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OCCURRENCE OF *ASPERGILLUS FLAVUS* AND AFLATOXIN B_1 IN PADDY AND IT MILLING FRACTIONS WITH REFERENCE TO NORMAL AND CONTROLLED MILLING ENVIRONMENT

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Keywords:

Storage fungi, *Aspergillus flavus*, Aflatoxin B_1, Parboiled rice, Raw rice, Husk, Brown Rice and Rice Bran

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ABSTRACT: The contamination of Rice and its by-products with storage fungi, especially toxigenic *Aspergillus flavus* and Aflatoxin B_1 leads to potential health hazard to human and domestic animals. Therefore, the present investigation was focussed on the comparative analysis of the fungal contamination with *A. flavus* and Aflatoxin B_1 in Raw and Parboiled paddy and its milling fractions like Husk, Brow Rice, Polished rice and rice bran in a normal rice mill and controlled mill environment. The results of the study indicated that the raw and parboiled paddy showed 100% contamination with fungi in untreated grains in which *A. flavus* occurred 63% and 77%, respectively. The level of Aflatoxin B_1 in untreated Raw and Parboiled paddy was 36 ppb and 50 ppb, respectively. The husk analyzed for the presence of fungal contamination showed 100%, 85%, and the *A. flavus* was 43%, 30% respectively for Raw and Parboiled grains. But there is no Aflatoxin B_1. Found in either of the husk samples. The brown rice exhibited almost closer to the untreated Raw and Parboiled paddy for both *A. flavus* contamination and the presence of Aflatoxin B_1. The Polished rice showed a low level of contamination with *A. flavus* and Aflatoxin B_1 in both normal and controlled milling environments. In Rice bran of Raw and Parboiled paddy, the levels of *A. flavus* ranging from 12,000 cfu/g to 28,000 cfu/g. However, the bran showed a moderate to a high level of Aflatoxin B_1 contamination as 120 ppb in Raw rice bran to 150 ppb in parboiled rice bran, whereas the parboiled rice bran of controlled mill environment showed only 10 ppb. Thus, the present study concludes that the controlled milling environment shows a lower level of contamination of fungi as well as aflatoxin B_1 than normal milling environment, which leads to requiring sanitation practice that may give a reduction of fungal load as well as Aflatoxin B_1 contamination in some extent.

INTRODUCTION: Rice is a semi-aquatic, annual grass that can be grown under a broad range of climatic conditions [1]. It is not only a part of many people's diet around the world but being the main energy source in some regions.

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Rice (*Oryza sativa* L.) is cultivated in almost all the states of India, contributing about 42% to the country's foodgrain production and provides a livelihood for about 70% of the population.

About 90% of rice is being produced in Asian countries, particularly China and India being the major producers [2]. The storage fungi and mycotoxins are the major causes of losses in agricultural commodities and its products from harvesting to storage [3, 4, 5, 6]. The rice is commonly called "paddy" when harvested because the grain is still covered by the husk.

After husk separation, the rice is called "brown rice" and it can be passed through polishing and sorting processes before being called "white rice" or polished rice, which is the most used form for food. Since rice production is affected due to changes in temperature, ongoing climate change can pose a risk to rice productivity and availability due to possible increases of abiotic and biotic stresses [7]. Rice is the primary source of carbohydrates, protein, fiber, some vitamins like the B complex & E, and minerals in which 75% of weight in with starch. Although the nutritional value of rice is very good but despite all this the problem is the contamination with fungi and fungal toxin production due to fungal attack on standing crops or during storage when humidity of atmosphere and moisture content storage and humidity atmosphere of the rice grain are higher.

The higher moisture content and humidity atmospheare of rice favour the growth and contamination with graph of Aspergillus, Penicillium which are generally called as storage fungi. The group species *A. glaucus*, *A. flavus*, *A. candidus*, *A. niger*, *A. nidulans*, *Penicillium citrinum*, and *P. funiculosum* are the major storage fungi in different forms of rice stored in different ways [8, 9, 10, 4, 11, 12, 13]. In rice, the *A. glaucus* dominated at 15.5% moisture content, and toxin producing *A. flavus* grow well at 18% moisture content was reported by [14].

