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Faculdade de Engenharia de Alimentos

LUÍSA FREIRE

FUNGI AND MODIFIED MYCOTOXINS IN GRAPES AND WINES: VARIABILITY OF FORMATION AND STABILITY DURING THE PROCESSING

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RESUMO

A ocratoxina A (OTA) é a principal micotoxina contaminante de uvas e seus derivados, sendo responsável por diversos efeitos prejudiciais à saúde. Além da OTA, algumas formas modificadas, geradas por reações ao longo do processamento, por micro-organismos ou pelo metabolismo de plantas, podem ocorrer. Neste sentido, o objetivo neste estudo foi determinar a variabilidade de multiplicação e produção de OTA e ocratoxinas modificadas por cepas de *Aspergillus carbonarius* e *A. niger*; e avaliar a formação e degradação de OTA e ocratoxinas modificadas da colheita ao vinho. As cepas de *A. carbonarius* e *A. niger* foram incubadas em meio à base de uva. A determinação de OTA foi realizada por cromatografia líquida de alta eficiência, e a detecção de ocratoxina modificadas por espectrometria de massas de alta resolução. A maior produção de OTA foi detectada no dia 15, e um declínio foi observado no dia 21. Entre os possíveis derivados buscados, identificamos apenas etilamida ocratoxina A produzido pela cepa CCDCA10443 (*A. niger*) no dia 21 no meio à base de uva. Uma suspensão de conídios de *A. niger* e de *A. carbonarius* foi inoculada em três variedades de uvas (Syrah, Touriga Nacional e Moscato Itália), em diferentes estádios de maturação. Foi observada uma correlação positiva entre os seguintes parâmetros físico-químicos das uvas: pH, sólidos solúveis totais, glicosídeos totais em glicose e antocianina total; e níveis de OTA produzidos por *A. niger* e *A. carbonarius.* Entre os possíveis derivados buscados, identificamos decarboxi ocratoxina A na variedade Moscato Itália nos estádios: início do pintor e 15 dias após o início do pintor; e etilamida ocratoxina A na variedade Syrah no estágio ótimo de colheita. Para detectar a formação de ocratoxinas modificadas na presença de cepas de *Saccharomyces cerevisiae,* foi utilizado a espectrometria de massas de alta resolução. Embora a presença de OTA não tenha influenciado nos parâmetros de crescimento das cepas de *S. cerevisiae*, entre os possíveis derivados buscados foram identificados: ocratoxina α, ocratoxina β, éster metílico de ocratoxina α, éster metílico de ocratoxina B, etilamida ocratoxina A, ocratoxina C, hidroxi ocratoxina A, éster metílico de hidroxi ocratoxina A e éster de celobiose ocratoxina A. Para avaliar o efeito das etapas de vinificação no destino da OTA bem como a formação de ocratoxinas modificadas em vinhos, foram elaborados vinhos branco, *rose* e tinto. As porcentagens de redução da OTA foram de 90,72, 92,44 e 88,15 % nos vinhos branco (Moscato Itália), *rose* (Syrah) e tinto (Touriga Nacional), respetivamente. Entre os possíveis derivados buscados, identificamos: ocratoxina β, éster metílico de ocratoxina α, éster metílico de ocratoxina B, éster metílico de ocratoxina A, etilamida ocratoxina A, ocratoxina C e éster de glicose ocratoxina A. Os derivados detectados neste estudo podem ter sido formados pelo próprio fungo, pela ação das

cepas de leveduras ou através de reações com componentes do meio de cultura, uva, mosto e vinho. Esses resultados indicam que a subnotificação dos níveis totais de micotoxinas nos alimentos pode ocorrer, aumentando a incerteza em relação aos riscos à saúde da população.

Palavras-chave: *Aspergillus carbonarius, Aspergillus niger,* micotoxina mascarada, ocratoxinas, vinho e vinificação.

ABSTRACT

Ochratoxin A (OTA) is the main mycotoxin contaminating grapes and its products, being responsible for several harmful effects on health. In addition to OTA, some modified forms may occur as a result of reactions taken place during processing, due to microbial transformation or the metabolism of plants. In view of this issue, this study aimed to determine the variability of fungal growth and production of OTA and its modified forms by strains of *Aspergillus niger* and *A. carbonarius*; and evaluate the variability of formation and degradation of OTA and its modified forms during winemaking. *A. carbonarius* and *A. niger* strains were incubated in grape-based medium. The determination of OTA was performed by high-performance liquid chromatography, and a high-resolution mass spectrometry was used for the detection of modified ochratoxin. The highest production of OTA was detected on day 15, and a decline on day 21 was observed. Among the targets sought, we identified only ethylamide ochratoxin A produced by strain CCDCA10443 (*A. niger*) on day 21 in grape-based medium. A conidia suspension of *A. niger* and *A. carbonarius* was inoculated on grapes of three varieties (Syrah, Touriga Nacional and Muscat Italia). A positive correlation was observed between the following grapes physicochemical parameters: pH, total soluble solids, total glycosides in glucose, total anthocyanin and OTA levels produced by *A. niger* and *A. carbonarius*. Among the elected targets, we identified the decarboxy ochratoxin A in Muscat Italia variety at veraison and 15 days after the beginning of veraison stages; and ethylamide ochratoxin A in the Syrah variety at the ripeness stage. To detect the formation of modified ochratoxins in the presence of *S. cerevisiae* strains, high resolution mass spectrometry was used. Although the presence of OTA did not influence growth parameters of yeast strains, among the elected targets we identified: ochratoxin α, ochratoxin β, ochratoxin α methyl ester, ochratoxin B methyl ester, ethylamide ochratoxin A, ochratoxin C, hydroxy ochratoxin A, hydroxy ochratoxin A methyl ester, and ochratoxin A cellobiose ester. White, rose and red wines were prepared to evaluate the effect of the vinification steps on the fate of OTA as well as the formation of modified ochratoxins in wines. The OTA reduction was 90.72, 92.44 and 88.15% in the white (Moscato Italy), *rose* (Syrah) and red (Touriga Nacional) wine, respectively. Among the targets sought, we identified: ochratoxin β, ochratoxin α methyl ester, ochratoxin B methyl ester, ochratoxin A methyl ester, ethylamide ochratoxin A, ochratoxin C and ochratoxin A glucose ester. These derivatives detected in this study may have been formed by the fungus itself, by the action of yeast or by reactions with components of the medium culture, grape, must and wine. These

results indicate that there may be underreporting of total mycotoxin levels in food, increasing uncertainty concerning health risks to the population.

Keywords: *Aspergillus carbonarius, Aspergillus niger,* masked mycotoxin, ochratoxins, wine and winemaking.

SUMÁRIO

INTRODUÇÃO

As uvas e seus derivados estão entre os principais alimentos considerados fontes de compostos fenólicos. Estes compostos, principalmente o resveratrol, apresentam propriedades antioxidantes, anti-inflamatórias, bactericidas e auxiliam na prevenção de doenças cardiovasculares (RIBÉREAU-GAYON et al., 2006; TOALDO et al., 2015). No entanto, embora o consumo de vinhos seja aliado a efeitos benéficos a saúde, os micro-organismos presentes nas uvas podem ter influência direta nestes. Além de afetarem a saúde das videiras e serem responsáveis pela deterioração das uvas, algumas espécies fúngicas também são capazes de produzirem compostos tóxicos à saúde humana, como as micotoxinas (ČEPO et al., 2018; FERRANTI et al., 2018).

As uvas viníferas estão suscetíveis à infecção por fungos filamentosos presentes no ambiente da lavoura, colheita e na elaboração dos vinhos. As principais espécies contaminantes pertencem aos gêneros *Alternaria*, *Acremonium*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium* e *Rhizopus*. No entanto, o gênero *Aspergillus*, além de estar associado à deterioração, é o principal gênero responsável pela presença de ocratoxina A (OTA), principal micotoxina contaminante de uvas e vinhos (ROUSSEAUX et al., 2014, FREIRE et al., 2017).

Os fungos estão presentes nos cachos desde o início da maturação das uvas. No entanto, devido às alterações nas características físico-químicas das uvas e a elevação da umidade relativa e temperatura no vinhedo, a ocorrência destes micro-organismos aumenta significativamente nos estádios próximos à colheita, favorecendo a produção de OTA pelas espécies *A. carbonarius* e *A. niger* (LEONG et al., 2006; QUINTELA et al., 2013; FREIRE et al., 2017).

A OTA é um metabólito estável, portanto pode permanecer ao longo do processo de vinificação, estando presente no produto final (CECCHINI et al., 2006; CSUTORÁS et al., 2013; CECCHINI et al., 2019). Após ingerida, esta micotoxina pode acumular-se no sistema circulatório, nos rins e nos tecidos adiposo e muscular e, pode provocar efeitos neurotóxicos, carcinogênicos, teratogênicos, imunotóxicos e nefrotóxicos em diversas espécies de animais. A OTA também tem sido associada à nefropatia endêmica dos Balcãs, doença renal crônica em seres humanos (CHEN et al., 2018; LUO; LIU; LI, 2018). Devido a estes fatos, a IARC (Agência Internacional de Pesquisa sobre o Câncer) classificou a OTA como um possível carcinógeno para humanos (grupo 2B) (IARC, 1993). Deste modo, a União Europeia fixou 2µg/kg de OTA máxima permitida em vinhos e sucos de uvas (EC, 2006). Este mesmo limite foi fixado no Brasil em 2011 (BRASIL, 2011).

O vinho tinto tem sido relatado com maiores concentrações de OTA que o vinho branco ou *rose*, muito provavelmente, devido aos diferentes processos de elaboração destes vinhos (LASRAM et al., 2008; GENTILE et al., 2016; DACHERY et al., 2017). A etapa de maceração das uvas é o principal processo responsável pela concentração da OTA nos vinhos, pois possibilita a migração da micotoxina presente na casca para o mosto durante a fase inicial da fermentação alcoólica. Tal processo é realizado no vinho tinto para potencializar a extração da cor; já no vinho *rose* e branco o tempo de contato entre a casca e o mosto é menor, havendo pouco contato entre eles (FERNANDES; VENANCIO, 2003; LASRAM et al., 2008; CSUTORÁS, et al., 2013; CECCHINI et al., 2019).

