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ORIGINAL ARTICLE

Aflatoxigenic *Aspergillus flavus* Contamination in Red Pepper – A Prototype Growth Study

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ABSTRACT
The present study focused on the growth of Aspergillus flavus, its level of contamination, and changes occurring due to contamination, in different forms of red pepper (whole, crushed and ground) during its storage period. The growth of Aspergillus flavus in red pepper was determined by means of total fungal count and content of ergosterol. The rate of production of aflatoxins by the fungus in red pepper was also monitored. The fungal colony-forming unit (CFU) count and content of ergosterol revealed that Aspergillus flavus growth was significantly higher in the crushed sample than that in whole and ground forms of red pepper. Similarly, the Aflatoxin content was high in crushed red pepper followed by ground and whole red peppers. The results revealed that crushed red pepper samples were highly susceptible to Aspergillus flavus growth and aflatoxin contamination, thereby indicating the crucial importance of its storage under appropriate and sterile conditions.
Key words: *Aspergillus flavus, Red pepper, Contamination, Growth, Total fungal colony count, Ergosterol, Aflatoxin*

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INTRODUCTION

Spices are a commodity that has been entwined with humankind since primitive times, defining mores and racial multiplicity. Among spices, Red pepper (*Capsicum annuum* L.) is an admired and extensively used commodity all over the world. The brilliant red colour, aroma, pungency and nutritional value of red pepper have been attributed to its volatile oils, carotenoids, capsaicinoids, protein, fibre and mineral contents [26]. *Capsicum* fruits are of significance due to their medicinal and pharmacological properties including antioxidant, anti-inflammatory, anticancerous and analgesic activities [18]. Presence of bioactive compounds mainly; flavonoids, phenolic acids, carotenoids, and vitamins C, E, A make red pepper an important functional food of pharmacological significance [11].

The presence of mycotoxins like aflatoxin due to the contamination of toxigenic fungi is a major food safety issue associated with red pepper processing and storage. *A. flavus*, *A. parasiticus*, *A. nomius*, *A. pseudotamarii*, *A. bombycis*, *A. ochraceoroseus*, and *A. australis* belonging to *Aspergillus* genera have been reported to produce aflatoxin. Among them, *A.flavus* is the main reported foodborne fungus and abundantly present in the tropical regions. Ingestion of foods contaminated with aflatoxins can result in aflatoxicosis. Aflatoxicosis can be of acute or chronic type [10]. In developed nations, the estimated average aflatoxin intake through dietary exposure is <1ng/kg body weight per day. However, it is >100 ng/kg body weight per day in the case of some sub-Saharan African nations [7]. Chronic exposure to

aflatoxins has been attributed to have a role in the development of cancer, impaired blood coagulation and protein formation, toxic hepatitis, immunosuppression etc. Aflatoxins are considered to be Group I carcinogen by the IARC and aflatoxin B1 is reported to be 1000 times more potent than benzopyrene based on TD_{50} parameter. Together, aflatoxin and hepatitis B exposure add to the possibility for the development of human hepatocellular carcinoma [14, 25].

Aflatoxin contamination in red pepper is a grave concern as it can affect primarily the producers by incurring huge economic losses and simultaneously affect the consumers, especially of the less developed countries by incurring health-related costs when human and animal health are affected. Aflatoxin level is by and large a function of the contaminant fungal strain. Understanding the growth pattern of contaminant fungi would thus be fundamental in predicting fungal growth and thus decisive in averting aflatoxin contamination [6, 25]. In the present study, we have tried to understand the growth pattern of toxigenic *Aspergillus flavus* in red pepper. The experimentally infected red pepper samples were observed for 30 days during which parameters like total fungal colony count, ergosterol content, protein content, moisture content, and aflatoxin production were analyzed to comprehend the extent of aflatoxin contamination in red pepper samples and to bring out a correlation between the various parameters and to explore its application in risk analysis concerning red pepper.

MATERIAL AND METHODS

SAMPLE

Samples of red pepper in whole, crushed and ground form were used for the study. Good quality red pepper samples were procured and steam sterilized (120ºC, 4.35psi). 150g of sterilized red pepper were transferred into sterile 2l conical flasks. For each sample type, five such conical flasks were maintained (i.e., Whole – 5, Crushed – 5, Ground – 5). The sterilized samples (one flask of each sample type) were randomly checked for their moisture content and fungal colony count before starting the experiment to rule out any microbial contamination present. The sterile samples had a moisture level in the range between 8% to 10% and the dilution plating of the samples revealed nil growth in the dilution plates. The remaining batch of sterilized red pepper samples (four flasks of each sample type) served as the substrate for performing the growth study. Out of the four flasks, three flasks maintained as replicates and one flask served as uninoculated control.