According to [15], Aspergillus species, which grow at a starting RH of 65% are common initiators of grain deterioration in storage and transit. Aflatoxins are produced as a by-product during the growth of fungi, *Apergillus flavus*, and *Aspergillus parasiticus*. Mycotoxins especially aflatoxins are toxic substances produced by fungi of the genus Aspergillus which grow on cereals and other agricultural crops [16]. Aflatoxins are of great concern with respect to public health due to their potential as powerful hepatotoxic [17]. Toxins produced by the toxigenic *A. flavus* strains are called Aflatoxins which are highly toxic, mutagenic, teratogenic, and carcinogenic. They are of economic and health importance because of their ability to contaminate agricultural commodities worldwide, in particular cereals and oilseeds, resulting in contaminated human food and animal feeds which cause a heavy loss in the market, including export. Consumption of food contaminated with Aflatoxins has been shown to produce human hepatic and extrahepatic carcinogenesis [18]. Aflatoxin exposure increases the risk of liver cancer, particularly hepatocellular carcinoma, and if the exposure is high, it can result in acute aflatoxicosis and death [20].

The growth and multiplication of toxin-producing *A. flavus* in the cereal grains start from the field at the standing crops stage and further increase during storage after harvesting [13]. Further contamination and growth with different species of grain spoilage fungi in rice were directly changed during paddy processing treatment [20, 21].

The changing pattern of mycoflora and occurrence of aflatoxin in milling fraction of rice were reported by [22]. The pattern of food grain spoilage fungi in paddy at different climatic areas was studied by Vincent [23] and found the higher humidity area like coastal region form the growth of fungi on a rice grain. Hence, the present study was focused on finding out the contamination of toxigenic *A. flavus* and aflatoxin B_1 in paddy and its milling fraction at a normal and controlled rice mill environment.

MATERIALS AND METHODS:

1. Collection of Paddy Samples: Specific lot of paddy sample, about 30 kg was collected from Tiruvannamalai District of Tamil Nadu. The paddy sample was collected directly from the formers field after threshing and drying. Approximately 10kg for each treatment was collected and kept in the laboratory for further study. About 10 kg was retained as raw paddy and 20 kg used for parboiling in a normal and controlled mill environment. In this study, a total 15 samples in Raw and parboiled paddy and it milled products, brown rice, polished rice, husk, and rice bran were collected for the analysis of fungi and aflatoxin B_1. The samples were collected epically in a cleaned polythene bags and tightly used for further analysis. The rice samples were collected from the paddy processing in the following ways:

- Raw paddy and milled products
- Parboiled paddy and its milled products from the rice mill in a normal/ regular environment.

- Parboiled paddy and its milled products from the rice mill in the controlled environment as cleaning and hygiene were maintained to prevent atmospheric contamination of fungi.

Paddy Processing and Milling Process: In paddy processing, Paddy is placed in the 'Hopper' of the huller. From the hopper, the paddy is fed continuously by a vibrating feeder, between two rollers: a fast roller having corrugations, and a slow synthetic roller having a smooth surface. Then, the removal of husk and brown rice during milling and polishing of brown rice into polished rice by removing outer layer is called rice bran. During polishing the bran passes out through the perforated screen and polished rice is discharged through the outlet. Parboiling is the process of soaking paddy for overnight and steamed for 60 minutes. Then the steamed (or) parboiled paddy is subjected to drying in an open yard. Then the dried parboiled paddy is between for milling process as hulling and polishing to obtain polished rice and parboiled rice bran to bring down the moisture to 16%.

Mycofloral Analysis:

(i) Sample Preparation: The paddy samples were mixed thoroughly by shaking, and a subsample was drawn for platting. The rice grain was directly plated on the nutrient agar medium without any treatment is called untreated grain, which harborn the fungi on the surface as well as infection on the grains to some extent. By this method, the Surface Contaminations' (sc) of the grains were allowed to grow on the agar medium [3]. Husk was collected after hulling process of paddy, where the brown rice and husk were removed. The brown rice was also collected separately for analysis. Rice brawn and polished rice samples were collected after the polishing process of the brown rice in the rice mill for analysis. Uniform random sampling was done for platting and analysis.