Entretanto, diversos estudos relatam a diminuição do teor de OTA durante a fermentação alcoólica devido ao metabolismo de detoxificação e a um mecanismo de adsorção pelas paredes das células das leveduras (CECCHINI et al., 2006; PETRUZZI et al., 2015; CHEN et al., 2018). Já na fermentação malolática é observada uma redução menor de OTA, devido a sua adsorção à parede celular das bactérias láticas e pelo metabolismo de detoxificação destes micro-organismos ou; pela adsorção da micotoxina pelas partículas sólidas suspensas (FERNANDES; VENACIO, 2003; DALIÉ; DESCHAMPS; RICHARD-FORGET, 2010). Adicionalmente, estudos sugerem que, as maiores reduções de OTA ocorrem na separação sólido-líquido, já que a maior concentração da micotoxina se encontra na casca (LASRAM et al., 2008; CECCHINI et al., 2019). Além disto, o tratamento de clarificação de vinhos tintos com carvão ativado também é eficaz na redução (GAMBUTI et al., 2005).

Estudos recentes concentram-se em avaliar os mecanismos de degradação e descontaminação de OTA ao longo da elaboração dos vinhos (MASSOUD et al., 2018; CECCHINI et al., 2019). Entretanto, sabe-se que a OTA pode ser modificada e transformada em outras moléculas. Devido a uma alteração em sua estrutura, polaridade, solubilidade e/ou massa molecular, estas moléculas formadas não são detectadas pelos métodos analíticos tradicionais desenvolvidos para detecção da micotoxina mãe (OTA) (BERTHILLER et al, 2013; FREIRE; SANT'ANA, 2018).

O termo "micotoxinas mascaradas" surgiu quando alguns casos de micotoxicoses não se correlacionaram com a quantidade de micotoxinas detectada nas matrizes analisadas. A toxicidade elevada poderia desta forma, ser atribuída à ocorrência de metabólitos tóxicos não detectados (GAREIS, 1994). Estes metabólitos foram recentemente denominados de micotoxinas modificadas e podem ser formados em diferentes estágios do processo produtivo dos alimentos (RYCHLIK et al., 2014).

As micotoxinas modificadas podem ser formadas por micro-organismos, gerados como parte do mecanismo de defesa da planta infectada, ao longo do processamento de alimentos e, em alguns casos, podem ser reconvertidos na micotoxina mãe durante o metabolismo de animais e humanos, tornando-se biodisponível novamente (BERTHILLER et al., 2013; DALL'ERTA et al., 2013; BRODEHL et al., 2014; PARIS et al., 2014; FREIRE; SANT'ANA, 2018). Estes derivados geralmente coocorrem com as micotoxinas livres sendo que, em alguns casos, a concentração de micotoxinas modificadas excede o nível da forma livre em alimentos processados (DE BOEVRE et al., 2012; BERTHILLER et al., 2013; MAUL; PIELHAU; KOCH, 2014).

Apesar de frequentes estudos da presença de OTA em uvas e vinhos e redução dos níveis da micotoxina ao longo da vinificação, estudos de vigilância, até o momento, não nos fornecem informações precisas sobre a formação de micotoxinas modificadas por micro-organismo; nas uvas; e ao longo do processamento. Alguns compostos formados podem possuir efeitos tóxicos semelhantes a micotoxina mãe. Além disto, após a ingestão, é possível que ocorra a liberação da micotoxina mãe no trato digestivo e esta seja reabsorvida, aumentando a exposição total do organismo às micotoxinas (HILDEBRAND et al., 2015). Tais fatos podem resultar em subnotificação da ocorrência e importância do nível total de micotoxinas presentes no alimento (STOEV; DENEV, 2013; FREIRE; SANT'ANA, 2018).

Neste sentido, o objetivo neste estudo foi determinar a variabilidade de multiplicação e produção de OTA e ocratoxinas modificadas por cepas de *Aspergillus carbonarius* e *A. niger*; e avaliar a formação e degradação de OTA e ocratoxinas modificadas da colheita ao vinho. Para tal, os seguintes objetivos específicos foram considerados:

❖ Determinar a variabilidade da multiplicação e produção de OTA por cepas de *A. carbonarius* e *A. niger* isoladas de uvas, bem como investigar a formação de micotoxinas modificadas por estes fungos em meio de cultivo a base de uva.

❖ Avaliar a influência do estágio de maturação e da variedade das uvas na produção de OTA por cepas de *A. carbonarius* e *A. niger* e na formação de micotoxinas modificadas.

❖ Avaliar a influência da presença de OTA nos parâmetros cinéticos de crescimento de leveduras e elucidar a formação de ocratoxinas modificadas na presença de cepas de *Saccharomyces cerevisiae* durante a fermentação.

❖ Avaliar o efeito das etapas de vinificação no destino da OTA e a formação de ocratoxinas modificadas em vinhos.

CAPÍTULO 1

Revisão de literatura: Modified mycotoxins: An updated review on their formation, detection, occurrence, and toxic effects.

Artigo publicado no periódico “Food and Chemical Toxicology”: Freire, L.; S. Sant’Ana, A. S. Modified mycotoxins: An updated review on their formation, detection, occurrence, and toxic effects. Food and Chemical Toxicology, 111, 189-205, 2018.

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Review

Modified mycotoxins: An updated review on their formation, detection, occurrence, and toxic effects

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ABSTRACT

Modified mycotoxins are metabolites that normally remain undetected during the testing for parent mycotoxin. These modified forms of mycotoxins can be produced by fungi or generated as part of the defense mechanism of the infected plant. In some cases, they are formed during food processing. The various processing steps greatly affect mycotoxin levels present in the final product (free and modified), although the results are still controversial regarding the increase or reduction of these levels, being strongly related to the type of process and the composition of the food in question. Evidence exists that some modified mycotoxins can be converted into the parent mycotoxin during digestion in humans and animals, potentially leading to adverse health effects. Some of these formed compounds can be even more toxic, in case they have higher bioaccessibility and bioavailability than the parent mycotoxin. The modified mycotoxins can occur simultaneously with the free mycotoxin, and, in some cases, the concentration of modified mycotoxins may exceed the level of free mycotoxin in processed foods. Even though toxicological data are scarce, the possibility of modified mycotoxin conversion to its free form may result in a potential risk to human and animal health. This review aims to update information on the formation, detection, occurrence, and toxic effects caused by modified mycotoxin.

1. Introduction

Mycotoxins are toxic secondary compounds synthesized under specific conditions by certain fungal species capable of growing in a wide variety of foods. The most common mycotoxins are: aflatoxins; ochratoxin A (OTA); citrinin; patulin; trichothecenes: deoxynivalenol (DON), T2 toxin (T2) and HT2 toxin (HT2); fumonisins and zearalenone (ZEN). These metabolites are produced mainly by fungi of the genera *Aspergillus*, *Fusarium*, *Penicillium*, and *Alternaria* (Anfossi et al., 2016).

Several fungal species can produce the same mycotoxin, and one single species can synthesize more than one mycotoxin. However, the growth of the toxigenic fungi does not necessarily imply mycotoxin production. Similarly, the absence of mycotoxin is not ensured by the elimination of fungi, as it may have been produced before the inactivation of the fungi (Turner et al., 2009).

The growth of toxigenic fungi and mycotoxin production can occur at all stages of production and processing (Yogendrarajah et al., 2014). Their growth is largely dependent on environmental factors such as microbial competition, nutrient availability and substrate structure, activity water, pH, temperature, relative humidity, presence of insects, and application of fungicides and pesticides (Anfossi et al., 2016; Hameed et al., 2013). However, such factors have a different influence on fungal growth and mycotoxin production. Despite this, it is known that these factors are usually stricter for mycotoxins production than for fungal growth (Garcia et al., 2009). Therefore, it is difficult to describe a set of environmental conditions that will foster both fungal growth and mycotoxin production (Cast, 2003).

Mycotoxins are commonly found in cereals, fruits, and spices. However, because they are stable compounds, mycotoxins tend to remain in the final product. As such, mycotoxins are found in processed products such as beer, breads, juices, chocolate, and wine, due to the use of contaminated raw materials (Kabak, 2009; Turner et al., 2009). Two or more mycotoxins can co-occur in foods, rising the total levels of mycotoxin present and negatively affecting human and animal health

Abbreviations: 15-ADON, 15-Acetyl-deoxynivalenol; 3-ADON, 3-Acetyl-deoxynivalenol; AME, alternariol monomethyl ether; AOH, alternariol; D3G, deoxynivalenol-3-glucoside; DOM-1, de-epoxy deoxynivalenol; DON, deoxynivalenol; DON-15-GlcA, deoxynivalenol-15-glucuronide; DON15S, deoxynivalenol-15-sulfate; DON-3-GlcA, deoxynivalenol-3-glucuronide; DON3S, deoxynivalenol-3-sulfate; DON-GlcA, deoxynivalenol-glucuronide; FB1, fumonisin B1; FHB, Fusarium head blight; HFB, hydrolyzed fumonisins; HT2, HT2 toxin; HT2-3G, HT2 toxin-3-glucoside; LC-MS/MS, Liquid chromatography tandem-mass spectrometry; MS/MS, tandem-mass spectrometry; NIV-3G, Nivalenol-3-glucoside; OTA, ochratoxin A; T2, T2 toxin; T2-3G, T-2 toxin-glucoside; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; ZEN, zearalenone; ZEN-14G, zearalenone-14-glucoside; ZEN-16G, zearalenone-16-glucoside; α-ZEL, α-zearalenol; α-ZEL-14G, α-zearalenol-14-glucoside; β-ZEL, β-zearalenol; β-ZEL-14G, β-zearalenol-14-glucoside

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Table 1
Major mycotoxin found in food and their modified forms.

