some species may invade and colonize plant tissues during all stages of production: cultivation, harvesting, drying, transport, in the processing and storage (Rodriguez-Amaya and Sabino, 2002; Galvano et al., 2005). The storage of grains such as rice, under inadequate conditions favor the growth of fungi, during development to produce secondary metabolites called mycotoxins that affect human and animal health (Tanaka et al., 2007).

For the development of fungi and their mycotoxins, they need favorable conditions, and the most important factors are: temperature, water activity and moisture content, pH, chemical composition of food, rate of oxygenation, storage period, degree of fungal contamination, physical conditions of grain or seeds, arthropods and microbial interaction (Boeing, 2003). The fungal genera *Aspergillus* and *Penicillium* are considered the main fungi contaminating grains such as rice, corn, wheat, sorghum, nuts and cotton seeds used in the formulation of foodstuffs (Rodriguez-Amaya and Sabino, 2002). Its capacity to grow at high temperatures and low water activity makes settlers of several crops (Moss, 1991).

Some species of the genus *Aspergillus* are important producers of mycotoxins, such as aflatoxins (AFs), ochratoxin (OTA) (Cast, 2003). Within the genus *Penicillium,* some species produced a variety of mycotoxins such as cyclopiazonic and penicilic acid, citreoviridin, citrinin, ochratoxin A, patulin, roquefortine and others (Pitt and Hocking, 2008). The ingestion of mycotoxins could cause various detrimental health effects by inducing different clinical signs and lesions, where these will be linked to the type of mycotoxin, dose and incubation period (Dilkin and Mallmann, 2006). The presence of fungi with the capability to produce mycotoxins in foods does not confirm the presence of these, but only the possibility of contamination. Moreover, the absence of these fungi does not ensure the food is clear of these compounds, because these toxins persist for a long time after the fungus has lost its viability (Yoshisawa, 2001).

The high frequency of consumption of rice and its by-products, as a potential source of mycotoxins there is a need for information on the microbiological quality and mycotoxin contamination in this cereal in areas of northern and northeastern Brazil. Therefore, the study aimed to evaluate the microbiological quality, the presence of fungi and mycotoxins in rice products.

MATERIALS AND METHODS

Forty samples were used (500 g each), 20 g rice flakes (marks: A, B, C and D) and 20 g of mass rice (marks: E, F, G and H) sold in different supermarkets in the City of Terezina - Piaui, Brazil. The data collection period was from January to May 2011. After collecting the samples, they were homogenized and mixed, quartered, to obtain 100 g samples for the analysis. The mycological evaluation was performed immediately, and aliquots were stored for the mycotoxin analysis.

Each sample (25 g) was diluted with 225 mL of peptone water 0.1% (wt/v). This mixture was shaken and decimally diluted (10^{-2} and 10^{-3}). Dilutions performed in peptonated water were incubated for 24 h at 37°C for the *Salmonella* presence as recommended by APHA (2005). The analysis of total and thermotolerant coliforms, as well as *Bacillus cereus* also was carried out following the methodology described by APHA (2005).

For fungi analysis, 0.1 mL aliquot of each dilution (duplicate) per sample was spread on the surface of solid media dichloran- Rose Bengal chloramphenicol-(DRBC) (Pitt and Hocking, 2007). The plates were incubated for 7 days at 25°C. All plates containing 10 - 100 CFU were counted and the results denominated in colony forming units (CFU) per gram of sample. At the last day of incubation, colonies of *Aspergillus* and *Penicillium*, after microscopic identification according to criteria proposed by Pitt and Hocking (2008), were transferred to malt extract agar (MEA) and incubated at 25°C for 7 days for subsequent species identification. For the identification of *Penicillium*, colonies were grown on Czapek yeast agar (CYA) at 5, 25 and 37°C MEA at 25°C and 25% glycerol nitrate agar (G25N) at 25°C. To identify *Aspergillus*, the cultures are grown on CYA (25 and 37°C), MEA (25°C) and Czapek yeast extract agar with 20% sucrose (CY20S) at 25°C. All the plates were incubated for 7 days. Each strain was identified according to the methods provided by Pitt (1988) and Klich and Pitt (1994). To determine producing strains of citrinin, we used the method described by Lin and Dianese (1976), if inoculating isolated *Penicillium citrinun* In Medium Coco CAM (Cocunut-Agar-Medium) and further read on cromatovisor to 366 nm.

The strains of *Aspergillus flavus*, were evaluated in their potential to produce mycotoxins using the method described by Soares and Rodriguez-Amaya (1989). *A. flavus* strains were grown on MEA plates at 25°C for 7 days, the mycelium was transferred to an Eppendorf micro-tube and 1000 µL of chloroform was added. The

Table 1. Content of coliforms, *Salmonella* spp., and *Bacillus* spp. in rice sub-products.