ASPERGILLUS FLAVUS

Aspergillus flavus, the fungus used in the present study was isolated from a visibly contaminated crushed red pepper sample. Pure isolate thus obtained was confirmed as *A. flavus* by means of morphological and molecular identification. Morphological identification was done by observing fungal growth and the colony diameter on various fungal media i.e., Czapek yeast extract agar (CYA), 25% Glycerol nitrate agar (G25N), Czapek yeast extract agar with 20% sucrose (CYA20S), Malt extract agar (MEA), *Aspergillus* differentiation medium (ADM) and Dichloran Rose Bengal Chloramphenicol agar (DRBCA) at 25ºC and Czapek yeast extract agar (CYA) at 37 ºC. Lactophenol cotton blue staining and scanning electron microscopy was also done. Molecular confirmation was done by PCR amplification of ITS fragments followed by rDNA - ITS sequencing. Bioedit software v 7.0.9 [9] was used to edit the sequences. The sequences were then blasted with GenBank Nucleotide Database (http://www.ncbi.nlm.nih.gov/BLAST/) applying the algorithm Blast N [1]. The output was sorted based on maximum identity with other genus/ species names in GenBank records. The sequence based identity that showed cut-off of 97% or greater was considered significant. The sequence with highest maximum identity to the query sequence was defined as the best hit. The fungal gene sequence was identified as of *A.flavus*. The gene sequences were deposited in GenBank and the accession number was obtained (GenBank Accession Number SUB3194471 *Aspergillus* MG388294).

Qualitative confirmation of aflatoxin producing capability of the *A.flavus* isolate was done by detecting the presence of blue fluorescence surrounding the colony growth on Coconut agar medium (CAM). For this, the isolate was first cultured on *Aspergillus* differentiation medium and then agar plug of the grown culture was taken and inoculated in the center of coconut agar plates. The plates were checked from 2nd day to 5th day for the appearance of blue fluorescence under long wavelength UV light of 365 nm. Appearance of a ring of blue fluorescence surrounding the fungal isolate was characteristic of aflatoxin production.

PREPARATION OF A.FLAVUS SPORE INOCULUM

A.flavus spore inoculum to be used for inoculation of red pepper sample was prepared from 7 days old culture on PDA slants. For the preparation, 10 ml of 0.1% Tween 80 was added to PDA slants and the culture scraped using a sterile loop. Using a Haemocytometer, the spore count in the suspension was estimated. 2 ml containing the spores (about 10^5 spores) was inoculated into flasks containing the red

pepper samples. An un-inoculated sample served as the control. Inoculated triplicate samples (Whole – 3, Crushed – 3 and Ground – 3) and control (Whole – 1, Crushed – 1, Ground – 1) were maintained at 28ºC and studied for the next 30 days period to find the extent of fungal growth and contamination in red pepper. For understanding the growth pattern of *A.flavus* in red pepper, determination of total fungal colony count and ergosterol estimation was done. Also, analysis of the aflatoxin content, changes in moisture content and protein estimation were monitored for 30 days. On the 0^{th}, 3^{rd}, 7^{th}, 14^{th}, 21^{st} and 30^{th} day of inoculation, the required quantity of samples was retrieved from the inoculated red pepper samples (triplicates) and analyzed for the various parameters.

GROWTH STUDY

Total fungal colony count

Total fungal colony count was determined using the dilution plating method. From the experimental set for each sample type, 2.5g of sample was withdrawn and added to 22.5 ml of Butterfield's phosphate-buffered dilution water. Sample dilutions of up to 10^{-6} were prepared by transferring 1ml of previous dilution to 9ml of diluent. All the dilutions were shaken 25 times in 30 cm arc for 7s. 1ml of each dilution was transferred at the centre of sterile marked Petri dish in triplicates. 15-20 ml of Dichloran18% glycerol agar (Cooled to 45± 1ºC) was poured on to each plate. The sample dilution and agar medium was uniformly mixed by alternate rotation and moving the plates back and forth. The plates were incubated upright in dark at 25ºC for 5 days without disturbing and counted after 5 days of incubation. The plates were re-incubated for another 48 hours if no growth was observed. Agar plates containing 10-150 colonies were counted and results expressed as CFUs/g or CFUs/ml based on average count of the triplicates [2].