Free mycotoxin	Major producing fungi	Matrix	Effects on health	modified mycotoxin
DON	*Fusarium culmorum; Fusarium graminearum* (Richard, 2012; Bottalico, 1998)	feed (Zhao et al., 2015), oat (Fredlund et al., 2013), cereals (Soleimany et al., 2012a), maize (Souza et al., 2008)	In animal: reduced growth and weight gain, feed refusal and emesis, affects system immune and intestinal functions. In human: nausea, diarrhea and vomiting (Bryden, 2012; Pinton et al., 2012; Pinton et al., 2009; WHO, 2002)	DON-3-glucoside; DON-Hexitol; DON-S-cysteine; DON-S-cysteinyl-glycine; DON-glutathione; DON-di-hexoside; "DON-2H"-glutathione; DON-malonylglucoside; 15-acetyl-DON-3-glucoside; 3-Acetyl-DON; DON-3-sulfate; DON-15-sulfate; 3-epimer-DON; norDON A, B and C; norDON-3-glucoside A, B, C and D; DON-3-glucoside-lactone; de-epoxy DON; DON-glucuronide; de-epoxy DON-3-sulfate; de-epoxy DON-15-sulfate;
ZEN	*Fusarium culmorum; Fusarium graminearum; Fusarium heterosporum* (Gajecka et al., 2011; Hussein and Brasel, 2001)	feed (Zhao et al., 2015), cereals (Soleimany et al., 2012b), breakfast cereals (Ibáñez-Vea et al., 2011), maize (Souza et al., 2008)	precocious pubertal changes, fertility problems and hyper estrogenic (Minervini and Dell'Aquila, 2008; Binder, 2007; IARC, 1993)	ZEN-16-O-β-glucoside; ZEN-14-O-β-glucoside; α-zearalenol; β-zearalenol; α-zearalenol-glucoside; β-zearalenol-glucoside; ZEN-4-glucoside; ZEN-4-sulfate; malonyl-glucosides (ZEN-MalGlc, α-ZEN-MalGlc, β-zearalenol-MalGlc); di-hexose- (ZEN-DiHex, α- zearalenol-DiHex, β-zearalenol-DiHex); hexose–pentose disaccharides (ZENHexPent, α-zearalenol-HexPent, β-zearalenol-HexPent); tri-hexose conjugate (β-zearalenol-TriHex); α-zearalenol-Sulfate; α-zearalenol; β- zearalenol
OTA	*Aspergillus* and *Penicillium* genera (Freire et al., 2017; Passamani et al., 2012; Serra et al., 2006)	wine (Freire et al., 2017), coffee (Leong et al., 2007), cocoa (Mounjouenpou et al., 2008), grapes (Lasram et al., 2007), cereals (Lee and Magan, 2000)	Immunosuppressive effects (Rossiello et al., 2008), teratogenic (Balasaheb et al., 2007), carcinogenic (Brown et al., 2007), mutagenic (Palma et al., 2007), neurotoxic (Sava et al., 2006) and genotoxic (Tozlovanu et al., 2006).	ochratoxin α; 4S-hydroxyochratoxin A; 4R-hydroxyochratoxin A; hydroxyochratoxin A-β-glucoside; ochratoxin A methyl ester; Ochratoxin α amide; 14-decarboxy-ochratoxin A;. Ochratoxin A mono- and disaccharide esters
T2 e HT-2	*Fusarium sporotrichioides* (Cast, 2003; Brown et al., 2001)	beer (Rubert et al., 2013), corn, wheat, barley, oats (Cast, 2003)	Inhibition protein synthesis and effects immunotoxins (WHO, 2002; Richard, 1991; Niyo et al., 1988).	HT2 toxin-3-glucoside; T-2 toxin-α-glucoside; T-2 toxin-β-glucoside; 15-acetyl-T2-tetraol-glucoside; hydroxy-HT2-glucoside; hydroxy-HT2-malonyl-glucoside; T2-triol-glucoside; dehydro-HT2-glucoside; HT2-diglucoside; HT2-malonyl-glucoside; 3-acetyl-HT2; 3-acetyl-T2; feruloyl-T2; HT2-sulfate
Fumonisin	*Fusarium proliferatum; Fusarium verticillioides; Aspergillus niger* (Varga et al., 2010; EFSA, 2005; Scott, 1993)	feed (Zhao et al., 2015), beer (Matumba et al., 2014), chili (Yogendrarajah et al., 2014), cereal (Soleimany et al., 2012a)	hepatotoxic, nephrotoxic (Binder, 2007), immunosuppressive (Marin et al., 2006), pulmonary edema in swine and leukoencephalomalacia in horses (IARC, 2002)	hidden fumonisins; N-(carboxymethyl) fumonisina B1; N-Acyl hydrolyzed fumonisin B1; bound hydrolyzed fumonisins

(Pedrosa and Borutoya, 2011).

A large variety of toxic effects in animals and humans has been observed due to the ingestion of food contaminated with mycotoxins, such as immunosuppression, carcinogenic, genotoxic, teratogenic or mutagenic effects (Zhu et al., 2015; CE, 2006). Nonetheless, the impact of mycotoxins on health depends on different factors, including levels of ingestion, the toxicity of the compound ingested, body weight, species and age of the individual. Other factors include the presence of other mycotoxins, exposure time, individual physiological condition and action mechanism of the compound (Richard, 2007; Hussein and Brasel, 2001). Besides, it is possible that the form mycotoxins are present, due to the transformation of their original molecules into different structures (modified mycotoxins), is not detected by traditional methods and may result in underreporting (Table 1).

The term "masked mycotoxin" arose in the mid-1980s, and the suspected presence was due to the occurrence of some cases of mycotoxicosis that did not correlate with some mycotoxins detected in the matrices analyzed. The high toxicity could thus be attributed to the occurrence of undetected forms, such as conjugated mycotoxins (Gareis, 1994). These metabolites, recently renamed as modified mycotoxins, undergo changes in their structure, polarity, solubility and molecular mass.

Modified mycotoxins can be produced by fungi as well as be formed as part of the defense mechanism of the plant infected with the toxigenic fungi. Furthermore, these metabolites can be formed along the processing of foods from contaminated raw materials and, in some cases, can be reconverted to the parent toxin during the animal and human metabolism (Berthiller et al., 2009a, 2013; Vendl et al., 2009).

Due to the lack of elucidation of their structures and lack of analytical standards, the modified mycotoxins are characterized as undetectable molecules through conventional analytical techniques used in the detection of free mycotoxin, which implies an underestimation of the total content of mycotoxins in contaminated foods. Even though toxicological data are scarce, the possibility of modifiedmycotoxin conversion to its free form may result in a potential risk to human and animal health. The concern is supported as the conversion of modified to free form may lead to increased bioavailability of mycotoxin (Paris et al., 2014; Berthiller et al., 2013; Dall'Erta et al., 2013; Tran et al., 2012).

Modified mycotoxins co-occur with free mycotoxins (Berthiller et al., 2013), and in some cases, the concentration of modified mycotoxin exceeds the level of free form in processed foods (Maul et al., 2014; De Boevre et al., 2012). Therefore, the understanding of the formation or degradation of free and modified mycotoxins constitute information of high relevance for the refinement of quantitative risk assessment models (Warth et al., 2015). The relevance of this knowledge is underpinned considering that the total levels of mycotoxins in foods are very likely to be higher than the levels detected if modified mycotoxins are present but not detected (Stoev and Denev, 2013).

It is necessary to develop reliable methods for the detection of modified mycotoxin, as well as to study their stability during the processing of foods, their fate in the digestive system and toxicokinetic and toxicodynamic studies (McCormick et al., 2015). Also, the understanding of the formation process of these metabolites and the knowledge of their structure and molecular mass can solve the analytical and technological problems associated with these metabolites (Maul et al., 2012).

This review aims to address the different phenomena involved in the formation of modified mycotoxins. The formation of modified mycotoxins in the field, during processing, as well as the possible toxic effects caused by their ingestion and the assessment strategies used to detect these compounds were discussed. Although the subject has been reviewed before (Rychlik et al., 2014; Berthiller et al., 2009a,b, 2013; Cirlini et al., 2012), findings and a crescent number of scientific paper recently published on the topic have been observed. Therefore, an update is needed that reflects the advances in the knowledge regarding modified mycotoxins. This information is key as they may assist in the development of strategies for reducing the risk of underreporting of the total mycotoxin levels present in food and consequently to safeguard animal and food health.

2. Modified mycotoxins

The first modified mycotoxin discovered, although the term "masked mycotoxin" had not yet been designated, was aflatoxin M1, a substance formed from the hydroxylation of aflatoxin B1 and eliminated in the milk of animals that consumed feed contaminated with this mycotoxin (Masri, 1967). After this discovery, Gareis (1994) detected another compound derived from ZEN in the 1980s. However, the discovery and detection of various forms of mycotoxin was intensified only in recent years, most likely from the advent of liquid chromatography coupled to mass spectrometry, the main technique used in the elucidation of these compounds (Rychlik et al., 2014).

Although the classification of different forms of mycotoxin is still confusing and needs further investigation, the term "masked mycotoxins" has been indicated to be used exclusively for compounds derived from mycotoxins that were formed by plants' defense mechanisms (Berthiller et al., 2013). However, it is known that mycotoxin-derived compounds can also be formed by other pathways, such as food processing and animal metabolism. Therefore, the term "modified mycotoxin" was recently coined to denote all forms derived from mycotoxins, regardless of their origin. This term includes compounds formed by reactions throughout processing, by microorganisms, or by the metabolism of plants (Rychlik et al., 2014; Berthiller et al., 2013).

