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Incidence of Fumonisin B$_2$ Production by *Aspergillus niger* in Portuguese Wine Regions

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ABSTRACT: Fumonisin B$_2$ (FB$_2$) was recently found to be produced by *Aspergillus niger*. When grape-derived products were subsequently analyzed, FB$_2$ contamination was found in raisins, must, and wine. This study evaluated 681 strains of black aspergilli species isolated from Portuguese wine grapes for FB$_2$ production when grown on Czapek yeast agar. FB$_2$ was not detected in *Aspergillus carbonarius* ($n = 75$) or *Aspergillus ibericus* ($n = 9$) strains, but it was detected in 176 (29%) of the strains belonging to *A. niger* aggregate ($n = 597$). The amount of FB$_2$ produced by these strains ranged from 0.003 to 6.0 mg/kg with a mean of 0.66 mg/kg. The Alentejo region had the lowest percentage (10%) of fumonisinogenic strains, whereas the Douro region had the highest percentage of fumonisinogenic strains (38%). Only 10 strains were found to produce FB$_2$ and ochratoxin A simultaneously.

KEYWORDS: *Aspergillus niger*, fumonisin, ochratoxin A, grapes, wine

■ INTRODUCTION

Aspergillus niger is a filamentous fungus involved in food biodeterioration, but it is also used to produce added-value compounds, such as citric acid and several enzymes with GRAS (generally regarded as safe) status. For many decades, it was considered to be a safe microorganism, and it was not known to produce mycotoxins until recently, when it was discovered that some *A. niger* isolates can produce low levels of ochratoxin A (OTA).[1] More recently, it was discovered that *A. niger* can produce fumonisin B$_2$ (FB$_2$).[2] FB$_2$ is the main fumonisin produced by *A. niger*, but others, such as fumonisin B$_4$ (FB$_4$) and fumonisin B$_6$ (FB$_6$), are also produced at lower levels.[3,4] According to Varga et al.,[5] FB$_2$ represents approximately 73% of the total fumonisin produced by *A. niger*. These findings raise the possibility of fumonisin contamination by *A. niger* in several agricultural commodities in which it is a frequent contaminant, for example, coffee beans,[6] cocoa beans,[7] grapes,[8,9] dried fruits,[10] and feedstuffs.[11] Therefore, more studies were needed to determine the incidence of mycotoxigenic isolates in commodities and to better understand its environmental distribution and toxicological impact.

One of the main problems associated with FB$_2$ production by *A. niger* is related to grape-based products (in particular, wine). *A. niger* is by far the most common species of *Aspergillus* present on grapes,[12] and FB$_2$ has been detected in grapes, raisins, must, and wines.[13−15] In wines, the levels found seem to be low, ranging from 0.4 to 2.4 μg/L according to Logrieco et al.[16] and from 1 to 25 μg/L according to Mogensen et al.[13] Among seven Portuguese wines analyzed, only one was contaminated with FB$_2$ (2.8 μg/L).[13] Nevertheless, as in other countries, the main black aspergilli found in Portuguese grapes are strains that belong to the *A. niger* aggregate.[17] Therefore, the potential for local strains to produce FB$_2$ needs to be evaluated.

For studies conducted between 2001 and 2003 on the incidence of ochratoxigenic strains in Portuguese wine grapes, approximately 700 black aspergilli isolates were collected, preserved in glycerol, and stored at −80 °C.[18] In this study, we investigate the levels of FB$_2$ produced by those strains, because they are still representative of the local black aspergilli found in the five main winemaking regions of Portugal. This study aims to evaluate the incidence and distribution of fumonisinogenic strains in those regions and to evaluate whether they correlate with the production of ochratoxin A.