Sample	Average Fungi CFU/g in Log_{10}	Coliforms at 35°C in NMP/g	Coliformes at 45°C in NMP/g	Absence/ presence *Salmonella* spp/25 g	Absence/ presence *Bacillus* spp/25 g
A (n=5)	3.65	1.98	1.20	Absence	Absence
B (n=5)	4.17	2.77	0.69	Absence	Absence
C (n=5)	3.12	2.01	0.90	Absence	Absence
D (n=5)	3.39	1.62	0.69	Absence	Absence
E (n=5)	3.58	1.09	0.47	Absence	Absence
F (n=5)	3.18	1.11	0.47	Absence	Absence
G (n=5)	2.96	0.92	0.47	Absence	Absence
H (n=5)	3.16	1.09	0.47	Absence	Absence

Table 2. Percentage (%) of filamentous fungi isolated from rice sub-products intended for human consumption.

Fungal genera	No. isolates	Frequency (%)
Aspergillus e teleomorfos	47	35.6
Penicillium	39	29.5
Cladosporium	24	18.2
Fusarium	09	6.8
Mucorales	07	5.2
Curvularia	03	2.4
Chaetosartorya	02	1.5
Emiricella	01	0.8
Total	132,0	100

mixture was stirred at 4000 rpm for twenty minutes, the mycelium was removed and the extract of chloroform evaporated at environmental temperature. The residue was redissolved in 200 µL of chloroform and extracts were tested for aflatoxin by thin layer chromatography (TLC). The extracts were analyzed by chromatography on silica gel plates 60 F254, TLC aluminum plates (20 x 20 cm, thickness 250 µm, Merck, Germany). The liquid carrier was chloroform : acetone (90:10 v / v). The detection limit of the method used is 5 µ/g.

The presence of mycotoxins (aflatoxins, ochratoxin A and zearalenone) in rice product samples were analyzed follow the methodology proposed by Soares and Rodriguez-Amaya (1989). Briefly, 50 g of sample was extracted with 270 mL of methanol and 30 mL of 4% of potassium chloride, after filtering the filtrate was clarified with 30% ammonium sulfate and celite. The mixture was filtered through qualitative filter paper and 150 mL of filtrate (clarified extract) was transferred to a separatory funnel. The toxins were extracted by liquid-liquid partitions with chloroform twice. The organic phase was combined and evaporated to dryness in-route steam at 80°C. The residue was dissolved in 200 µL of benzene. For toxin identification and quantification silica gel plates 60 F254, TLC aluminum plates (20 x 20 cm, thickness 250 µm, Merck, Germany) were used. The plate was development with toluene-ethyl acetate-formic acid (60:40:0.5). For the visualization of aflatoxins B1, B2, G1 and G2 and ochratoxin A plates were placed under UV lamp 366 nm. For zearalenone the silica gel plate was sprayed with 20% aluminum chloride in 75 % ethanol and heated at 110°C for five minutes.

The results of the counts were transformed to log10, and correlated analysis of variance was performed followed by the test for comparison of average SNK significance ($p < 0.05$) using SIGMASTAT statistical package (1994).

RESULTS AND DISCUSSION

The levels of coli forms at 35 and 45°C, *Salmonella* and *B. cereus* in rice derived samples are shown in Table 1. All this parameters are below the limits established by law RDC No. 12, of January 2001 (Brazil, 2001). Many pathogens can be associated with seeds and grains of rice, harming the health quality of their products (Guimarães et al., 2010). Thus, monitoring the quality of their products is of great importance, specifically in relation to microbiological standards, since these features allow an assessment of the conditions of processing, storage, distribution, service life and the risk to consumer health.

The counting of yeast and filamentous moulds was performed by means of enumeration of fungal propagules and expressed as colony forming units per gram of analyzed sample (CFU/g) (Table 1). The fungal counts varied from 2.96 to 4.17 CFU/g. There was no significant difference between the different marks of corn flour analyzed ($p<0.05$).

The presence of fungi in food can cause modifications in the organoleptic characteristics such as: taste, smell and appearance, leading to a significant decrease in food quality (Cast, 2003). Table 2 show the occurrence of filamentous fungi in rice products sold in commercial establishments from Teresina, PI, Brazil. All analyzed brands, both rice flakes as rice dough, were contaminated by different genera of fungi, some potentially able to produce mycotoxins, which may have a potential risk to human health. 132 fungal colonies were isolated, which were distributed in eight genera of fungi. The genus most frequently isolated was *Aspergillus* spp. and its teleomorphs (35.6%), followed by *Penicillium* spp. (29.5%) and *Cladosporium* spp. (18.2 %). Guimarães et al. (2010), using two techniques for fungal detection,

Table 3. Relative frequency (%) of *Aspergillus* species isolated from rice sub-products intended for human consumption.

