ORIGINAL ARTICLE

Mycotic and aflatoxin contamination in *Myristica fragrans* seeds (nutmeg) and *Capsicum annum* (chilli), packaged in Italy and commercialized worldwide

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Keywords

Aflatoxins • Moulds • Spices

Summary

Aflatoxins are secondary metabolites of moulds known to be carcinogenic for humans, and therefore should not be ingested in high doses. This study aimed to determine the level of mould and aflatoxin contamination in dehydrated chilli and nutmeg imported from India and Indonesia, respectively, packaged in Italy, and commercialized worldwide.

We tested 63 samples of chilli (22 sanitized through heat treatment and 41 not heat-treated) and 52 samples of nutmeg (22 heat-treated and 30 not heat-treated) for aflatoxin, moulds and moisture content.

Heat-treated samples were less contaminated than untreated samples. Spices in powder form (both chilli and nutmeg) were more contaminated than whole ones. In untreated spices, we observed a positive correlation between mould and moisture content. Of the powdered nutmeg and chilli samples, 72.5% and 50% tested positive for aflatoxin contamination, with a range of 0-17.2 μg kg^{-1} and 0-10.3 μg kg^{-1}, respectively.

The steam treatment of spices would be useful in reducing the initial amount of moulds. Although the risk from the consumption of spices contaminated with aflatoxins is minimal, owing to the small amount used in food, preventive screening of the whole food chain is very important, especially because the most frequently identified toxin was B_1, which is the most dangerous of the four toxins (B1, B2, G1, G2).

Introduction

Aspergillus, *Penicillium* and *Fusarium* are ubiquitous, saprophytic moulds. They may contaminate natural foods and animal feeds, producing mycotoxins that exert toxic effects on human and animal health [1]. Mycotoxins are produced by the secondary metabolism of moulds, the most common being the aflatoxins (AFs). These are mainly produced by two species of environmental filamentous fungi, *A. flavus* and *A. parasiticus*, and rarely by *A. nomius* [2], which can grow in many types of food, particularly in cereals.

Fungal growth in food is favoured by poor conditions in the producing countries, temperatures of 25-30°C, humidity between 88% and 95%, and water activity values greater than 0.78. Furthermore, in these environmental conditions, mycotoxins are very likely to be produced [3, 4].

Since 1993, AFB_1 and a natural mixture of aflatoxins have been classified as "carcinogenic to humans" (group 1) by the International Agency of Research on Cancer [5], while AFM_1, a metabolite of AFB_1, has been classified as "probably carcinogenic to humans" (group 2A). Today, AFs are well known to be toxic, mutagenic, carcinogenic, immunosuppressive, and teratogenic agents [5], capable of crossing the placental barrier [6]. Extensive experimental evidence shows that AFs can induce liver cancer in most species, including humans, notably among HBV carriers, as AFs and hepatitis B virus are co-carcinogens [7].

Mycotoxins can be found in a large variety of foods, such as cereals, fruit, infusion herbs, spices, coffee, cacao, fodder, etc. Fungal contamination can occur throughout the production chain, from the harvesting, drying and storage phases to product transportation [8]. Zinedine et al. [1] reported that about 50% of samples of some Moroccan cereals and spices were contaminated by mycotoxins. In a survey on cereals and cereal products conducted in the UK retail market, the Food Survey Information sheet [9] reported that the vast majority of the samples (71.8%) contained mycotoxins, although at levels below the regulatory limits for contamination in Europe [10]. However, the survey also showed that only 7 samples from the 220 analysed (3.2%) were found to contain levels of mycotoxins above the regulatory limits laid down in EU legislation [10], and in most cases these levels were only marginally above the limit.