Aflatoxin analysis

For aflatoxin analysis, the American Spice Trade Association Analytical Method 24.2 was followed [16]. Aflatoxin content present in the inoculated chilli samples were determined by LC-MS/MS (HPLC-Agilent-1260 infinity with autosampler and MS MS) using Syncronics C-18, 5µm, 4.6 × 100 mm column. 2 g of the homogenized red pepper sample was mixed with 1g of sodium chloride and 20 ml of acetone: water at a ratio of 80:20 and blend at high speed for 3 minutes and centrifuged at 6000rpm for 10 minutes at 10ºC. The blender contents were filtered through a prefolded filter paper and the pH adjusted to 7.4 using 2M sodium hydroxide. 10 ml of the filtrate was pipetted and diluted with 100ml phosphate buffered saline solution (PBS) and mixed thoroughly. Immuno affinity columns to be used were adjusted to room temperature. After removing the caps from the column top, they were fixed in the vaccum manifold. 100 ml of the diluted filtrate was passed through the column at a flow rate of 2-3ml per minute. Once the entire sample had passed through the column, the column was rinsed with 10ml of HPLC water at a flow rate of 6ml per minute. The rinse was repeated using 10ml PBS. Further air was passed through the column to remove any residual liquid. Using 0.5ml of 100% methanol, the bounded aflatoxins were eluted from the column at a flow rate of 1 drop per second and following elution, 0.5 ml of Millipore water was passed through the column and collected in amber glass vial. The clear solution was diluted to 10 times with initial mobile phase and the sample extract was ready. 20µl of the sample extract was injected into LC-MS/MS containing the mobile phase of Methanol: 0.1% formic acid in water.

Ergosterol analysis

2g of the sample was saponificated (1h reflux to 70ºC) with 2g KOH in 25 ml of methanol in the presence of 100µl of butylated hydroxytoluene (BHT) as antioxidant mixture was extracted with petroleum ether. After the saponification they were left to cool. The saponified mixture was extracted with petroleum ether. The residue from evaporated petroleum ether extract was dissolved with 1ml of methylene chloride-isopropanol. The clear solution was taken out and diluted to 10 times with initial mobile phase and injected into HPLC UV. For HPLC analysis a ZORBAX Eclipse C18 column was used with a water, methanol and acetonitrile gradient. The detection wavelength was 280 nm [13].

Moisture analysis

5g of the sample was transferred to distilling flask and sufficient volume of toluene was added to cover the sample completely and boiling chips also added. After assembling the apparatus, the trap was filled with toluene. A loose non-absorbing cotton plug was inserted into the top of the condenser, to prevent condensation of atmospheric moisture to the condenser. After bringing to boil, reflux at about 2drops per second was done until most of the water got collected in the trap and then the reflux rate was increased to 4 drops per second. After 2 hours the reading was noted and refluxing continued until two consecutive readings 15 min apart showed no change so that the total extraction was 3 hours. Any water held up in the condenser was dislodged with a brush/wire loop. The condenser was carefully rinsed with 5ml toluene. Volume of water in the trap was read. The moisture content was reported to an accuracy of 0.0% by weight [15].

Protein analysis

Total protein content in the sample was quantified using Lowry's method [12]. The amount of protein in the sample was estimated from the standard curve of Bovine Serum Albumin (BSA) versus absorbance. The sample total protein content was expressed as equivalent microgram bovine serum albumin per 0.1 ml sample (µg protein/0.1 ml).

Maximum specific growth rate

From the obtained values of total fungal colony count and ergosterol content, maximum specific growth rate (μ_{max}) was calculated using the equation; $\mu_{max} = \frac{\ln x2 - \ln x1}{t3 - t2}$ [21]. Lag phase was calculated from the intercept of the tangent at the inflection with the lower asymptote [3].