***Aspergillus* species**	**No. of strains**	**Frequency (%)**
A. flavus	15	31.9
Eurotium spp.	07	14.9
A.ostianus	07	14.9
A.clavatus	05	10.6
A.fumigatus	03	6.4
A.niger and agregados	03	6.4
A. terreus	02	4.3
A.niveus	02	4.3
A. candidus	01	2.1
A. paradoxy	01	2.1
A.oryzae	01	2.1
Total	47	100

Table 4. Relative frequency (%) of *Penicillium* species isolated from rice sub-products intended for human consumption.

***Penicillium* species**	**No. of strains**	**Frequency (%)**
P. citrinum	21	53.8
P. restrictum	07	17.9
P. corylophilum	03	7.7
P. decumbens	03	7.7
P. implicatum	02	5.1
P. citreonigrum	01	2.6
P. paxilli	01	2.6
P. purpurogem	01	2.6
Total	39	100

found that genera *Penicillium, Aspergillus*, *Cladosporium*, *Fusarium* and *Trichoderma* were present in samples of polished white rice.

Out of 47 *Aspergillus* spp. strains isolated, 7 belong to *Aspergillus* teleomorph Eurotium. The most frequent species was *A. flavus* with 31.9% followed by *A. ostianus* (14.9%). The relative frequencies of species *A. niger* and aggregated (6.4%), *A. fumigatus* (6.4%) were low, however, the presence of such species is significant because these species can produce mycotoxins (Abarca et al., 2001). Other *Aspergillus* species were identified at a lower frequency as *Aspergillus terreus, Aspergillus clavatus, A. terreus, Aspergillus niveus, Aspergillus candidus, Aspergillus paradoxy* and *Aspergillus oryzae*.

Among the *Aspergillus* species, we can observe in Table 3 that the 30% of the strains was *A. flavus*. The presence of *A. flavus* in foods poses a potential hazard because it can cause disease in workers who are directly in contact with it, such as aspergillosis (Akan et al., 2002), allergies and respiratory problems by contact and inhalation of conidia. Also, the potential to produce aflatoxin, if the stored conditions are not appropriate, is significant. All strains of *A. flavus* were analyzed for their ability to produce aflatoxins using two techniques, culture and chromatographic method, but none of the strains evaluated show ability to produce aflatoxins. In a study done by Guimarães et al. (2010), in polished rice and parboiled, it was found that 50% of the strains of *A. flavus* were positive in coconut agar technique in the aflatoxin production (but the ability was not checked by other methods.

According to data from the UN Food and Agriculture Organization (FAO, 2006), with appropriate conditions, species of the genus *Aspergillus* spp. can grow groundnut, corn and other grains and produce mycotoxins. Thus, from the results, it may be suspected that these products in their processing steps have not offered sufficient conditions for the fungi to produce aflatoxins.

Eight *Penicillium* species were observed within a total of 39 strains, *Penicillium citrinum* (53. 8%) was the more frequent, followed by *Penicillium restrictum* (17.9%). Other species were also identified in a lesser frequency as *Penicillium corylophilum, Penicillium decumbens, Penicillium citreonigrum, Penicillium implicatum* and *Penicillium paxilli* (Table 4).

P. citrinum is one of the most common fungal species in Brazilian foods, and the responsible for the citrinin contamination, a toxin nephrotoxic (Oliveira et al., 2006). All strains of *P. citrinum* isolated were tested in their ability to produce citrinin, using the agar coconut technique, none of the strains produced the toxin.

All the samples of rice products were analyzed for the presence of mycotoxins (aflatoxins, ochratoxin A and zearalenone), however, with the method used, the presence of any of them was not evidenced. In a survey carried out by Nunes et al. (2003) in different types of rice (coarse, parboiled and white polished), the authors detected samples contaminated with ochratoxin A and zearalenone. Silva et al. (2008) analyzed the aflatoxin contamination in the rice used in a government department by thin layer chromatography and toxin was not detected, but when high performance of liquid chromatography was used in the same samples, aflatoxins were detected in 23.07% of the samples. In this study, aflatoxins were derived in a post-column electrochemical reactor KOBRACELL mark and screened by fluorescence detection with a wavelength of 425 to 360 nm and B1 and B2, G1 and G2 to 455 nm, and the quantization limit of the technique 0, for each aflatoxin 5 mg/kg.

It is recommended that survey will be conducted routinely in rice, not only fungi but also bacteria, because only with this control, the consumer providing quality products can be guaranteed.

Conclusion

The rice products (rice dough and rice flakes) commercialized in Terezina had satisfactory, hygienically and sanitary conditions by the techniques, the presence of products in the established standard by the relevant legislation was not found. Mycotoxins were not detected in the samples.

Conflict of interests

The author(s) did not declare any conflict of interest.

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