■ MATERIALS AND METHODS

Chemicals and Reagents. Sodium cyanide, 2,3-naphthalenedicarboxaldehyde (NDA), boric acid, and an FB$_2$ standard (OEKANAL, 50 mg/L in acetonitrile/water) were obtained from Sigma-Aldrich (Sintra, Portugal). Isocratic grade acetonitrile and methanol, acetic acid, and NaOH were obtained from Merck (Lisbon, Portugal). The FumoniTest WB immunoaffinity columns (IAC) used in this study were obtained from Vicam (USA).

Biological Materials and Growth Conditions. Strains were isolated between 2001 and 2003 from Portuguese wine grapes from five different Portuguese wine regions. These strains were identified and preserved at −80 °C in glycerol by Serra.[18] They include 75 strains of *Aspergillus carbonarius*, 9 strains of *Aspergillus ibericus*, and 597 strains belonging to the *A. niger* aggregate. All of the *A. carbonarius* strains were ochratoxigenic (mean of 1.13 mg/kg), but only 4% of the *A. niger* aggregate strains produce OTA (mean of 0.14 mg/kg). None of the *A. ibericus* strains were found to produce OTA.[18] *A. niger* ex-type strain NRRL 326 (= CBS 554.65^T = ATCC 16888 = MUM 03.01) and *A. niger* strain NRRL 3 (= CBS 120.49 = ATCC 9029 = MUM 92.13) are known to produce FB$_2$ and were used as positive controls.[2] The reference strain *Aspergillus tubingensis* CBS 134.48 (= MUM 06.152) was used as a negative control.

The strains were revived in MEA (Blakeslee's formulation)[19] for 7 days in the dark at 25 °C and then subcultured into Czapek yeast agar (CYA)[19] and incubated at 25 °C for 8 days in the dark. CYA medium was

chosen to allow comparison of the results with the OTA production levels that were previously reported for these strains.[18] Twenty-five strains from the *A. niger* aggregate (with different levels of FB_2 production) were selected to be subcultured into a medium containing 50% grape juice (GJ50), prepared as described previously,[20] and were incubated as described above.

FB_2 Extraction and Determination. FB_2 was extracted from colonies using five plugs that were 7 mm in diameter (mean weight = 0.707 g) and 1 mL of methanol/distilled H_2O (3:1, v/v), as reported previously.[2] Samples were dried at 50 °C with a gentle stream of nitrogen, and then the dried residues were derivatized with NDA[21] by adding, in the following order, 200 μL of methanol, 200 μL of 0.05 M borate buffer (pH 9.5, adjusted with 2N NaOH), 100 μL of sodium cyanide (0.13 mg/mL in distilled water), and 100 μL of NDA (0.25 mg/mL in methanol). After vortexing, samples were heated at 60 °C for 15 min in a thermostated bath, cooled to room temperature, diluted with 1.4 mL of acetonitrile/distilled H_2O (3:2, v/v), and analyzed by HPLC with fluorescence detection (λ_{ex} = 420 nm and λ_{em} = 500 nm). Batches of 20 samples were prepared and injected within 12 h of derivatization. When the FB_2 peak saturated the detector, samples diluted with acetonitrile/distilled H_2O (3:2, v/v) were reanalyzed. The HPLC apparatus was composed of a Varian 9002 pump, a Marathon Basic autosampler with a 50 μL loop, a Jasco FP-920 fluorescence detector, and a Galaxie chromatography data system. The chromatographic separation was performed with a 30 min isocratic run on a C_{18} reversed-phase YMC-Pack ODS-AQ analytical column (250 × 4.6 mm i.d., 5 μm), fitted with a precolumn of the same stationary phase. The mobile phase was composed of acetonitrile/water/acetic acid (60:40:1, v/v/v) that was filtered and degassed with a 0.2 μm membrane filter (GHP, Gelman). The flow rate was set to 1.0 mL/min, and the column temperature was 28 °C (Technochroma).