This change in terminology is necessary since, due to advances in the development of techniques of extraction, identification, detection, and quantification and production of analytical standards, some of the modified mycotoxins are likely to be detected (Rychlik et al., 2014).

In the literature, some references classify modified mycotoxins based on the form in which the parent mycotoxin binds itself to the food matrix. When the mycotoxin reacts with food components through covalent bonds, it is called "conjugated". On the other hand, when the mycotoxin links to food components through noncovalent bonds (physical entrapment) it is called "hidden." Despite this, all forms are considered "bound" for being linked to the matrix (Berthiller et al., 2009a,b, 2015; Dall'Asta et al., 2009).

A recent definition has classified these compounds as free, matrix-associated, and modified mycotoxins. Free mycotoxins are the compounds arising from the secondary metabolism of toxigenic fungi (OTA, DON, ZEN). Modified mycotoxins have had their structure modified by chemical (process) or biological (plants, animals, microorganisms) phenomena. The so-called matrix-associated mycotoxins are formed when the compound forms a complex with the food matrix or are physically bond, or yet, are covalently bound to the matrix without a reaction, due to processing or a biological phenomenon (Rychlik et al., 2014).

The identification and quantification of modified mycotoxins are mostly performed by *in vitro* studies using artificial contamination of the matrix (food/plant) with the parent mycotoxin, due to the inexistence of official standards and methodologies for detection. So far, only the deoxynivalenol-3-glucoside (D3G) standard, a derivative of DON, exists commercially. These studies favor knowledge on the formation route of the modified mycotoxins from the parent mycotoxin and their destination in plants, along with the processing and within the digestive system. However, some recent studies have detected modified mycotoxins in naturally contaminated foods (Table 2).

In addition to the occurrence of these metabolites in fresh foods, due to detoxification processes from the metabolism of plants (Berthiller et al., 2006), the modified mycotoxins can also occur in products subjected to physical and chemical processes (Dall'Asta et al., 2008; Park et al., 2004). Their modification is also attributed to the metabolism of animals and humans (Vendl et al., 2009) (Fig. 1).

Table 2
Occurrence of modified mycotoxin in foods.

Matrix	Technique	Modified mycotoxin	Levels (μg/Kg)/(μg/L)[a]	References
wheat	LC-MS/MS	D3G	50–200	Berthiller et al. (2005)
corn	LC-MS/MS	D3G	< 20 - 70	
wheat	LC-MS/MS	D3G	76–1070	Berthiller et al. (2009b)
corn	LC-MS/MS	D3G	10–763	
corn products	LC-MS/MS ELISA	bound fumonisins	NR	Dall'Asta et al. (2008)
corn	LC-MS/MS	hidden fumonisins	54–982	Dall'Asta et al. (2009)
beer	LC-MS/MS	D3G	< 2.5–37	Kostelanska et al. (2009)
corn	LC-MS/MS	Total fumonisins	< 25 - 44274	Dall'Asta et al. (2012)
corn	ELISA	Total DON	120–750	Tran et al. (2012)
malt	LC-MS/MS	D3G	12.9–186	Zachariasova et al. (2012)
baked goods	LC-MS/MS	D3G	43–399	
beer	LC-MS/MS	D3G	6–82.1	
beer	LC-MS/MS	D3G	< 3.5–81.3	Varga et al. (2013)
corn	LC-MS/MS	hidden fumonisins	NR	Oliveira et al. (2015)
durum wheat	LC-MS/MS	D3G	< 50 - 850	Palacios et al. (2017)

[a] NR - Not reported: levels were reported only in charts.

2.1. Modified mycotoxin by plants

Plants infected with mycotoxin-producing fungi, through their defense mechanism, can alter the structures of mycotoxins by conjugation with organic molecules or by hydrolysis, reduction and oxidation reactions, also altering their polarity. These reactions may reduce their toxicity, and facilitate their sequestration in the plant vacuole or apoplast. These derivatives may also be incorporated into cell wall components (Berthiller et al., 2013; Bowles et al., 2006; Coleman et al., 1997; Sewald et al., 1992). However, the efficiency of the detoxification process of mycotoxins in plants depends on the effectiveness of the enzyme produced by the plant in reaching and acting on the mycotoxin (Higa-Nishiyama et al., 2005).

Detoxification mechanisms involve three main phases: chemical modification (phase I and II metabolism) and compartmentalization (phase III metabolism), resulting in the formation of masked mycotoxin (Berthiller et al., 2013). Phase I reactions involve hydrolysis, reduction or oxidation of the molecule. Typically, these reactions occur with lipophilic xenobiotics and are catalyzed by esterases, amidases, P450 monooxygenases, and peroxidases. However, these reactions do not always reduce the toxicity of the original xenobiotic, and may in some cases increase toxicity (Berthiller et al., 2013; Cole and Edwards, 2000; Coleman et al., 1997; Coupland, 1991). These reactions occur mainly by making the lipophilic xenobiotics reactive to phase II. Hydrophilic xenobiotics already have reactive groups and, therefore, there is no need to undergo phase I metabolism (Coleman et al., 1997).

The reactions in phase II are characterized by the detoxification itself, due to conjugation reactions. These reactions occur through the binding of hydrophilic biomolecules to the xenobiotic, catalyzed by glycosyl-, malonyl- and glutathione-S-transferases. Compounds such as glucose, fatty acids, glutathione and hemicellulose bind to the mycotoxin molecule, increasing its solubility and facilitating its sequestration in phase III, thereby reducing its phytotoxicity (Berthiller et al., 2013; Bowles et al., 2006; Coleman et al., 1997; Lamoureux et al., 1991). However, some mycotoxin derivatives may still be toxic to cellular components, although they normally have their toxicity quite reduced as compared with the parent toxin. This occurs due to the increased polarity of the molecule, facilitating their transport through ATP-dependent glutathione-conjugate transporters, which allows their elimination or storage (Coleman et al., 1997). In phase III, the modified mycotoxins are stored in the vacuole, apoplast or irreversibly bound to the cell wall. In this way, the products are not able to exert toxic effects on the plant (Schröder et al., 2007; Bourbouloux et al., 2000; Coleman et al., 1997). The β-linked glucose-conjugates of trichothecenes, D3G and HT2 toxin-3-glucoside (HT2-3G), and zearalenone, zearalenone-14-glucoside (ZEN-14G), are the most common compounds (Gratz, 2017; Broekaert et al., 2015a; Meng-Reiterer et al., 2015).

2.1.1. Modified trichothecene

D3G, one of the most common modified forms of DON, is formed through the plant defense mechanism, in which glycosyltransferase enzymes bind an endogenous glucose molecule to the hydroxyl group of carbon 3 of the DON molecule. This binding limits the interaction of the group responsible for the toxicity of mycotoxin with the target cellular

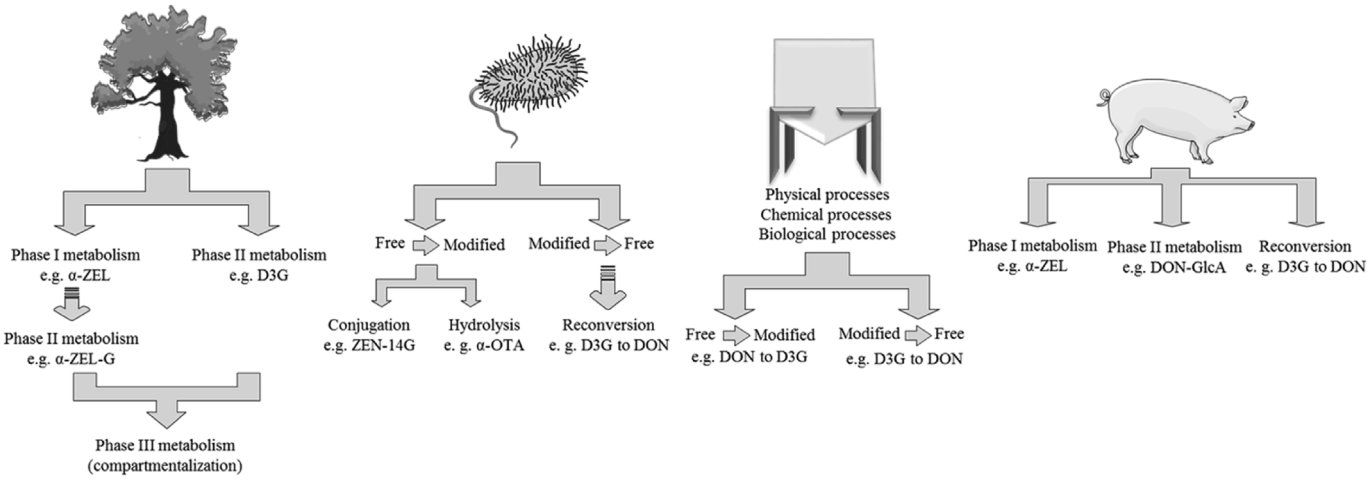

Fig. 1. Formation of modified mycotoxin by plants, microorganisms, processing and animals (images with permission Les Laboratoires Servier).

components, thereby reducing its toxicity. In addition, it alters the polarity of the molecule, favoring the exit of the cytosol, due to access to membrane-bound transporters. The conjugated mycotoxin is then stored in the plant vacuole or apoplast or bound to the cell wall (Ovando-Martínez et al., 2013; Maul et al., 2012; Berthiller et al., 2005; Poppenberger et al., 2003; Jones and Vogt, 2001).

The ability to convert DON to D3G, 3-Acetyl-deoxynivalenol (3-ADON) and 15-Acetyl-deoxynivalenol (15-ADON) was shown to be distinct for different previously contaminated cereals. A higher conversion of DON to D3G was observed in spelt, followed by wheat and barley and a smaller transformation in corn and rye. No DON conversion was seen in oats. This conversion occurred intensely throughout the germination. In this stage, there is a high concentration of glucose and enzymes that can favor the glycosylation process, aiding in the formation of D3G (Maul et al., 2012).