In Europe, there are two specific regulations regarding mycotoxins: one concerns the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs [11], while the other establishes maximum levels of certain contaminants in food-

stuffs [10, 12, 13], such as AFB_1 in spices at 5 µg/kg and total aflatoxins at 10 µg/kg.
In Italian cuisine, chillies, the fruit of the *Capsicum annum* plant of the *Capsicum* genus (family: *Solanaceae*), are among the most frequently used spices. The chilli is appreciated for its pungency, caused by the presence of capsaicinoids, which are known to have chemo-preventive, anti-carcinogenic [14, 15], antioxidant [16], anti-inflammatory [17], antiviral, antibacterial, and antifungal properties [18]. Capsaicinoids are not stable in dehydrated chillies; they can lose their activity through oxidation [19]; consequently, chilli powder can lose up to 5% of its capsaicinoid content each month of storage. Capsaicinoids are present in different amounts in chilli varieties and cultivars [20]. Their concentration ranges from 0.001% to 0.01%, in fresh red pepper varieties, especially paprika, from 0.1% to < 1% in strong chilli varieties [21]. Pino et al. [22] found that the content of capsaicinoids varied between 0.42% and 0.66%. Capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide) and dihydrocapsaicin (8-methyl-*N*-vanillylnonanamide) account for about 77-98% of capsaicinoids present in chillies [21], followed by minor capsaicinoids, such as nordihydrocapsaicin, homocapsaicin, homodihydrocapsaicin, nonivamide and other compounds [23, 24, 25].
Another spice often used in Italian cuisine is nutmeg, a dried ovoid seed of *Myristica fragrans* Houtt (family: *Myristicaceae*). Nutmeg is widely used both as a food spice and in alternative medicine, as it has been reported to have antidiarrheal [26], anti-inflammatory, anti-cancer [27], antioxidant, antibacterial and antifungal [28] properties. Nutmeg contains a mixture of hydrophobic and volatile compounds; among these, the most relevant are monoterpene hydrocarbons, followed by oxygenated monoterpenes, and others such as β-caryophyllene, which is reported to be anti-inflammatory and antifungal [29].
The aim of this study was to investigate the occurrence of aflatoxins and moulds, and to measure moisture content, in dehydrated chillies and nutmeg imported from India and Indonesia, packaged in Italy, and commercialized worldwide, in order to evaluate the health risk related to the consumption of these aflatoxin-laden foods, which are widely used in cooking worldwide.

Methods

A total of 115 samples of commercial spices, all imported and packaged by "Drogheria e Alimentari Spa" (Scarperia e San Piero, FI, Italy), were analysed for the presence of aflatoxins and moulds, and for the quantification of moisture. The samples analysed were divided as follows: 63 samples of chilli (13 samples in whole form and 50 samples in powder) and 52 samples of nutmeg (12 samples in whole form and 40 samples in powder). For convenience, crushed samples (28 chilli and 13 nutmeg) were included within those in powder form.
In order to reduce microbial contamination of the samples, and consequently to avoid microbial multiplication during the storage of packaged products, 22 chilli and 22 nutmeg samples had been subjected to steam treatment (100°C for a few minutes) by the supplier before shipment to the Italian factory (Drogheria & Alimentari Spa).

Detection of moulds

Analyses were performed in conformity with Standard ISO 7218:2007/Amd1 [30] and ISO 21527-2 [31]. A 25 g portion of each sample was aseptically taken, placed in 225 ml of Buffered Peptone Water (BPW) (Oxoid Spa, Rodano, MI, Italy), and homogenized by means of a Stomacher for 60 s at normal speed. Three subsequent decimal dilutions in BPW were prepared. A 0.5 ml portion of each dilution was streaked with Yeast Extract Dextrose Chloramphenicol Agar (YEDC) (Oxoid Spa, Rodano, MI, Italy) and incubated for 5 days at 25 ± 1°C. After incubation, colonies were counted and results were expressed as CFU *per* g of foodstuff (CFU/g).