FB_2 levels were determined by measuring the peak area and comparing it to the calibration curve from FB_2 standards of 200, 100, 20, 10, and 1 μg/L. Standards were injected regularly and added to the calibration curve (*n* = 10). The limit of detection (LOD) and the limit of quantification (LOQ) were calculated from the residual standard deviation (σ) of the regression line of the calibration curve and its slope (*S*) using the following equations: LOD = 3.3 × (σ/S) and LOQ = 10 × (σ/S).[22]

To confirm the identity of FB_2, extracts from 10 strains were submitted for purification using immunoaffinity columns (FumoniTest WB). Briefly, the dried residues were obtained as described above, resuspended in 1 mL of methanol, and diluted with 10 mL of phosphate-buffered saline (PBS). The pH was adjusted to 7.0 with 6 N HCl, and the total volume was loaded onto IAC at a rate of one to two drops per second. Then columns were washed with 10 mL of PBS, FB_2 was eluted with 2 mL of methanol into clean vials, and samples were evaporated to dryness, derivatized with NDA, and analyzed by HPLC, as previously described.

Association between FB_2 and OTA Production. To determine whether there was an association between the productions of FB_2 and the production of OTA, a 2 × 2 contingency table test was used. A low association between variables is indicated when φ is close to 0, and a strong association is indicated when φ is close to 1. A *p* value of ≤0.05 (two-tailed) was considered to be significant. The statistical package SPSS Statistics, version 19.0, was used to perform the statistical analysis.

■ RESULTS AND DISCUSSION

FB_2 had a retention time between 27 and 28 min in chromatograms. The calibration curve was linear in the range of 1−200 μg/L, with an R^2 of 0.99997 and a relative standard deviation (RSD) of the slope of 13%. Using this method, we measured LOD and LOQ values of 2 and 6 μg/kg, respectively. Typical
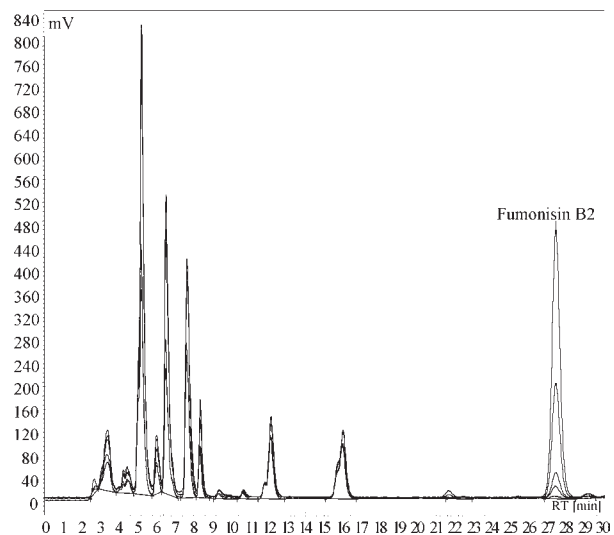

Figure 1. Overlaid chromatograms of FB_2 standards injected (1−200 μg/L).

chromatograms obtained for standards injected are depicted in Figure 1. Analysis of the stability of the NDA-derivatized FB_2 in standards and *A. niger* cultures found only a 4% reduction in FB_2 concentration after 12 h at room temperature.

As expected, the reference strains *A. niger* NRRL 326 and NRRL 3 produced FB_2 (0.789 and 1.710 mg/kg of culture substrate, respectively), whereas the *A. tubingensis* strain CBS 134.48 did not. Using IAC, the production of FB_2 by black aspergilli was confirmed in cultures of *A. niger* NRRL 326, 01UAs337, 02UAs95, and 03UAs192. On the contrary, also using IAC, no FB_2 was found to be produced by *A. tubingensis* CBS 134.48, *A. niger* 01UAs115, *A. ibericus* 03UAs267, and 03UAs89, and *A. carbonarius* 01UAs293 and 02UAs146. Typical chromatograms obtained for some strains are shown in Figure 2.