RESULTS AND DISCUSSION

The variations in the total fungal count (CFU) in whole, crushed and ground forms of pepper are given in Figure 1. In order to deduce the fungal growth kinetics, the fungal colony count in logarithmic scale was plotted against the time. The total fungal colony count results showed that the highest fungal contamination during the study occurred in crushed red pepper (reaching 10^4 CFU/g). In the case of ground red pepper, the count increased for 14 days and then decreased. Smita and Mishra, 2009 [24] has reported a similar trend for ground red pepper in which the fungal counts increased up to day 25 and then decreased. Uninoculated control showed nil growth. The maximum specific growth rate (μ_{max}) was calculated from the slope value of the kinetic curve. The μ_{max} values were in a decreasing order of crushed red pepper (0.17 Day^{-1}) > ground red pepper (0.05 Day^{-1}) > whole red pepper (0.02 Day^{-1}). The absence of a lag period for crushed red pepper sample was suggestive of crushed red pepper as an excellent substrate for the growth of *A.flavus*. For ground red pepper, the growth phase started after a short lag period of 1.75 days. A lag phase of 2.5 days was seen for the whole red pepper.

The ergosterol content was also more for the crushed sample. It increased from 0th Day to 30th Day in crushed and whole red pepper sample whereas for ground sample, the increase was observed till Day 14 and it reduced by Day 21 and 30. In order to deduce the fungal growth kinetics, the ergosterol content in the logarithmic scale was plotted against the time (Figure 2). The maximum specific growth rate was calculated (Crushed red pepper μ_{max} = 0.23 Day^{-1}, Whole red pepper μ_{max} = 0.04 Day^{-1} and Ground red pepper μ_{max} = 0.09 Day^{-1}). The μ_{max} calculated based on ergosterol content was higher for Crushed red pepper>Ground red pepper>Whole red pepper. Aflatoxin content increased in all the red pepper samples and was comparatively higher in crushed red pepper samples (Table 1). With regard to total protein content, there was a slight reduction for all the red pepper samples starting from Day 0 to Day 30; an initial average of 7.7mg/g reduced to 5.9mg/g for crushed red pepper, 7.0mg/g to 5.3 mg/g for whole red pepper and 7.9 to 7.4 mg/g for ground red pepper. In the case of moisture content, a slight increase was observed during the period. In crushed red pepper, an initial moisture content of 11.3% increased to 13% by Day 30, for whole samples 8.3% initial moisture content increased to 10.4% and 6.3% moisture content increased to 7% in ground samples.

Ergosterol, the primary sterol constituting the cell membranes of filamentous fungi had been widely used for estimating fungal biomass [8]. Ergosterol estimation results in the present study showed a good positive correlation with the total fungal colony counts obtained for all the red pepper samples (Crushed red pepper - 0.995, Ground red pepper – 0.986, Whole red pepper – 0.982). Both the fungal colony count and ergosterol content was higher for crushed red pepper. The distinctive presence of this sterol constituent and the amount determined in the tested red pepper sample types make it a good indicator of *A.flavus* invasion. Ergosterol estimation could well be employed as a prospective measure of growth of *A. flavus* in red peppers.

From the aflatoxin content determined, it could be noted that in all the inoculated sample types, aflatoxin kept increasing from Day 0 to Day 30. Also, surprisingly, the Aflatoxin B2 content was more compared to aflatoxin B1in the case of all red pepper samples. Probably it is because the *A.flavus* strain used in the study produced aflatoxin B2 more compared to aflatoxin B1. A similar fact that certain *A.flavus* strain could produce more aflatoxin B2 than B1 had been reported earlier by Dutton *et al*., 1985 [5]; Papa, 1977 [17]*;* and Schroeder *et al*., 1973 [23]. Aflatoxin is a secondary metabolite which is formed from polyketides produced by *A.flavus* [20]. Secondary metabolites are produced by metabolically inactive cells and hence are considered a non-growth associated product [22]. But here, aflatoxin production was observed alongside the increase in fungal count and ergosterol content indicating that aflatoxin formation is related to both growth rate and cell concentration. In crushed red pepper an exponential increase in total fungal count and ergosterol content was seen up to Day 7 and thereafter only a slight increase in fungal count and ergosterol content was seen probably due to the nearing of stationary phase. For ground

and whole red pepper, an exponential increase in total fungal count and ergosterol content was observed till Day14. Secondary metabolites are usually produced when the cell nears the end of the log phase and enters the stationary phase. This could be the reason for the tenfold increase in aflatoxin content of crushed red pepper after Day7 and the increase in aflatoxin content of ground and whole red pepper forms.

TABLE 1. 0th day, 3rd day, 7th day, 14th day, 21st day and 30th day aflatoxin analysis of inoculated red pepper samples.