Due to the different chemical composition of these cereals, it is possible that in spelt there is a greater induction of UDP-glycosyltransferases genes, the main enzyme active in detoxification. This enzyme is commonly observed in wheat infected by *Fusarium,* the main fungal species responsible for the accumulation of DON in cereals (Steiner et al., 2009 . Desmond et al., 2008).

D3G formation also seems to be affected by parent toxin levels (DON) and plant susceptibility to *Fusarium* head blight (FHB). A positive correlation between the increase in DON and D3G concentration is observed up to a concentration threshold of the parent mycotoxin. Higher DON concentrations seem to alter the efficiency of DON to D3G conversion, which indicates a limitation in the glycosylation capacity of the plant when it is highly contaminated, resulting in lower levels of D3G. Concerning plant resistance to FHB, a lower incidence of *Fusarium* and, therefore, a lower concentration of DON, will result in higher levels of D3G in response to infection by the fungus (Ovando-Martínez et al., 2013; Kostelanska et al., 2011a).

Several DON biotransformation products in different wheat lines have been identified: deoxynivalenol-hexitol, deoxynivalenol-S-cysteine, deoxynivalenol-S-cysteinyl-glycine, deoxynivalenol-glutathione, deoxynivalenol-di-hexoside, D3G, "deoxynivalenol-2H"-glutathione, deoxynivalenol-malonyl-glucoside and 15-acetyl-DON-3-β-D-glucoside. Most likely, wheat uses glycosylation reactions and glutathione pathway for DON detoxification, through the action of glycosyltransferases and glutathione-S-transferases, respectively. It is suggested that these adducts of cysteines, glutathione, and glucose avoid the interaction of these biotransformation products with the ribosomes, reducing the inhibition of protein synthesis, one of the main toxic effects of DON (Kluger et al., 2015; Fruhmann et al., 2014).

In wheat plants artificially contaminated with *F. graminearum* suspension and DON solution, the modified forms of DON deoxynivalenol-3-sulfate (DON3S) and deoxynivalenol-15-sulfate (DON15S) were also detected. DON15S was considered a moderate inhibitor of plant ribosome activity, whereas DON3S was not able to inhibit protein synthesis. These compounds are also regarded DON detoxification products through the plant defense mechanism, due to their lower toxicity as compared with the parent toxin (Warth et al., 2015).

The ability to metabolize xenobiotic substances in conjugated forms seems to be dependent on several factors such as the initial concentration of the parent mycotoxin, composition of the food and the presence of enzymes active in detoxification, and there may be different possible forms of conjugation, such as conjugated glucosides and sulfates. It is possible that the production and action of UDPglycosyltransferases and glutathione-S-transferases are directly proportional to DON concentration. However, a correlation between DON levels and the incidence of FHB in the field cannot yet be postulated. Further experiments under natural conditions are necessary so that the correlation between mycotoxin levels and disease severity found in laboratory-conducted experiments can be confirmed in the field (Audenaert et al., 2013).

The conjugation of fusarenon-X- and nivalenol with glucosides resulting in fusarenon-X-glucoside and nivalenol-glucoside, respectively, was also observed in wheat grains infected with *Fusarium.* This conjugation may also be related to the action of UDPglycosyltransferases enzymes produced by the plant (Nakagawa et al., 2011).

Similar to DON, another trichothecene, T2 toxin, is also responsible for the inhibition of protein synthesis. It also affects cell division and causes immunotoxic effects (WHO, 2002; Richard, 1991; Niyo et al., 1998). However, the modified mycotoxin from this mycotoxin, T2 toxin-α-glucoside, and T2 toxin-β-glucoside did not show phytotoxicity to algae *Chlamydomonas reinhardtii,* while the parent T2 toxin inhibited the growth of cultured cells (McCormick et al., 2015). Possibly, the algae defense mechanism against T2 acts similarly to the DON detoxification process in plants, in which a glucose molecule binds to the toxin and blocks the active group of the parent toxin, limiting its action.

Plant metabolic fate of T2 and HT2, in artificially contaminated wheat, found as main HT2-derived metabolites: 15-acetyl-T2-tetraol-glucoside, hydroxy-HT2-glucoside, hydroxy-HT2-malonyl-glucoside, T2-triol-glucoside, dehydro-HT2-glucoside, HT2-diglucoside, HT2-3G, HT2-malonyl-glucoside, T-2,3-acetyl-HT2 and T2-derived metabolites: hydroxy-HT2-glucoside, hydroxy-HT2-malonyl-glucoside, T2-triol-glucoside, dehydro-HT2-glucoside, HT2-diglucoside, HT2-3G, HT2-malonyl-glucoside, HT2, 3-acetyl-T2, feruloyl-T2, were detected (Nathanail et al., 2015).

The kinetics of the identified metabolites, throughout the development of the plant, demonstrated that the detoxification mechanism of the plant acts by converting T2 to HT2 quickly through deacetylation. Simultaneously, the HT2 formed was further metabolized through glycosylation to HT2-3G in T2-treated only assays. In plants infected with HT2, HT2-3G was the main metabolite found. However, after reaching its maximum concentration, its levels decreased, possibly due to subsequent metabolism or by covalent bound to the plant matrix. Other metabolites detected had a similar behavior. From the metabolites formed, it is possible to predict that the plant defense mechanism acts through glycosylation and C3-acetylation of both toxins, and (iso) ferulic acid conjugation of T2. However, those responsible for the toxicity of the molecules, C12-C13 epoxy group, were unaffected, reflecting the need for toxicological studies of the modified mycotoxin formed (Nathanail et al., 2015).

2.1.2. Modified alternariol

The conversion of alternariol (AOH) and alternariol monomethyl ether (AME) to conjugated metabolites in plants were analyzed using culture suspension of BY-2 tobacco cells. AOH and AME were not detected in the culture medium, which demonstrates a possible uptake of mycotoxins by the cells or metabolization into derivative compounds. After cell disruption, some of the toxins were detected. However, most of the mycotoxins were metabolized by conjugation with glucose. The presence of free hydroxyl groups in the molecule of these toxins enables the conjugation with sugars molecules, facilitating their storage in the plant (Hildebrand et al., 2015; Sandermann, 1992).

All AOH conjugates: 9-O-β-D-glucopyranosyl-AOH; 3-O-β-D-glucopyranosyl-AOH; 9-O-{β-D-glucopyranosyl (1 → 6)-β-D-glucopyranosyl}AOH; 9-O-(6-O-malonyl-β-D-glucopyranosyl) AOH; 3-O-(6-O-malonyl-β-D-glucopyranosyl)AOH; and the AME conjugates: 7-O-β-D-glucopyranosyl-AME; 3-O-β-D-glucopyranosyl-AME; 3-O-(6-O-malonyl-β-D-glucopyranosyl)AME; 3-O-(4-O-malonyl-β-D-glucopyranosyl) contained β-glucopyranose bound to the hydroxyl group of the mycotoxins in question (Hildebrand et al., 2015). Because they are not capable of excreting these formed metabolites, the conjugates can be stored in the plant vacuole or apoplast as soluble conjugates, or irreversibly bound to cell wall as insoluble conjugates. Therefore, both conjugates formed are considered a mechanism of detoxification of plants against contamination by mycotoxins produced by fungi (Hildebrand et al., 2015). However, toxicological studies must be performed to evaluate the actual contribution of these derivatives to the

overall toxicity of the food.

2.1.3. Modified ZEN

ZEN is a mycotoxin that has demonstrated effects of precocious puberty, fertility problems and symptoms of hyperestrogenism (Minervini and Dell'Aquila, 2008; Binder, 2007; IARC, 1993). This toxicity is related to its lactone ring and the C-4 hydroxyl group (El-Sharkawy and Abul-Hajj, 1988). Change in these structures can lead to a detoxification process (McCormick, 2013).

ZEN detoxification by rice plant leaves was evaluated *in vitro*. A reduction in toxin levels was observed in genetically modified plants, while in wild plants there was no change. This property can be ascribed to zhd101 gene expression; however, the metabolites formed were not identified (Higa-Nishiyama et al., 2005). In transgenic corn containing genes encoding enzymes with detoxification activities, similar results were observed (Igawa et al., 2007). However, the performance of enzymes derived from genetic modifications may be questioned due to their non-homogenous distribution over the plant and its different activity under different physiological conditions (Duvick, 2001).

In ZEN-treated barley seedlings, the metabolites ZEN-16-glucoside (ZEN-16G) and ZEN-14G were detected in the roots, although in small amounts. Suspensions of wheat cells added with ZEN were also tested for toxin masking potential. Only 13% of the initial concentration was converted to ZEN glycosides, and it is possible that the toxin was converted to other undetected metabolites, such as malonyl-glucoside and diglucoside (Paris et al., 2014).

In a model plant *Arabidopsis thaliana,* the UDP-glycosyltransferase gene was identified, which is responsible for encoding the enzyme capable of converting ZEN-14G from ZEN. Although this molecule has no toxic effects *in vitro,* it may be converted to ZEN along the digestion, its toxicity returning (Poppenberger et al., 2006). ZEN was also converted to ZEN-14G and ZEN-16G, through the action of the enzyme HvUGT14077, a UDP-glucosyltransferase, which catalyzes O-glucosylation at C-14 and C-16, with a preference of 14-glucoside synthesis (Michlmayr et al., 2017).