Quantitative determination of aflatoxins B1, B2, G1, G2

Sampling procedures were performed according to Regulation (EC) n° 401/2006 [11]. All organic solvents, methanol and acetonitrile, were HPLC-grade. A 25 g portion of each sample was extracted with 100 ml of acetonitrile/water (84:16 v/v) by means of a sonicator (Falc instruments, Treviglio, BG, Italy) for 20 minutes. The solution was filtered through filter paper and 5 ml of filtrate was purified with "Mycosep 228 AflaPat" (Romer Labs, Tulln, Austria). The eluate was directly used to perform HPLC analysis (Varian – Chicago, IL, USA). The analytical procedure was performed in accordance with Bononi et al. [32].
The HPLC instrument was equipped with a fluorescence detector and a post-column derivatization system (coring cell). Aflatoxins were detected at the excitation and emission wavelengths of 365 nm and 435 nm, respectively, as per Golge et al. [33]. The column was an OmniSpher C18 (250 mm x 4.6 mm I.D., 5 µm particle size), and was maintained at 40°C during the analysis, which was performed at a flow rate of 0.8 mL/min. The mobile phase was a mixture of water/methanol/acetonitrile (61/23/16, v/v/v). HNO_3 (4M) and KBr (23.8g/l) were always added to the water, which was the derivatizing agent. The retention time of aflatoxins was approximately 12-23 min and the total run time was 30 min.
Calibration curves were constructed by using fortified samples at 4 incremental concentrations of total aflatoxins: standards for AFB_1, AFB_2, AFG_1, AFG_2 were purchased from Or Sell (Limidi di Soliera, MO, Italy). The uncertainty value was calculated according to NMKL [34]. The validation parameters assessed were: recovery (80-95%), limit of detection (LOD = 0.13 µg/kg for B1, G1 and G2 and LOD = 0.07 µg/kg for B2), limit of quantification (LOQ = 0.4 µg/kg for B1, G1 and G2 and LOQ = 0.2 µg/kg for B2), repeatability (intra-day precision RSDr = 0.068 for B1 and 0.142 for B2), and reproducibility (inter-day precision RSDR = 0.088 for B1 and 0.174 for B2).

Determination of moisture content

The moisture content of samples was detected by measuring the weight loss of 1-2 g of the samples in a thermobalance (Ohaus, Switzerland) at 80°C. At the end of the process, the instrument showed the percentage of moisture of the sample on a wet-weight basis.

Statistical analysis

The regression used for the correlation was linear, and the Pearson coefficient (p) was used to evaluate the behaviour of the variables. Microsoft Office Excel 2007.

Results

Detection of mould and aflatoxin content

Mycotic contamination was lower than the detection limit (< 10 CFU/g) in 39.7% of chilli samples and in 30.8% of nutmeg samples (Tab. I).

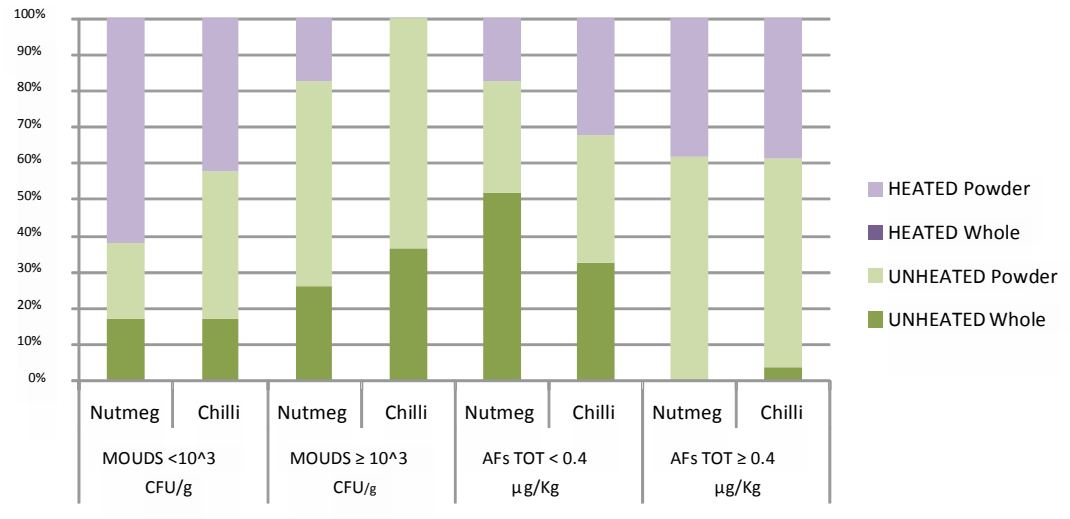
The percentages of samples with high contamination (≥ 10^3CFU/g) were higher in nutmeg (44.2%) than in chilli (17.5%) (Tab. I). Our results showed that in heat-treated samples there was a statistically significant reduction in the presence of mould: this reduction was more marked in nutmeg than in chilli samples ($p = 0.0001$ and $p = 0.067$, respectively). Few nutmeg (18.2%) and no chilli samples had mould concentrations higher than 10^3CFU/g. Undetectable AF content (< 0.4 µg/kg) was found in 58.7% and 44.2% of chilli and nutmeg samples, respectively (Tab. I). The percentage of samples with contamination equal to or higher than 5 µg/kg was 4.8% for chilli and 7.7% for nutmeg. Interestingly, higher levels of aflatoxin contamination (concentration > 1 µg/kg) were found in treated samples (data not shown): a possible explanation for this could be that treatment abated the competitive effect by reducing the number of moulds, thereby promoting toxinogenesis by surviving moulds.