(ii) Plating, Incubation, Observation and Identification: Grains treated above were spread out in a sterile filter-paper-lined petri dish to absorb excess moisture. From these, 100 grains were picked up randomly one by one using sterile forceps and plated on the Czapex-dox agar (50 sucrose) medium. A paper template was used under the plates for even distribution of 25 grains in each plate for enumeration of fungi from rice bran samples; the serial dilution plating technique was used and inoculate in CDA medium. The plates were incubated at 30 ± 1 ºC for 7 days observed daily from the 3rd to 5th day, and in some cases up to the 7th day after inoculation. The morphological and micro-scopical characters of fungal colonies that grew out were observed for identification of the individual species, as described in the standard method.

3. Analysis of Aflatoxin B_1:

Extraction and Estimation of Aflatoxin B_1 in the Rice Samples: Samples of various products of parboiled and raw rice were analyzed for aflatoxin B_1 by the standard method [25]. Fifty grams of samples were blended with 150 ml of methanol in a blender at high speed for 3 min and filtered through Whatman No. 1 filter paper. Thirty ml of the methanol extract was taken into a 250 ml separating funnel, and 60 ml of 20% ammonium sulphate was added followed by 30 ml of hexane. The separating funnel was shaken for 30 sec, and the layers were allowed to separate.

The upper hexane phase was discarded and 5 ml of methylene chloride was collected at the lower end of the funnel. The lower phase was removed into a small beaker and evaporated over a steam bath. The residue was re-dissolved in 0.5 ml benzene: acetonitrile (98:2), and the solution was designated as the assay sample. Here paddy samples were ground in a mixer grinder. Duplicates were made in every analysis of the experimental study for plating of fungi and analysis of aflatoxin B_1. Statistical calculations were made to avoiding the deviation (SD) and error in experiments.

RESULTS AND DISCUSSION: In the present study, about 30 kg of Ponni variety paddy samples were collected from Tiruvannamalai District of Tamil Nadu, and the occurrence of fungi in the paddy and its milled products such as i. Paddy, ii. Husk, iii. Brown rice, iv. Polished rice and v. Rice bran were studied for qualitative as well as quantitative patterns. The details on the species of fungi enumerated from the above samples were presented in **Table 1** & **2**. Totally 26 species of fungi observed from the above samples, including one species non - sporulating fungi. The total number of fungi and *A. flavus* colonies in the above rice samples were presented in **Table 3** to **8**.

TABLE 1: DETAILS OF SAMPLES USED IN THE ANALYSIS OF FUNGI AND AFLATOXIN B_1

S. no.	Sample details	RR	PBR		Total no.of samples
			NE	CE	
1	Paddy	1	1	1	3
2	Husk	1	1	1	3
3	Brown Rice	1	1	1	3
4	Polished Rice	1	1	1	3
5	Bran	1	1	1	3
Total		5	5	5	15

- Raw rice NE - Normal milling environment, Pbr- Parboiled rice, CE - Controlled milling environment

TABLE 2: QUALITATIVE OCCURRENCE OF FUNGI IN PADDY, BRAN AND OTHER PRODUCTS

S. no.	Name of species	Paddy	Bran
1	*Aspergillus candidus*	+	+
2	*A. clavatus*	-	-
3	*A. flavipes*	+	-
4	*A. flavus*	+	+
5	*A. fumigatus*	-	+
6	*A. glaucus*	+	+
7	*A. nidulans*	+	+
8	*A. niger*	+	+
9	*A. ochraceus*	-	-
10	*A. repens*	-	-
11	*A. restrictus*	+	-
12	*A. tamarii*	+	-
13	*A. terreus*	+	+
14	*Penicillium chrysogenum*	-	+
15	*P.citrinum*	+	+
16	*P. funiculosum*	+	-
17	*P. oxalicum*	+	-
18	*P. purpurogenum*	-	-
19	*Absidia corymbifera*	+	-
20	*Cladosporium cladosporiodes*	-	+
21	*C. oxysporum*	-	+
22	*Mucorsp*	+	+
23	*M. racemosus*	+	+
24	*Rhizopusstolonifer*	+	+
25	*Trichoderma*	-	+
26	*Non sporulating fungi*	+	+

(i) Paddy: The paddy grains from all the samples as in raw paddy and parboiled paddy in a normal environment showed 100% fungal contamination on the surface, where the parboiling and drying in the open atmosphere. When the paddy is parboiled in the controlled environment, it shows, absence of fungi at the surface level after drying. In the same way, the *A. flavus* occurred in the above samples as 70% in raw paddy and 75% parboiled paddy. Whereas no contamination with fungi, including *A. flavus* occurred in parboiled paddy from a controlled environment. The results of the large number of paddy samples were reported by 11. In the above study, paddy samples of both from storage atmosphere and standing crops in the field showed 100% fungal contamination, which is true in the present study on the observation from paddy samples. The results of the fungal occurrence in the paddy samples showed in **Table 3**.