Likewise, Berthiller et al. (2006) evaluated the formation of phase I and II metabolites from ZEN in model plant *Arabidopsis thaliana.* In the plant extracts, ZEN, α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), α-zearalenol-glucoside, β-zearalenol-glucoside, ZEN-4-glucoside, ZEN-4-sulfate, malonyl-glucosides (ZEN-MalGlc, α-ZEN-MalGlc, β-ZEL-MalGlc), di-hexose- (ZEN-DiHex, α-ZEL-DiHex, β-ZELDiHex), hexose–pentose disaccharides (ZENHexPent, α-ZEL-HexPent, β-ZEL-HexPent) and tri-hexose conjugate (β -ZEL-TriHex) were identified. The proposed conversion pathway indicates that phase I metabolites (α-ZEL and β-ZEL) were later converted to phase II metabolites (α-ZEL-glucoside e β-ZEL-glucoside). Moreover, after the formation of monoglucosides, through conjugation in phase II metabolism, it is suggested that the formation of malonylglucosides and disaccharides occurs from the respective monoglucosides formed previously.

2.1.4. Modified OTA

The detoxification process of OTA in plants also seems to be related to the action of enzymes in the cleavage or conjugation of the parent mycotoxin resulting in modified mycotoxin. The biosynthesis of glutathione synthetase, the enzyme responsible for the metabolism of glutathione, increased considerably in *Arabidopsis thaliana* tissues containing OTA, suggesting the involvement of glutathione in OTA detoxification. Glutathione is considered an antioxidant and substrate in the detoxification of xenobiotics responsible for oxidative stress in plants. The levels of glutathione-S-transferase and glutathione peroxidase also increased. These enzymes are involved in the conjugation reactions, which make the toxin more polar, facilitating its transport and storage, reducing toxicity to plants (Wang et al., 2014).

Modification of OTA to derived compounds in plant-cell suspension cultures was studied by Ruhland et al. (1996). The metabolism of OTA occurred differently in the various cultures tested. After one day of incubation, the almost complete metabolism of OTA was observed in carrots, tomatoes, and corn, whereas for soybeans three days were required, while for wheat, barley and potatoes, 13 days were required for transforming 90% of OTA in their derivatives. Ochratoxin α, 4S-hydroxyochratoxin A, 4R-hydroxyochratoxin A, hydroxy-ochratoxin A-β-glucoside, ochratoxin A methyl ester and other unidentified polar metabolites were detected. This difference in metabolization is most likely due to genetic issues unique to each culture involving the production of enzymes capable of acting on mycotoxin, reducing its toxicity.

However, compounds such as 4-hydroxyochratoxin A have similar toxicity to ochratoxin A (Creppy et al., 1983). In addition, OTA glycosides, such as hydroxychratoxin A-β-glucoside, can be cleaved by the metabolism of humans and animals, returning to the original toxicity due to the release of the free toxin. Therefore, despite the toxin detoxification effort, these compounds may act synergistically with OTA by raising levels of total mycotoxins in the food and the risk to human and animal health (Ruhland et al., 1996).

2.2. Modified mycotoxin by microorganisms

In addition to the plant infected with mycotoxin-producing fungi, some strains of yeast, bacteria and filamentous fungi also can modify mycotoxins, especially conjugate sulfate, and glucoside esters, through their defense mechanisms and enzymatic activity (Brodehl et al., 2014; Berthiller et al., 2013). It is possible that up to 50% of the parent mycotoxin will form adducts with the matrix of the microorganism or with components of the culture medium and therefore are not detected by conventional methodologies (Kakeya et al., 2002). The presence of these compounds may lead to the underreporting of total levels of mycotoxins in food products, especially in fermented products such as wines, beer, and shoyu, since the microorganisms used in the preparation of these foods are capable of producing such compounds. However, the mechanisms of these modifications have not yet been systematically studied (Brodehl et al., 2014; Berthiller et al., 2013).

2.2.1. Modified mycotoxin by yeast

Trichomonascus clade species showed the ability to biotransform T2 into T2 toxin-α-glucoside (by glycosylation); into 3-acetyl T-2 toxin (through acetylation by the action of acetyltransferases); and into neosolaniol (by removal of the C-8 isovaleryl group by the action of isovaleryl esterase). This transformation is considered a detoxification against the deleterious effects of the toxin. The defense mechanism of microorganisms acts on C-3 hydroxyl group of the parent mycotoxin, reducing its toxicity (McCormick et al., 2012).

ZEN mycotoxin has been converted to α-ZEL, β-ZEL, ZEN-14G and ZEN-16G by a strain of *Saccharomyces cerevisiae* (Paris et al., 2014). *Clonostachys rosea* is also able to detoxify ZEN by the action of enzyme zearalenone lactonohydrolase (*zhd101*). A dose-dependent induction of *zhd101* gene expression was observed in the presence of ZEN (Kosawang et al., 2014). This enzyme cleaves the ZEN molecule to 1-(3,5-dihydroxy-phenyl)-10′-hydroxy-1′-undecen-6′-one, reducing its toxicity (Kakeya et al., 2002).

The action of the yeast *Saccharomyces pastorianus* on D3G, T2, and HT2 during fermentation reduced toxins by 9–34%. This reduction is associated with both biotransformation and a physical binding on the cell walls. D3G was not reconverted to DON. However, the yeast was able to transform DON into D3G during fermentation. 15-ADON and 3-ADON were also formed from DON. HT2-sulfate, 3-acetyl-HT2, and T2 appeared in the assays containing HT2. HT2 and 3-acetyl-T2 were the metabolites identified throughout the fermentation process containing T2. Acetylation of DON, HT2, and T2 was the main biotransformation route. This alteration may affect the ability of the toxin to inhibit protein synthesis (Wu et al., 2013). However, deacetylation may also be responsible for the modification of free mycotoxins, such as the conversion of T2 to HT2 by the loss of the C-4 acetyl group (Nathanail et al., 2016).

2.2.2. Modified mycotoxin by filamentous fungi

The fungal metabolism is also able to produce and excrete some modified mycotoxin, such as 3-ADON and 15-ADON, found in food contaminated by species of the genera *Fusarium*. Those metabolites are formed from 3,15-diacetyl deoxynivalenol and are DON biosynthetic precursors from their deacetylation (Leonard and Bushnell, 2004).

Besides these, some fungi that do not produce mycotoxins can modify the parent mycotoxin resulting in conjugates glucosides and sulfates through ZEN metabolization, such as the formation of ZEN glucosides by *Rhizopus* and *Thamnidium* species, and ZEN sulfates by *Fusarium* spp. and *Aspergillus* spp. (Jard et al., 2010; Plasencia and Mirocha, 1991; El-Sharkawy, 1989; Kamimura, 1986). These conjugates can be reconverted to ZEN through the action of enzymes (sulfatases) or chemical hydrolyzes. Therefore, both industrial processing and the digestive process of animals and humans can lead to the release of the ZEN molecule, which may have health effects, such as its estrogenic activity (Berthiller et al., 2009a; Plasencia and Mirocha, 1991).

Rhizopus spp. and *Aspergillus oryzae* strains proved able to metabolize ZEN to conjugated derivatives (ZEN-14-Sulfate, α-ZEL-Sulfate, ZEN-16G, ZEN-14G, and α-ZEL). Almost all of the initial ZEN concentration was converted after three days of incubation. However, the adsorption of ZEN by the fungal cell wall should be considered. It is estimated that 20% of the toxin reduction is due to adsorption. Therefore, the reduced levels of the toxin are due to both adsorption on the microorganism cell wall and the transformation process of the parent mycotoxin into its derivatives (Brodehl et al., 2014). Similar results were found by Varga et al. (2005) in evaluating the conversion of ZEN to modified mycotoxin by species of the genera *Rhizopus*.

α-ZEL and β-ZEL are also natural metabolites of fungal species. Although they are considered to be metabolites from the defense mechanism of the microorganisms and the β-ZEL derivative has lower toxicity as compared with ZEN, α-ZEL demonstrated tenfold higher toxicity than the parent mycotoxin (Metzler et al., 2010).

Another conjugate, 15-Monoacetoxyscirpenol 4-α-glucopyranoside, also seems to be produced by fungal metabolism. The species *Fusarium sulphureum* demonstrated the ability to produce it, but its toxic effects are not yet known (Gorst-Allman et al., 1985).

Besides, some fungal species have shown to be able to convert the modified mycotoxins to free mycotoxin through their enzymatic complex. Cellulase, produced by *Trichoderma* species, and cellobiase, by *Aspergillus* spp., demonstrated the ability of enzymatic hydrolysis of D3G with the release of a glucose molecule and DON (Berthiller et al., 2011). β-glucosidase from *Aspergillus niger* and *Phanerochaete chrysosporium* were also able to hydrolyze D3G (Michlmayr et al., 2015). Nevertheless, the degradation of conjugates with glutathione did not lead to the parent mycotoxin again, resulting in other degradation compounds. However, conjugations with epoxides, lactones or aldehyde groups seem not to be hydrolyzed and, therefore, are irreversible (Berthiller et al., 2013).

2.2.3. Modified mycotoxin by bacteria

An aerobic bacterium (*Marmoricola* sp.), previously isolated from wheat grain, was tested as a decontamination agent of DON in preharvest grains affected by FHB. A considerable reduction was detected, possibly due to the tolerance of the microorganism to the environment and its ability to metabolize DON. However, metabolites formed from detoxification have not been identified (Ito et al., 2012).

Microorganisms belonging to the genera *Acinetobacter, Leadbetterella,* and *Gemmata* present in plant and soil samples were indicated as responsible for converting DON to 3-keto-4-deoxynivalenol. When incubating these cultures with wheat contaminated with DON, 3-epimer-DON derivate was also observed (Wilson et al., 2017), which is possibly less toxic (He et al., 2015), although still maintaining the epoxide ring, considered as the group responsible for the toxicity of the molecule (Ikunaga et al., 2011).

In contrast, the reconversion of modified mycotoxin to parent mycotoxin is also observed. D3G, a derivative of DON, was hydrolyzed by β-glucosidase of *Lactobacillus brevis* and *Bifidobacterium adolescentis* (Michlmayr et al., 2015; Berthiller et al., 2011).