In order to study the different occurrences of moulds and AFs, the trend in contamination was plotted as a function

Tab. I. Moulds and total aflatoxins in chilli and nutmeg samples (total, unheated, heated).

		chilli			nutmeg		
Contamination		N total samples (%)	N unheated samples (%)	N heated samples (%)	N total samples (%)	N unheated samples (%)	N heated samples (%)
Moulds (CFU/g)	< 10	25(39.7)	13(31.7)	12(54.5)	16(30.8)	2(6.7)	14(63.6)
	10 - < 10^2	13(20.6)	7(17.1)	6(27.3)	1(1.9)	0(0)	1(4.5)
	10^2 - < 10^3	14(22.2)	10(24.4)	4(18.2)	12(23.1)	9(30.0)	3(13.6)
	10^3 - < 10^4	8(12.7)	8(19.5)	0(0)	16(30.8)	12(40.0)	4(18.2)
	≥ 10^4	3(4.8)	3(7.3)	0(0)	7(13.4)	7(23.3)	0(0)
Total Aflatoxins (µg/Kg)	< 0.4	37(58.7)	25(61.0)	12(54.5)	23(44.2)	19(63.3)	4(18.2)
	0.4 - < 3	19(30.2)	12(29.2)	7(31.9)	21(40.4)	9(30)	12(54.5)
	3 - < 5	4(6.3)	2(4.9)	2(9.1)	4(7.7)	2(6.7)	2(9.1)
	≥ 5	3(4.8)	2(4.9)	1(4.5)	4(7.7)	0(0)	4(18.2)
	Total	63 (100)	41(100)	22(100)	52(100)	30(100)	22(100)

Fig. 1. Mould and aflatoxin content of heat-treated and non-heat-treated samples.



of the granulometry (whole and powdered) of the two spices: mycotic contamination higher than 10^3CFU/g was found in 11 chilli samples, 63.6% of which were ground (Fig. 1).
The mean level of mould contamination in powdered chilli samples was a little lower ($1.6 \times 10^3 \pm 3.9 \times 10^3$ CFU/g) than in whole chillies ($4.7 \times 10^3 \pm 1 \times 10^4$ CFU/g). This difference is greater if we consider the contamination level ≥ 10^4 CFU/g: 15.4% of whole chilli samples fell within this range, whereas only 2.0% of the ground samples did.
The two types of nutmeg samples displayed similar levels of mould contamination: whole ($5.8 \times 10^3 \pm 1 \times 10^4$ CFU/g) and powder ($8.4 \times 10^3 \pm 1 \times 10^4$ CFU/g). If we consider the contamination level higher than10^4 CFU/g, the results were 8.3% and 15.0% of whole and powder samples, respectively. In this case, it was interesting to subdivide ground nutmeg into crushed samples (7.7%) and fine powder samples (18.5%). Interestingly, and probably owing to the absence in nutmeg of antimycotic compounds, such as capsaicinoids, none of the whole nutmegs (Tab. II) and only 7.7% of chilli samples presented AFs (data not shown), whereas they were all contaminated by moulds. Ultimately, Table II shows that there was a statistical difference between powder and whole samples ($p < 0.0001$) regarding the aflatoxin content of both spices.