	Average aflatoxin B1, B2 and Total aflatoxin content (µg/l)											
Day	WRP				CRP				GRP			
	B1	B2	Total	C	B1	B2	Total	C	B1	B2	Total	C
0	2.8	3.1	5.9	0	13.8	14.2	28.0	0	0	0.7	0.7	0
3	8.4	10.8	19.2	0	39.6	43.2	82.8	0	0	3.1	3.1	0
7	82.8	83.4	166.2	0	139.1	136.8	275.9	0	4.5	44.9	49.4	0
14	146.6	146.9	293.5	0	2353.1	2182.3	4535.4	0	13.6	85.5	99.1	0
21	157.6	159.0	316.6	0	2722.1	2553.2	5275.3	0	27.1	99.3	126.4	0
30	171.2	186.8	358.0	0	3170.3	2912.7	6083.1	0	44.1	118.7	162.8	0

WRP – Whole red pepper, CRP – Crushed red pepper,GRP – Ground red pepper, B_1 – Aflatoxin B_1, B_2 – Aflatoxin B_2, C - Control

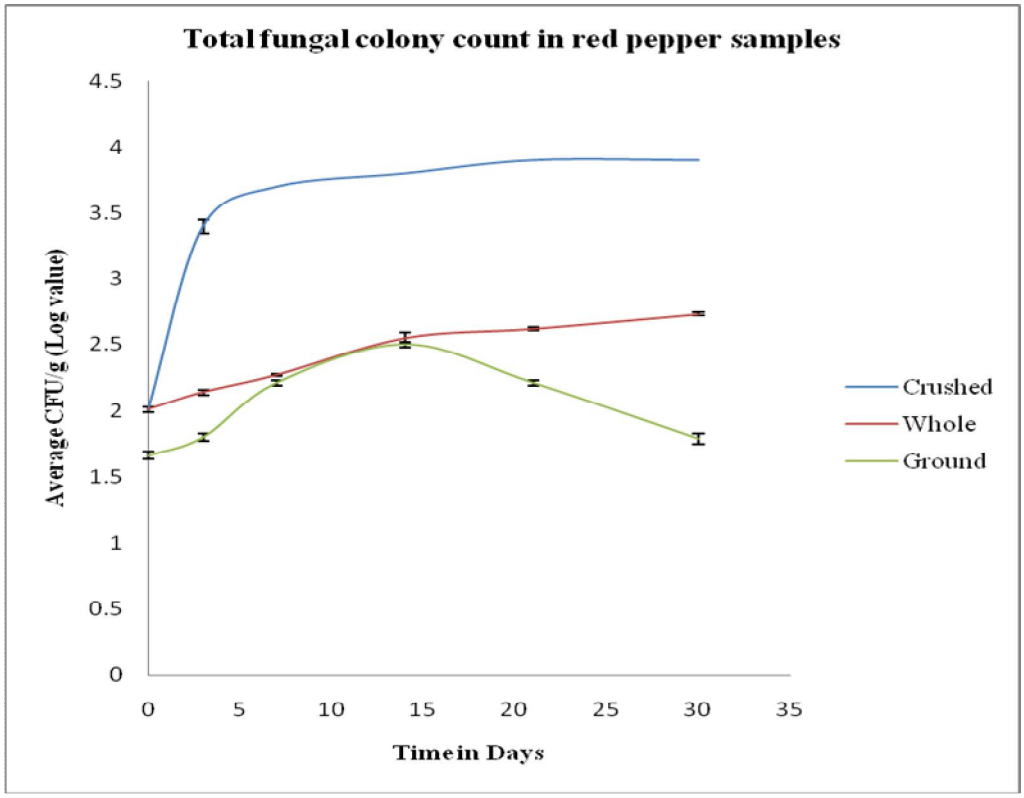

FIGURE 1. Total fungal colony count in red pepper samples

Red pepper, an economically important commodity, majorly produced in tropical environments, is predisposed to aflatoxin contamination. Factors like; substrate aeration, temperature, moisture content, and relative humidity are crucial contributing factors but the same factors may influence the fungal growth differently. Additionally, conditions that are favorable to fungal growth may not be favorable for aflatoxin production. But it is the initial presence of aflatoxigenic *A.flavus* in red pepper that results in further fungal growth and aflatoxin production. The best approach would therefore be in preventing the growth of *A.flavus* thereby indirectly averting aflatoxin production [4].