Of the strains isolated from Portuguese grapes, FB_2 was not detected in *A. carbonarius* or *A. ibericus* but was detected in 176 (29%) of the strains belonging to the *A. niger* aggregate (Table 1). These results are in agreement with a recent study, in which only 7 of 30 *A. niger* strains isolated from grapes (23%) were found to produce FB_2.[23] According to the same authors, at least one essential gene involved in FB_2 production is lacking in the nonproducers. Additionally, like us, they found no FB_2 was produced by *A. carbonarius* strains. In other studies, the percentage of FB_2-producing strains found was higher (between 60 and 77%); nevertheless, the small number of strains analyzed may account for the observed discrepancy.[4,14,24] The same occurred in our study, in the Madeira wine region, where only four strains were tested and 75% of strains were found to produce FB_2. The amount of FB_2 produced by strains from Portuguese grapes ranged from 0.003 to 6.0 mg/kg (mean = 0.66 mg/kg; median = 0.021 mg/kg), which is lower than the levels reported by Susca et al., who reported strains that produced 0.1−293.0 mg/kg (mean = 51.3 mg/kg; median = 17.5 mg/kg),[23] and those reported by Varga et al., who found production levels between 0 and 14.4 mg/kg (mean = 4.0 mg/kg; median = 3.2 mg/kg).[5] The small number of strains analyzed (30 and 20, respectively) may explain the differences.

It was also found that FB_2 produced by strains varied greatly (from ppb to ppm). Namely, 57% of the fumonisinogenic strains produced <0.1 mg/kg of FB_2, 21% produced between 0.1 and
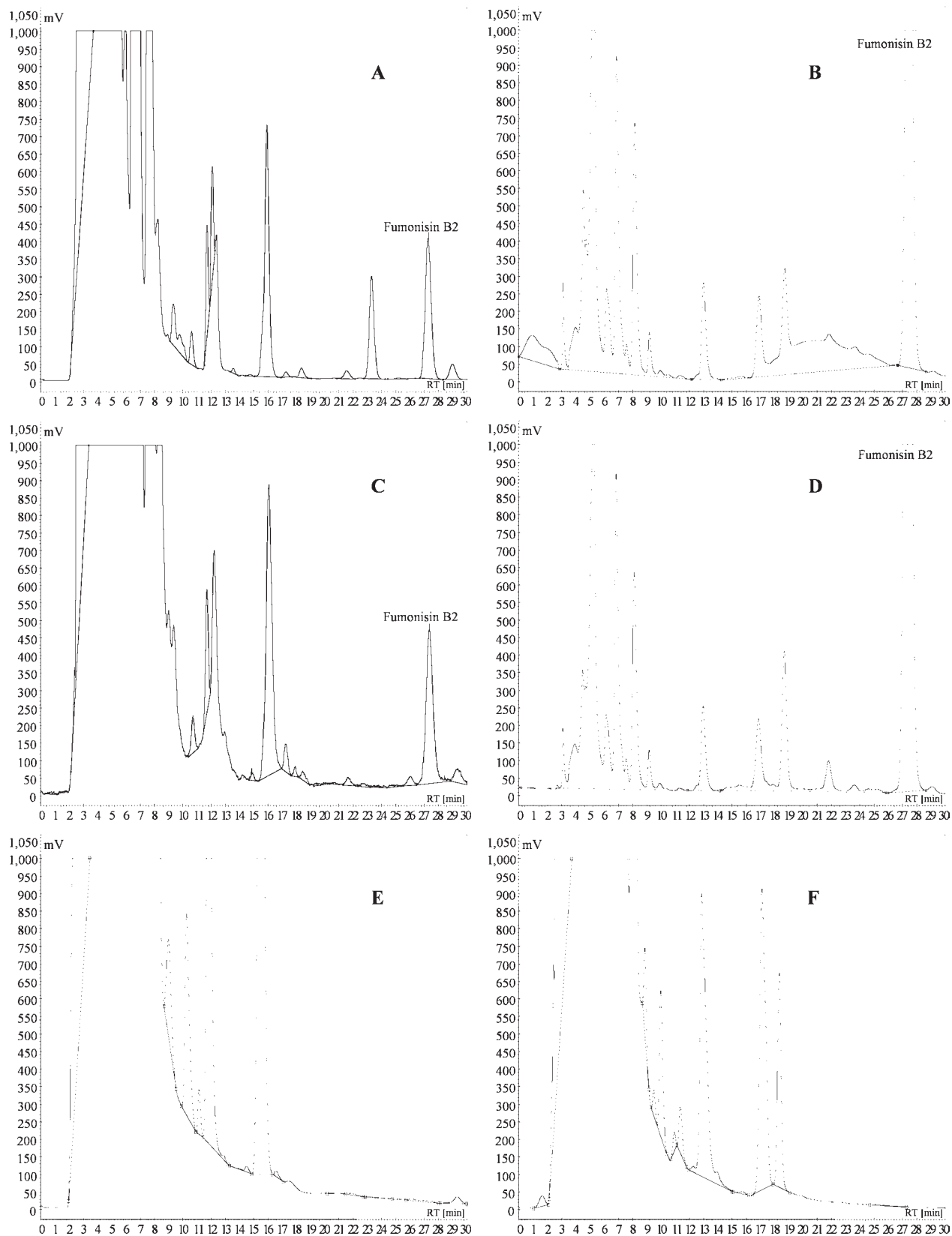
Figure 2. Typical chromatograms obtained for the determination of FB$_2$: (A) positive control *A. niger* NRRL 3; (B) *A. niger* NRRL 3 analyzed with IAC; (C) *A. niger* 01UAs337 from grapes; (D) *A. niger* 01UAs337 analyzed with IAC; (E) negative control *A. tubingensis* CBS 134.48; (F) *A. niger* 01UAs115 from grapes.