Influence of moisture on non-heat-treated nutmeg and chilli

A positive correlation (linear correlation r = 0.19 and line gradient m = 998.4) was found between moisture and mould content in non-heat-treated nutmeg samples (Fig. 2), prevalently due to whole samples (linear correlation r = 0.28 and line gradient m = 1349.8).
A negative correlation was also found between moisture and mould content in chilli samples (Fig. 3) (linear correlation r = -0.17 and line gradient m = -779.63), mostly due to powder samples (linear correlation r = -0.44 and line gradient m = -1215.8), which showed a higher moisture content (mean = 6.61; SD = 1.42) than whole ones (mean = 6.31; SD = 1.50). A positive correlation (Fig. 4) was found between aflatoxin production and moisture content in non-heat-treated nutmeg samples (linear correlation r = 0.10 and line gradient m = 0.0593), prevalently in ground samples (linear correlation r = 0.32 and line gradient m = 0.2274). No correlation was found in chilli samples. The moisture content of products was always low; only one sample of nutmeg had a moisture content of 12.68%; the mould concentration of this sample was high (3.9×10^4CFU/g).
Whole chilli samples presented lower moisture content than powdered ones; together with the lesser availability of nutrients, this caused a slowdown in the multiplication of mould. Indeed, the percentage of samples with a detectable level of mould contamination was lower for whole chilli samples than for powdered ones.

Mycotoxin occurrence

Only six samples exceeded the B_1 limit of 5 μg kg^{-1} set by European Commission Regulations [13]: three (5.77%) samples of heat-treated nutmeg powder, two (3.17%) of untreated chilli powder, and one (1.59%) of heat-treated chilli powder. We did not find any sample contaminated with aflatoxin G2, and aflatoxin G1 was only found in three powder samples (two nutmeg and one chilli).

Discussion

The quantification of moulds and aflatoxins in chilli and nutmeg is important because of their widespread culinary use and consequent ingestion by consumers, especially since storing spice products for long periods of time is one of the predisposing factors for aflatoxin production. Given that moulds produce mycotoxins, it is very important to evaluate the quality of spices that are shipped around the world [4].
Similar levels of contamination were found by Hammami et al. [35] and Mandeel [36].
We could conclude that the antimycotic effect of capsaicinoids in chilli was more marked in ground samples

Tab. II. Number and percentage, moisture and AF content of each mould contamination range.

Unheated samples	Mould contamination Range	Samples N	Samples %	Moisture mean %	Moisture range %	AF mean μg/Kg	AF range μg/Kg
Chilli powder	< 10	7	17.1	7.16	5.74-8.22	2.06	0-10.3
	10-10^3	14	34.2	6.47	4.94-7.95	1.03	0-3.9
	≥ 10^3	7	17.1	7.21	5.4-9.58	1.2	0-7.0
Whole chilli	< 10	6	14.6	6.01	3.14-6.94	0.23	0-1.4
	10-10^3	3	7.3	5.93	5.82-6.9	0	0
	≥ 10^3	4	9.7	7.07	5.09-9.61	0	0
Nutmeg powder	< 10	2	6.7	9.84	9.38-10.3	1.1	0-2.1
	10-10^3	4	13.3	7.91	4.02-9.9	2.5	0-4.8
	≥ 10^3	12	40.0	9.04	4.71-11.1	0.6	0-2.0
Whole nutmeg	< 10	0	0	-	-	-	-
	10-10^3	5	16.7	10.99	9.83-12.0	0	0
	≥ 10^3	7	23.3	9.00	5.32-12.7	0	0

Fig. 2. Correlation between moulds and moisture contents in total (blue/grey) and whole (black) non-heat-treated nutmeg samples.

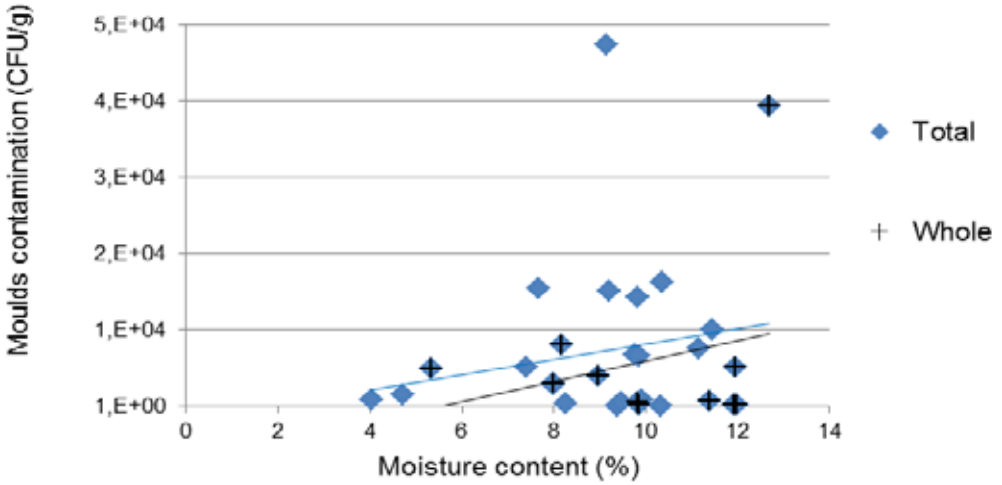


Fig. 3. Correlation between moulds and moisture contents in total (blue/grey) and powder (black) non-heat-treated chilli samples.