The β-glucosidase of *Bifidobacterium adolescentis* was also able to act on glucosides of DON (D3G), nivalenol (nivalenol-3-glucoside (NIV-3G)) and HT2 (HT2-3G) present in samples of cereals and beers, releasing their parent mycotoxin. Therefore, despite the beneficial effects on health due to the use of this microorganism as probiotics in food, the presence of these can also increase the bioavailability of metabolites with toxic effects for humans and animals, such as mycotoxin (Michlmayr et al., 2015).

2.3. Modified mycotoxin by processing

Although mycotoxins are considered stable to processing, they may be partially removed or modified through physical processes such as thermal treatment, peeling and milling; chemical processes such as alkali and acid treatment; and biological processes such as fermentation. However, these modifications are not entirely clear (Suman and Generotti, 2015; Berthiller et al., 2013; Bullerman and Bianchini, 2007; Kuiper-Goodman, 2004).

The reduction of parent mycotoxin levels during the processing is partly due to physical removal, degradation, its transformation into new forms, or its association with food components. Therefore, the reduction of free mycotoxin levels does not indicate a decrease in total levels of mycotoxin and cannot be related to risk mitigation of processed food intake (Humpf and Voss, 2004).

2.3.1. Modified fumonisins

In chemical treatments using alkali, there is hydrolysis of fumonisins resulting in hydrolyzed fumonisins (HFB) (Humpf and Voss, 2004). However, studies on the fate of fumonisins over thermal and alkali processes may show an underestimation of levels due to lower recoveries of analytical methods for the determination of modified mycotoxins (De Girolamo et al., 2001). Furthermore, the toxic effects of HFB are not yet fully understood, although studies indicate a reduction in toxicity after hydrolysis (Norred et al., 1997; Gelderblom et al., 1993).

Besides hydrolysis, the modification of this toxin has been explained by the formation of covalent bonds via the side chains of fumonisin tricarballylic acid and the hydroxyl group of sugars or amino and sulfidric groups of proteins (Dall'Asta et al., 2009).

Bonds between the amine group of fumonisin B1 (FB1) and reducing sugar molecules of the food can also occur during thermal processes, resulting in derived compounds such as N-(carboxymethyl) fumonisin B1 (Howard et al., 1998). However, little is known about the toxicity of N-substituted fumonisins, although some studies indicate a reduction in toxicity due to blocking of the amino groups, now bound to a sugar molecule. However, another derivative, N-Acyl HFB1, showed greater toxicity than HFB1 to cells (Desai et al., 2002; Humpf et al., 1998). Therefore, further studies are required to determine the conditions for the formation of these compounds and their toxicity.

The type of process used in the production of the final product can also directly influence total levels of fumonisins. Thermally processed foods, such as corn flakes, tend to contain bound fumonisins, while derived foods processed with alkali and thermally, such as snacks, tend to contain low contamination. This event may be related to the bond between fumonisin and fatty acids, which causes the mycotoxin to be extracted into the cooking oil, resulting in low contamination of the final product (Park et al., 2004).

2.3.2. Modified trichothecene

The simulation of food components using α-D-glucose as sugar model, methyl-α-D-glucopyranoside as starch model and amino acid derivatives N-α-acetyl-L-lysine methyl ester and BOC-L-cysteine methyl

ester as protein model, was performed to evaluate the thermal degradation of mycotoxin produced by fungi of the genera *Fusarium*. All models resulted in the reduction of DON, but the greatest degradation was observed in the model containing N-α-acetyl-L-lysine methyl ester at 150 °C for 10 min. Moreover, only this model showed appreciable amounts of three degradation products. This degradation possibly occurs due to alkaline catalysis by the ϵ-amino group of the model compound, because DON is unstable to alkaline hydrolysis. This reaction can promote the opening of the 12,13-epoxy group. However, it is possible that pyrolysis or polymerization reactions are also responsible for the reduction in DON levels (Bretz et al., 2006).

Throughout the production of bakery products, changes in the parent mycotoxin and modified mycotoxin levels are observed, although the results are still controversial and further studies are needed. The milling process to produce flour is known to reduce the DON levels in wheat products through physical removal, due to the separation of the outer layers of the grain. A non-homogenous distribution of toxins is observed, and an increased concentration of modified mycotoxin is present in the bran (Kostelanska et al., 2011a).

During the production of cookies, DON levels were shown to be affected by the cooking time, roasting time and temperature. On the other hand, D3G concentrations increased up to 48% after fermentation, followed by reduction after cooking and roasting. These results indicate a possible conversion of DON to D3G by microorganisms used in the fermentation, and a possible degradation of the toxin by thermal processing (Generotti et al., 2015).

In contrast, during the baking, DON levels were not significantly influenced by the dough fermentation process. An early decrease in D3G levels (13%) was observed after kneading the dough and an increase of only 8% after fermentation (Kostelanska et al., 2011a).

Baking in the oven helped reduce both DON and D3G. It is possible that DON has been degraded in NorDON A, B, and C, and D3G in norD3G A, B, C, D, and D3G-lactone, since these compounds were detected in bread after thermal treatment. When the D3G standard was submitted to thermal treatment, the degradation products NorDON A, B, and C were also detected. These results indicate that although D3G is formed along the bread processing, the bond between glucose and DON, the constituent of the D3G molecule, can be hydrolyzed during the thermal treatment, resulting in low concentrations in the final product. Furthermore, other transformations may also occur resulting in degradation products and other modified mycotoxins (Kostelanska et al., 2011a).

Bakery improvers also influence DON and D3G levels. The D3G levels in bread containing bakery improvers were higher as compared with levels present in the non-fortified loaves of bread, although there was thermal degradation in the cooking process. These improvers contained in their composition glycolytic enzymes which may have aided in D3G separation from the polysaccharides present in the bread. However, it is possible that such enzymes only act in the hydrolysis of the bond between polysaccharides and D3G and not between glucose and DON (Kostelanska et al., 2011a).

Enzymes can also act directly on the D3G molecule, reconverting it to the parent mycotoxin DON. The addition of enzymes during the baking process demonstrated a 15% increase of DON in the fermentation and 35% during baking when xylanase was added. This enzyme releases the DON molecules that are bound to the walls of cereals through hydrolysis. The addition of α-amylase also caused an increase in the concentration of DON due to hydrolysis of DON molecules bound to the starch from the dough. The use of cellulases, proteases and glucose oxidases can also promote an increase in the concentration of DON in the fermentation stage. However, lipase did not affect DON concentration, most likely due to a low DON interaction with lipids present in the food. In some cases, DON content in the final product was greater than in the flour, which indicates an additional risk to products manufactured from contaminated raw materials and underreporting of total levels of mycotoxin in the final product (Vidal et al., 2016).

Opposite results were found for D3G levels. After fermentation, both the dough with added enzyme and the control had a lower D3G level. However, in the dough added with glucose oxidase, an increase in the D3G level was observed. After heating, an increase was also seen in the dough containing xylanase, α-amylase, cellulase, and lipase. It is possible that D3G bound to flour components were released during cooking with the aid of the enzymatic activity (Vidal et al., 2016).

According to Wu et al. (2017), the rise in temperature and the cooking time will directly influence the reduction of DON levels; however, the initial increase in temperature may activate enzymes present in the raw material capable of transforming DON in D3G or release D3G that is bound to oligosaccharides. Nevertheless, data on the influence of fermentation on D3G formation are still controversial. Furthermore, its concentration seems to increase in processes with milder temperatures for a short period, while a decrease is observed in more drastic processes.

The formation of modified mycotoxin also occurs throughout the production of beers, but the concentration of this mycotoxin in the beer was difficult to predict because it depends on the initial concentration contained in the raw material and the stages of the production process (Bullerman and Bianchini, 2007).

During the production process of beer, an increase of 880 and 630% in D3G level in the malt and beer was observed, respectively, compared with the initial D3G concentration detected in barley. According to Lancova et al. (2008), this increase may be related to the occurrence of enzymatic activity during the germination of barley grains, wherein the enzymes would release the mycotoxins bound to polysaccharides resulting in new metabolites such as D3G, a similar process that occurs along the baking.

A considerable increase in the concentration of DON and D3G along the production of beers was also detected by analyzing samples of malt, sweet wort and beer. An increase of up to 530, 650 and 210% of DON, ADONs, and D3G, respectively, were detected (Kostelanska et al., 2009).

In a similar study, higher levels of deoxynivalenol oligo-glucosides were detected during malting and production of sweet wort. Such contamination is explained by the enzymatic activity present in these stages, which in addition to promoting the hydrolysis of conjugated DON bound to the starch and dextrin of cereals, also allows DON glycosylation with glucose forming deoxynivalenol oligo-glucosides. An increase of DON during the malting process was also observed. The authors suggest that the conditions of this stage are favorable for the development of toxigenic fungi and production of mycotoxins, with the possible synthesis of new molecules of DON and therefore an increase in their concentration (Zachariasova et al., 2012). However, it is also possible that the enzymes present in this stage can have released the DON from their glucosides.

D3G concentration was higher compared with DON levels in all raw malt. However, none of these toxins were detected in roasted malt. Possibly along the roasting, the toxins have been degraded to other compounds not detectable by the testing methodology. D3G levels were also higher in all mix-malts samples and beers analyzed. However, ADONs were detected in only 63% of the intermediate liquid products. A considerable increase in the levels of DON and D3G in beers as compared with malt was also observed in this study. This increase may be related to the milling stage that takes place in aqueous conditions, and at a temperature of about 40 °C. This procedure may facilitate an additional release of non-extractable mycotoxins that may be bound to the food matrix and had not been previously detected (Kostelanska et al., 2011b).

Alcohol content also seems to be a factor that influences the mycotoxin concentrations in beers. In commercial beer samples, 74% were positive for the presence of D3G, while 64% for DON. A higher concentration of DON and D3G was detected in beers with higher alcohol content, probably due to a greater amount of wort in the fermentation required to achieve higher levels of alcohol. On the other hand, the non-

alcoholic beers contain low concentrations of mycotoxins, indicating a low release of toxins contained in the raw material to the final product, due to the absence of alcohol. Alcohol may work as an extractor of the mycotoxins adsorbed in yeast cells and strongly bound to the raw material (Kostelanska et al., 2009). However, this correlation was not found by Kostelanska et al. (2011b).