TABLE 3: OCCURRENCE OF TOTAL FUNGI, *A. FLVUS* AND AFLATOXIN B_1 IN DIFFERENT PADDY SAMPLES

S. no.	Sample details	Parameters analyzed		
		Total grains infected	No. of grains With *A. flavus*	Occurrence of AFB_1 (ppb)
1	Raw paddy (RP)	100	70	36
2	Parboiled paddy (N E) (Pbp)	100	75	50
3	Parboiled paddy (C E) (Pbp)	0	0	0

RP - Raw paddy, NE - Normal milling environment, Pbp- Parboiled paddy, CE - Controlled milling environment, AF B_1- aflatoxin B_1, ppb-Parts per billion

(ii) Husk: In the husk, the contamination of fungi on surface-level from all 3 samples as raw paddy, parboiled paddy in an open environment, and parboiled paddy in a controlled environment almost reflect the paddy samples. The percentage of infestation of paddy samples observed as 100 in raw paddy, 85 in parboiled paddy, and absent in parboiled paddy under a controlled environment. Regarding the occurrence of *A. flavus*, 50% on raw paddy, 35% on the parboiled sample in an open environment, and is 0% in parboiled rice in a controlled environment. Whereas, for the analysis of aflatoxin B_1, all the 3 samples showed negative results *i.e.* 0ppm.

This result indicates the aflatoxin B_1 is present/ produced in the nutritive portion of the paddy as alueron layer, which covers the brown rice. Hence, it is concluded that the husk is not supporting the production of Aflatoxin B_1 because it does not contain any nutrients.

The studies on storage fungi, especially Aspergillus species in paddy processing by 22, show the husk contains less number of fungi from open mill environment, and no fungi from a controlled environment is reflecting in the present study. The results of fungi in the husk showed in **Table 4**.

TABLE 4: OCCURRENCE OF TOTAL FUNGI, A.FLVUS AND AFLATOXIN B_1 IN DIFFERENT HUSK SAMPLES

S. no.	Sample details	Parameters analyzed		
		Total grains infected	No. of grains with *A. flavus*	Occurrence of AFB_1 (ppb)
1	Raw paddy (RP) – Husk	100	50	0
2	Parboiled paddy (Pbp) (N E)-Husk	85	35	0
3	Parboiled paddy (Pbp) (C E) – Husk	0	0	0

RP - Raw paddy husk, NE - Normal milling environment, Pbp- Parboiled paddy, CE - Controlled milling environment, AFB_1- aflatoxin B_1 Ppb-Parts per billion

(iii) Brown Rice: In brown rice, the overall fungal contamination was for total fungi observed as slightly lower than the whole paddy from all the sample sources as 85%, 82%and 45%, and 35% of *A. flavus*. The proportion of *A. flavus* did not markedly differ between raw and parboiled samples except in the sample from the control environment, where it was generally lower (0%). However, the percentage of *A. flavus* in untreated grains was consistently lower in the samples milled in the milled environment than in commercial mills of the normal environment even though the difference was not very high **Table 4**. But, the amount of aflatoxin B_1 in brown rice is slightly higher than the rough rice (paddy) from the above samples. This may be due to the production of aflatoxin is concentrated in edible portions and is absent in husk because it does not contain nutrients. The results of fungi in brown rice showed in **Table 5**.