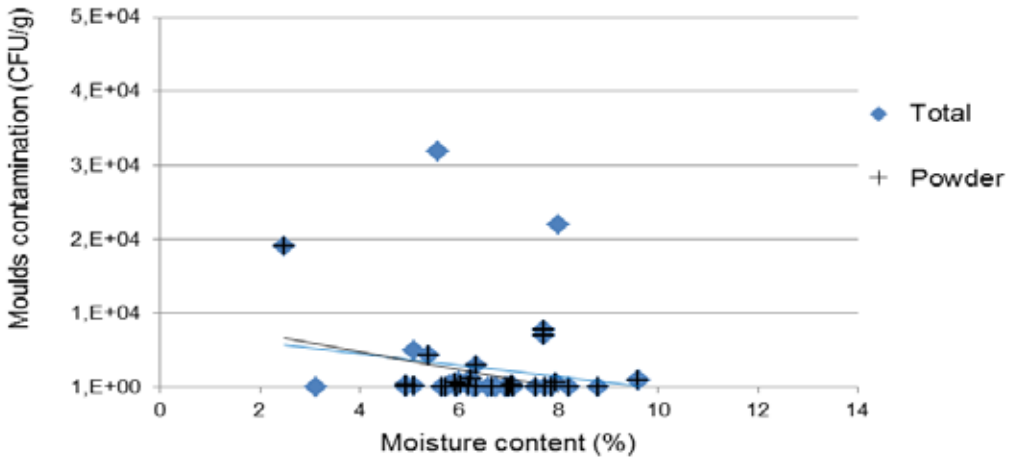
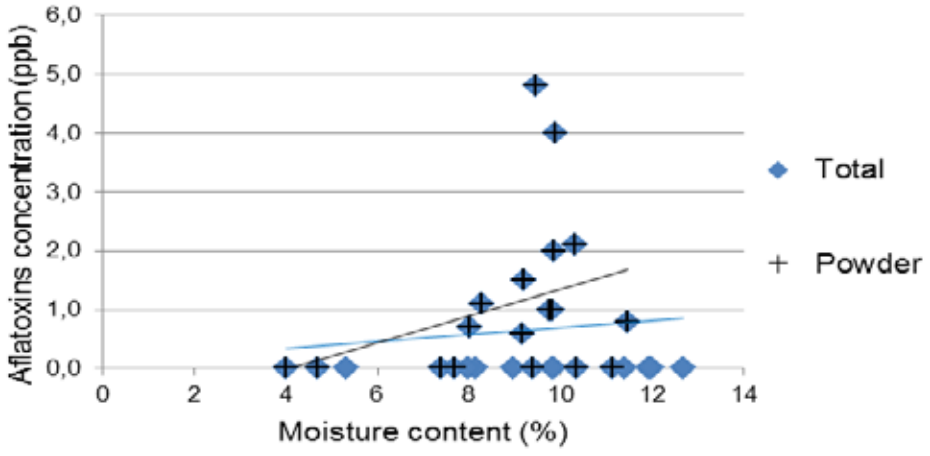


Fig. 4. Correlation between aflatoxins and moisture contents in total (blue/grey) and powder (black) non-heat-treated nutmeg samples.



Tab. III. Aflatoxins detected in nutmeg and chilli samples (heated and unheated).