1 mg/kg FB$_2$, and 22% produced FB$_2$ at levels >1.0 mg/kg. By comparing levels of FB$_2$ production with the levels of OTA produced by the ochratoxigenic strains,[18] we find that approximately half of the strains produce FB$_2$ at levels comparable to the

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Table 1. Number of A. niger Aggregate Strains That Produce FB$_2$ When Grown on CYA and Its Distribution and Levels in the Main Portuguese Wine Regions

wine region	no. of strains tested	no. of strains that produce FB$_2$				FB$_2$ (mg/kg)	
		<0.1 mg/kg	0.1−1 mg/kg	>1 mg/kg	total	mean	median
Douro	239	56	12	23	91	0.75	0.02
Ribatejo	198	39	19	5	63	0.28	0.01
Alentejo	135	5	3	6	14	1.48	0.75
Vinho Verde	21	0	2	3	5	1.09	1.29
Madeira	4	0	1	2	3	1.39	1.38
all	597	100 (17%)	37 (6%)	39 (6%)	176 (29%)	0.66	0.021

Table 2. Percentages of OTA and FB$_2$ Producer Strains from the A. niger Aggregate Found in Each Wine Region

wine region	OTA$^+$ (%)	FB$_2^+$ (%)	OTA$^+$ + FB$_2^+$ (%)	φ	p value
Douro	8.4	38.1	3.3	0.012	0.853
Ribatejo	2.5	31.8	1.0	0.028	0.691
Alentejo	0.7	10.4	0	−0.030	0.732
Vinhos Verdes	4.8	23.8	0	−0.125	0.567
Madeira	0.0	75.0	0	a	a
total	4.4	28.8	1.7	0.036	0.382

^{a} No statistics were calculated because the OTA is a constant.

levels of OTA produced by A. niger (<0.1 mg/kg) and that the other half produce levels of FB$_2$ comparable to the levels of OTA produced by A. carbonarius (>0.1 mg/kg). Additionally, the incidence of strains that produce >0.1 mg/kg of OTA or FB$_2$ is approximately the same (11%). Therefore, we predict that the exposure risk associated with FB$_2$-producing strains in Portuguese wine grapes is similar to the one posed by OTA-producing strains. However, the average tolerable daily intake (TDI) for FB$_2$ (2 μg/kg bw/day) is 400 times higher than the TDI for OTA (5 ng/kg bw/day).[25,26]