TABLE 5: OCUURENCE OF TOTAL FUNGI, *A. FLAVUS* AND AFLATOXIN B_1 IN DIFFERENT BROWN RICE SAMPLES

S. no.	Sample details	Parameters analysed		
		Total grains infected	No. of grains *A. flavus*	Occurrence of AFB_1 (ppb)
1	Raw paddy (RP) -Brown rice	85	45	70
2	Parboiled paddy (pbp) (N E)-Brown rice	82	35	60
3	Parboiled paddy (pbp) (C E)- Brown rice	0	0	0

RP - Raw Paddy, NE - Normal milling environment, Pbp - Parboiled paddy, CE -Controlled milling environment, AFB_1- aflatoxin B_1 Ppb-Parts per billion

(iv) Polished Rice: The observation for the fungal contamination in polished rice shows 35% in raw paddy and 72% in parboiled paddy which is more (or) less reflect the results of the brown rice but slightly lower.

The Proportion of *A. flavus* occurrence is 50% lower in both the above samples. However, the polished rice samples showed no contamination with aflatoxin B_1, which indicates that the toxin may be present on the nutrient-rich out layer of rice grains which is removed during polishing.

Earlier reports on the analysis of raw and parboiled polished rice samples for the contamination of fungi showed the presence of fungi which coincide

which the present study [6]. Hence, there is a possibility of the removal of fungal metabolites through rice bran. However, the parboiled paddy and its products from a controlled environment show absence of fungi and Aflatoxin B_1 in **Table 6**.

TABLE 6: OCUURENCE OF TOTAL FUNGI *A. FLAVUS* AND AFLATOXIN B_1 IN DIFFERENT POLISHED RICE SAMPLES

S. no.	Sample details	Parameters analysed		
		Total grains infected	No. of grains A. *flavus*	Occurrence of AFB_1 (ppb)
1	Raw paddy (RP) -Polished rice	35	20	0
2	Parboiled paddy (Pbp) (N E) - Polished rice	72	10	0
3	Parboiled paddy (Pbp) (C E)- Polished rice	0	0	0

R - Raw Paddy, NE - Normal milling environment, Pbp - Parboiled paddy, CE- Controlled milling environment, AFB_1- aflatoxin B_1 Ppb-Parts per billion.

(v) Bran: In rice bran, the levels of *A. flavus* occurred from 12,000 to 28,000 cfu/g in both raw and parboiled rice bran from a normal mill environment. In the rice bran prepared from the parboiled rice of a controlled milled environment, it shows absence of *A. flavus*. The remarkable result is the presence of high level of aflatoxin B_1 as 120 ppm, 150 ppm in raw and parboiled rice bran of normal environment. This indicates that the bran carrying all the fungal products, including toxins from rice grain, during milling process. The parboiled rice bran from a controlled mill environment shows no aflatoxin B_1 contamination. The analysis of a large number of rice bran samples raw and parboiled rice from rice mill showed a significant level of contamination which fungi and aflatoxin (Jayaraman & kalyanasundaram, 1990). The above observation was also true of polished rice, which had a fairly high overall fungal contamination, but a low proportion of *A. flavus* are shown in **Table 7**.

TABLE 7: OCUURENCE OF TOTAL FUNGI, A. *FLAVUS* AND AFLATOXIN B_1 IN DIFFERENT RICE BRAN SAMPLES

S. no.	Sample details	Parameters analyzed	
		Total grains infected	No. of grains A. *flavus*
1	Raw paddy (RP) -Polished rice	35	20
2	Parboiled paddy (Pbp) (N E) - Polished rice	72	10
3	Parboiled paddy (Pbp)(C E)- Polished rice	0	0

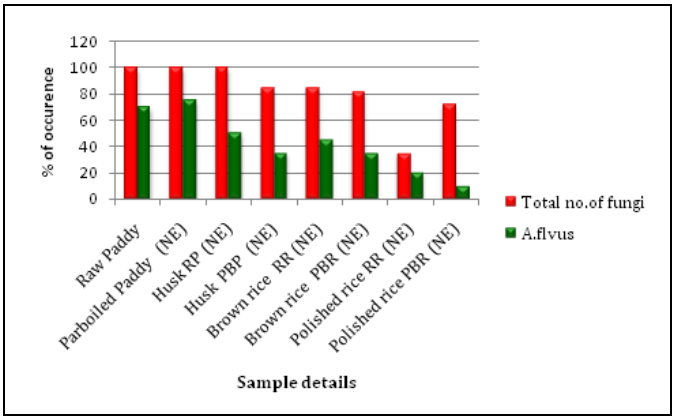
RP - Raw paddy, NE- Normal milling environment, Pbr- Parboiled paddy, CE - Controlled milling environment, AFB_1- aflatoxin B_1 Ppb-Parts per billion.