Samples		Powdered nutmeg	Whole nutmeg	Powdered chilli	Whole chilli
AFs negative	N	11	12	25	12
	%	27.5	100	50.0	100
B_1 positive	N	21	0	22	1
	%	52.5	0	44.0	8.33
	Mean	2.02 ± 1.81	ND	1.95 ± 1.68	0.11 ± 0.39
$B_1 + B_2$ positive	N	6	0	2	0
	%	15.0	0	4.0	0
	Mean B1	3.18 ± 3.45	ND	6.85 ± 4.59	ND
	Mean B2	0.48 ± 0.41	ND	0.25 ± 0.07	ND
$B_1 + G_1$ positive	N	1	0	0	0
	%	0.25	0	0	0
	B1	3.1	ND	ND	ND
	G1	0.9	ND	ND	ND
$B_1 + B_2 + G_1$ positive	N	1	0	0	0
	%	0.25	0	0	0
	B1	14.8	ND	ND	ND
	B2	1	ND	ND	ND
	G1	1.4	ND	ND	ND
G_1 positive	N	0	0	1	0
	%	0	0	2.0	0
	G1	ND	ND	1.0	ND

ND: Not detected (below the detection limit)

than in whole ones because the moulds were more exposed to these molecules. As can be seen, ground chillies were also those with the highest percentage (75.0%) of samples with detectable contamination (≥ 10 CFU/g). This can be explained by the fact that ground samples had the highest nutrient availability. Another aspect to consider is that the antimycotic effect of capsaicinoids is not sufficient to prevent mycotic multiplication in highly contaminated samples.

Higher percentages of mould-contaminated samples in powdered than in crushed spices may be due to the greater surface area and availability of nutrients in ground spices (as opposed to whole ones), together with the absence in nutmeg of antimycotic compounds, such as capsaicinoids.

As Kaaya & Eboku [37] reported with regard to cassava products, we found a positive correlation (linear correlation r = 0.19 and line gradient m = 998.4) between moisture and mould content in non-heat-treated nutmeg samples (Fig. 2), prevalently due to whole samples (linear correlation r = 0.28 and line gradient m = 1349.8). It is plausible that the negative correlations observed in chilli samples (Fig. 3) were due to the presence of capsaicinoids, which could affect mould growth and consequently toxinogenesis. No correlation was found between aflatoxin production and mould contamination in non-heat-treated nutmeg samples, while a weak positive correlation was found in chillies. It is encouraging to note that the moisture content of the products analysed in this study was always lower than 12%; as a percentage greater than 12% allows microbial growth [38].

Given that aflatoxins are considered of great concern by health authorities, we detected the content of total aflatoxins, B_1, B_2, G_1 and G_2 in our samples (Tabs. II and III). Our results were almost always below the limit of 5 μg kg^{-1} for B_1 and 10 μg kg^{-1} for total AFs, according to European Commission Regulations [13] for spices, and lower than those found by other authors [35, 39, 40].

Conclusions

The current study showed a high incidence of mould, in both chilli and nutmeg. These moulds can multiply and produce AFs if spices are preserved in a critical condition for a long time, as may happen in the case of products that are commercialized worldwide. Interestingly, nutmeg samples were more contaminated by mould than chilli samples; this could be due to the antimycotic effect of capsaicinoids. Most studies have reported that a high mycotoxin content is due to the susceptibility of spices to fungal contamination and multiplication resulting from environmental and packaging conditions, such as high humidity and temperature [41]. We also saw that it could be useful to steam-treat spices before packaging, in order to reduce the initial amount of moulds. A possible way to reduce moisture inside packages could be to use appropriate materials, such as sorbents; the reduction in moisture would then increase the replication time of mould, and consequently inhibit AF production. However, we did not test this in this study. Positive correlations were found between moisture and mould and between moisture and aflatoxins only in untreated nutmeg samples; in untreated chilli samples, the presence of capsaicinoids probably negatively affects these correlations.

Concerning the occurrence of moulds and aflatoxins, we did not find any correlation (untreated samples).
Although the risk from the consumption of spices contaminated with aflatoxins is minimal, owing to the small amount used in food, preventive screening of the whole food chain remains very important, especially since the most frequently identified toxin was B_1, which is the most dangerous of the four toxins (B1, B2, G1, G2).

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Authors' contributions

ALN, GP conceived, designed and coordinated the research. MO and SR collected data. MO and GP optimized the informatics database and performed the statistical analyses. GP, CC, MO, SR and ALN evaluated the results. GP wrote the manuscript. All Authors revised the manuscript and gave their contribution to improve the paper. All authors read and approved the final manuscript.

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