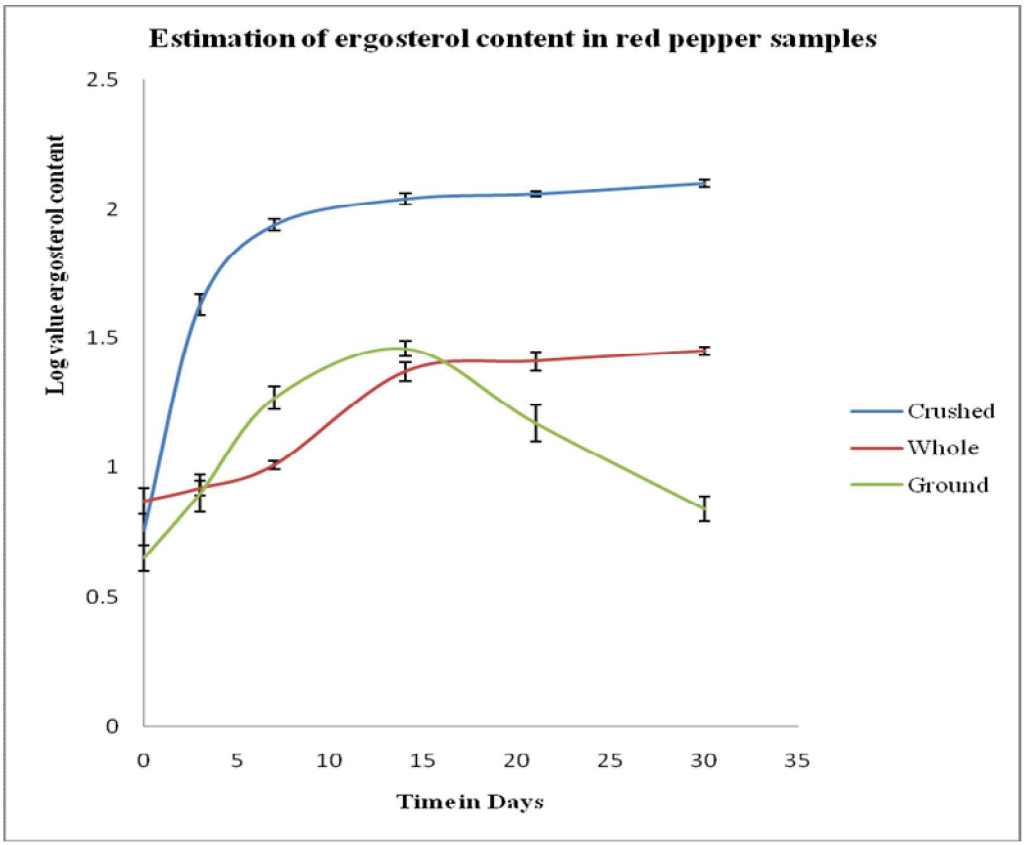

FIGURE 2. Estimation of ergosterol content in red pepper samples

The results obtained in the present study with the inoculated red pepper samples are a caveat on how quickly fungal growth can occur in a contaminated lot and the extent of aflatoxin contamination it could cause. The results of total fungal colony count, ergosterol content, and aflatoxin content showed a positive correlation which indicates that the parameters could be considered for application in predictive modelling studies of red peppers. The maximum specific growth rate based on the total fungal colony count and ergosterol content was comparatively higher for the crushed red pepper. Additionally, the absence of a lag period for crushed red pepper samples indicated that crushed red pepper provided a nutritive medium for fungal growth followed by the ground and whole red pepper. This observation points out that among the red pepper samples, the crushed form would be more susceptible to *A.flavus* growth and aflatoxin contamination. The structure of food can also be considered a decisive factor in the survival and growth of *A.flavus*. The physical barrier like wax coating usually helps to prevent the invasion of microbes in whole red pepper. However, both ground and crushed forms of red pepper are open to fungal attack. Compared to ground form, the pepper in crushed form has more void space between the particles. The increased aeration coupled with the availability of space for the growth of the fungus might be the reason for the faster growth kinetics of the fungi in crushed form. The storage of crushed pepper in compressed form with minimum voids between the particles might help to reduce the fungal growth and increase shelf life.

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COMPETING INTERESTS

The authors have declared that no competing interest exists.

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