(vi) Aflatoxin B_1 in Paddy and its Milled Products: The occurrence of aflatoxin B_1 (AFB_1) in paddy samples ranging from 36 ppb (raw paddy) to 50 ppb (parboiled paddy in a normal environment), whereas parboiled paddy from control environment show no aflatoxin B_1. The husk from all the paddy samples showed an absence of aflatoxin B_1. The aflatoxin B_1 occurrence in the brown rice samples of raw paddy and parboiled paddy from a normal environment was 70 ppb and 60 ppb, respectively. The polished rice of both raw and parboiled rice show free of aflatoxin B_1 which may due to the removal of toxins present in the nutrient layer on the endosperm. But, the rice bran samples of raw rice and parboiled rice contain a higher amount of aflatoxin B_1 as 120 ppb and 150 ppb, respectively.

TABLE 8: COMPARATIVE OCCURRENCE OF TOTAL FINGI, *A. FLAVUS* AND AFLATOXIN B_1 IN PADDY AND ITS MILLED PRODUCTS

S. no.	Sample details	Raw rice (Rr)		Parboiled rice (pbr)			
				Pbr (NE)		Pbr (CE)	
		A. flavus (%)	AFB_1 (ppb)	*A.flavus (%)*	AFB_1 (ppb)	*A .flavus (%)*	AFB_1(ppb)
1	Paddy	70	36	75	50	0	0
2	Husk	50	0	35	0	0	0
3	Brown rice	45	70	35	60	0	0
4	Polished rice	20	0	10	0	0	0
5	Bran	12,000 (cfu/g	64	28,000 C(fu/g)	220	0	0

The occurrence of a higher amount of aflatoxin B_1 in parboiled rice bran and a lower amount from raw rice bran were reported by 5, 26 in their survey study, which corroborates the present investigation. The contamination of aflatoxin B_1 in paddy samples from raw paddy and parboiled paddy were reported by [23, 24]. Similar results were observed from the present study of paddy samples. **Table 8** shows the overall presence of fungi, toxigenic *A. flavus*, and aflatoxin B_1 in different samples of paddy and it milled products in both the environment.



GRAPH 1: COMPARATIVE OCCURRENCE OF TOTAL FUNGI AND *A. FLAVUS* IN PADDY, HUSK, BROWN RICE AND POLISHED RICE SAMPLES PROCESSED IN NORMAL ENVIRONMENT Note- Rice sample from control environment show absence of fungi including *A. flavus* and aflatoxin B_1

CONCLUSION: It is concluded that from the present study as the paddy & milled fraction show the higher population of fungi in the raw paddy and parboiled paddy prepared from the normal environment. When compared with a controlled environment maintained by avoiding external air contamination, the presence of *A. flavus* and aflatoxin B_1 contamination in the above samples are not reflecting proportionately. The presence of a high level of aflatoxin B_1 in parboiled rice samples in few cases might be due to nutritional changes and supportive effect on aflatoxin production by toxigenic *A. flavus* already present in the paddy. The higher level of aflatoxin B_1 in rice bran might be the presence of aflatoxin B_1 only in the outer layer of grains, *i.e.*, aleurone layer, which contains higher nutrients. Since cattle feed and other food products contain a considerable percentage of rice bran, there is a possibility of the presence of aflatoxin B_1 in respective products, which possess health hazards to humans and animals apart from the mill environment.

It can be suspected that a considerable amount of toxin may be produced during the parboiling process, *i.e.*, during the soaking and drying stages. A comparison of aflatoxin content of paddy and bran (**Table 2** and **6**) might give an impression of much higher levels of toxin in bran. However, considering that bran forms a lower amount by weight of paddy, the actual levels ought to have been much higher. The levels of *A. flavus* as well as Aflatoxin B_1 were much lower in fractions of milled rice in the controlled environment than in the normal environment of commercial mills. Hence, sanitation practice and controlled environment of rice mill may help in the control of fungus and aflatoxin B_1 to some extent or not completely. Thus, the eco-friendly detoxification of aflatoxin B_1 in the raw materials before being the final product for consumption will be helpful for the safety of human health.

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