The highest incidences of fumonisinogenic strains were found in the Douro (38%) and Ribatejo regions (32%) (Table 2). Nevertheless, the majority of strains ($\approx$20%) produced FB$_2$ at levels <0.1 mg/kg, and the mean levels produced were low (0.75 and 0.28 mg/kg, respectively). On the contrary, the Alentejo region had a lower percentage of FB$_2$-producing strains (10%) but had the highest levels of FB$_2$ production (mean = 1.48 mg/kg). Production means >1.0 mg/kg were obtained from strains from the Vinho Verde and Madeira wine regions. As reported previously, ochratoxigenic black aspergilli are predominantly found in grapes from Portuguese wine regions with a typical Mediterranean climate (Douro and Alentejo).[17] Nevertheless, fumonisinogenic strain incidence in these two regions is very different. Therefore, with the present data, we cannot establish a connection between the typical microclimate characteristics of the regions and the presence of fumonisinogenic strains.

The amount of FB$_2$ produced by Portuguese strains was also evaluated in a grape-based culture medium. The levels of FB$_2$ produced when the 25 selected strains were grown on GJ50 were between 0.008 and 0.15 mg/kg (mean = 0.04 mg/kg; median = 0.02 mg/kg).

The same strains produced levels of FB$_2$ between 0.1 and 6.0 mg/kg (mean = 1.44 mg/kg; median = 0.84 mg/kg) in CYA. On average, when grown in GJ50, strains produce 97% less FB$_2$. Similar results have been found for OTA; a 76% decrease in production of OTA by black aspergilli was found when they were grown on GJ50.[20] Nevertheless, a direct relationship between the amount of toxin produced on GJ50 and its production on grapes cannot be drawn, because grapes were found to support A. niger growth and FB$_2$ production at levels between 0.15 and 2.5 mg/kg and between 0.17 and 7.8 mg/kg.[4] It is important to note that all nutrients present in GJ50 come from grape juice, but it is likely that the high water content and low nutrient content of the culture medium lead to low FB$_2$ production. Nevertheless, the levels of FB$_2$ found in grape-derived products were not as high as the levels produced by A. niger in culture medium, grapes, or raisins. According to the few publications available, natural levels of FB$_2$ were between 0.01 and 0.4 mg/L in must,[14] between 1 and 25 μg/L in wine,[13] and between 0.4 and 2.4 μg/L in red wine.[16]

In this study, we determined whether there was a correlation between FB$_2$ and OTA production by the A. niger aggregate strains; 10 strains (2%) were found to produce both mycotoxins (Table 2). A 2 × 2 contingency table was used to determine whether there was a correlation. The φ obtained was closer to 0 than to 1, indicating that little association exists, and the p value obtained was >0.05, which confirmed the lack of a significant association. Therefore, no significant association between the production of FB$_2$ and OTA among the Portuguese A. niger aggregate strains was observed.

In conclusion, our findings confirm the potential risk of FB$_2$ in Portuguese wine grapes. Nevertheless, the toxicological risk seems not to be higher than the one posed by OTA because only 6% of the local A. niger aggregate strains produce >1 mg/kg. Similar incidences and levels of production exist for OTA from A. carbonarius strains in Portuguese wine grapes, and OTA is not frequently found in Portuguese wines. Of 340 wines analyzed, OTA was detected in only 69 and was found at concentrations <0.5 μg/L.[27] Furthermore, it is known that the winemaking process itself contributes to lower the levels of OTA because most of the mycotoxin is eliminated with the solid residues from fermentation.[28] The same may also happen with FB$_2$, because a similar process was observed in the fermentation of FB$_1$-contaminated corn. That is, after the fermentation process, only 15% of the original FB$_1$ was found in the aqueous phase of the mash.[29] Therefore, the percent of FB$_2$ that is eliminated during wine fermentation should be investigated in future studies.

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