Different types of beer (pale, wheat, dark, bock, non-alcoholic beer and shandy) from 38 countries were evaluated for the presence of DON, D3G, and 3-ADON. No samples contained 3-ADON, 93% contained D3G and 77% contained DON, though at low levels averaging 6.9 μg/L and 8.4 μg/L, respectively. A positive correlation between alcohol content and mycotoxin levels was also found in this study. However, it was not possible to determine a relationship between the different types of beer and mycotoxin levels. Additionally, it was also not possible to find a correlation between DON and D3G levels, preventing a prediction of modified mycotoxins levels (D3G) from levels of parent mycotoxin (DON) (Varga et al., 2013). Differences in the technology used in the beers production and quality of raw material are also factors that will influence the levels of mycotoxins in beer (Kostelanska et al., 2009).

Drastic thermal treatment in potato inoculated with *Fusarium sambucinum* was effective in reducing another trichothecene, 4,15-diacetoxyscirpenol, and its conversion to the derivative DAS-M1. The thermal process may aid in reducing trichothecene toxicity since *in vitro* and *in vivo* studies show a lower toxicity of DAS-M1 as compared with the parent mycotoxin. However, it takes long cooking times, which may considerably affect the organoleptic characteristics of the product. Therefore, such processes are not suitable as strategies to reduce the total toxicity of the food (Shams et al., 2011).

2.3.3. Modified OTA

Ochratoxin α amide, a thermal degradation product of OTA, was not observed after the extrusion process of wheat-based products, demonstrating the stability of the toxin in processes with temperature rise to 180 °C. However, the degradation of OTA to ochratoxin α amide may occur during heating at 240 °C (Bittner et al., 2015). Besides to the formation of ochratoxin α amide, 14-(R)-ochratoxin A, 14-decarboxy-ochratoxin A, and ochratoxin α were detected as well in the products after the thermal treatment, such as roasting coffee (Bittner et al., 2015; Cramer et al., 2008).

However, low levels of degradation compounds found in previous studies do not explain the high reduction of OTA after the coffee roasting. Therefore, a similar study was carried out by Bittner et al. (2013) to clarify if the OTA is degraded during the roasting process or binds to food components. Ochratoxin A mono- and disaccharide esters were detected, confirming the bonding ability between the carboxyl group of ochratoxin A and the primary hydroxyl group of the carbohydrates of coffee during roasting.

Interestingly, higher levels of OTA were detected following preparation of the drink in comparison with roasted coffee, which can be associated with a possible cleavage of the bond between the carbohydrate and OTA and release of the toxin throughout the infusion (Studer-Rohr et al., 1995). Furthermore, due to the higher solubility of the grain carbohydrates after roasting (due to hydrolysis of polysaccharides), saccharide esters of OTA can also be released in the infusion, increasing the level of toxin present. However, the toxic effects of these derivatives have not been reported yet (BITTNER et al., 2013).

In addition to the processing conditions, factors such as the level of contamination of raw materials, the variety and the physicochemical characteristics of the matrix and the environmental conditions will also affect the levels of modified mycotoxins present in the food and its derivatives (Zachariasova et al., 2012).

2.4. Modified mycotoxin: effects on health

The toxicity of the modified mycotoxin to humans and animals is still unknown. However, there is a potential risk of release of parent mycotoxins by hydrolysis during food processing and digestion in human and animal organisms (Berthiller et al., 2013; Bowles et al., 2006). Due to this risk, The Panel on Contaminants in the Food Chain of the European Food Safety Authority decided that during risk assessment, the modified mycotoxin must be considered with the same toxicity of its parent mycotoxin (EFSA, 2014).

In addition, the classification of derivatives formed in modified mycotoxins or in detoxification products is only possible through studies that decipher their destination along food processing and digestion, thus attesting the release, or not, of the parent mycotoxin and the reduction of the toxicity of the formed compound (Berthiller et al., 2013).

2.4.1. In vitro and in vivo modified mycotoxin metabolism

Fumonisins present low absorption and bioavailability in the organism, despite this, studies show toxic effects after food intake even with low levels of this toxin, which suggests the contribution of fumonisins derivatives in these effects. Probably, this mycotoxin binds itself to carbohydrates or proteins from food during thermal processes and is reconverted to the parent mycotoxin after ingestion (Berthiller et al., 2013; Dall'Asta et al., 2008).

In vitro digestion of raw corn samples contaminated by fumonisins was performed by Dall'Asta et al. (2009) with the aim of finding out if the fumonisins are bound to proteins via covalent bonds or by complexation since the free fumonisins can only be released by complexation. The concentration of free fumonisins, after digestion, had an increase of 30–50% as compared to the initial levels detected in corn. These results indicate that the binding mechanism of the fumonisins is due mainly to the association with sugars and proteins since the release of the parent mycotoxin occurred along the digestion.

The modified trichothecenes (D3G, T-2 toxin-glucoside (T2-3G) and NIV-3G) and modified ZEN (ZEN-14G, α-zearalenol-14-glucoside (α-ZEL-14G) and β-zearalenol-14-glucoside (β-ZEL-14G)) showed to be stable to hydrolysis by the artificial digestive juice. Epithelial cells also did not demonstrate *in vitro* the ability to perform the hydrolysis of these compounds (Gratz et al., 2017).

McCormick et al. (2015) performed an artificial test simulating digestion to evaluate the behavior of T-2 toxin-glucosides anomers. They found that T-2 toxin α-glucoside and T-2 toxin β-glucoside remained unchanged after incubation with artificial human saliva. Similar results were found by Dall-Erta et al. (2013) when assessing the behavior of glucosides of DON and ZEN in digestion. These results suggest that, if the modified mycotoxin are reconverted into the parent mycotoxin, this reconversion will most probably happen in other steps of digestion, such as in the stomach or intestine.

This hypothesis was proven by Berthiller et al. (2011) when evaluating the stability of D3G on contact with hydrochloric acid, artificial stomach juice, artificial intestine juice, enzymes, and intestinal bacteria. D3G showed to be stable to hydrochloric acid and artificial stomach juice containing pepsin. The enzyme β-glucosidase and the artificial intestine juice containing amylase also did not show any activity on the toxin under the tested conditions. A partial conversion of D3G to DON, on the other hand, was observed in the presence of cellulase, an enzyme present in the intestine of ruminants.

Additionally, *B. bifidum*, *B. longum*, *C. freundii*, *E. avium*, *E. coli*, *L. amylovorus*, *L. crispatus*, *L. fermentum*, *L. gasseri*, *L. paracasei* and *L. rhamnosus* were unable to convert D3G to DON. As for *E. casseliflavus*, *E. faecalis*, and *E. gallinarum,* they showed little conversion ability. However, *E. cloacae*, *E. durans*, *E. faecium*, *E. mundtii*, *L. plantarum* and *B. adolescentis* showed efficient conversion. These microorganisms can act synergistically in the gastrointestinal tract of animals and humans by increasing the levels of DON. Besides, the consumption of fermented products containing probiotics can also assist in this conversion (Berthiller et al., 2011).

Therefore, despite some modified mycotoxin not being affected by stomach conditions, in the intestine, lactic acid bacteria can cleave the bond between sugar or protein molecules and toxin molecules,

reconverting the mycotoxin to its free form, and thus returning its toxicity. Due to the possibility of absorption of part of the parent mycotoxins in the intestine, such cleavage should be considered in toxicity studies (Dall-Erta et al., 2013; Berthiller et al., 2011).

The metabolism of the T-2 toxin-α-glucoside, T-2 toxin-β-glucoside, and T2 by bacteria found in human feces was also assessed. T2 and T2 glucosides were degraded significantly after 24 h, being T2 transformed, mainly HT2 (83%). Only 30% of the initial content of T-2 toxin-α-glucoside and T-2 toxin-β-glucoside were detected after 24 h. The concentrations of compounds generated after the degradation of the α and β forms were different. T-2 toxin α-glucoside was converted to T2 (13%), HT2 (30%) and in other non-elucidated metabolites (33%). While T-2 toxin-β-glucoside was degraded in T2 (58%) and HT2 (12%), which may indicate a greater toxic potential of T-2 toxin-β-glucoside, since there was a greater conversion to T2. These results suggest a risk of underestimation of the total levels of T2 and the need to monitor their conjugated forms in foods (McCormick et al., 2015).

The hydrolysis of D3G, T2-3G, ZEN-14G, α-ZEL-14G, β-ZEL-14G, and NIV-3G by human fecal microbiota was also observed (Gratz et al., 2017). Some bacteria present in animal and human microbiota are relevant in the reconversion of modified mycotoxins to the parent mycotoxin due to the production of hydrolytic enzymes capable of cleaving the bonds between glucosides and the toxin. Species such as *Sphingomonas paucimobilis* (Marques et al., 2003) and *Oenococcus oeni* (Michlmayr, 2010) produce β-glucosidase that have such hydrolysis ability and can enable the conversion along the digestion (Paris et al., 2014).

In addition to the conversion of modified mycotoxin to the parent mycotoxin by intestinal microbiota, bovine plasma, fetal bovine serum (FBS), whole blood, and serum albumin also showed the ability to hydrolyze ZEN-14G to ZEN, besides biotransformation to α-ZEL and β-ZEL. Proteins present with (pseudo) enzyme activity are possibly responsible for hydrolysis (Dellafiora et al., 2017). Despite the liver being considered the main responsible for the bioactivation of ZEN by the metabolism of several species (Malekinejad et al., 2006), the systemic circulation can also be a possible contributor to the high levels of ZEN in the organism.

Absorption and hydrolysis of 3-ADON and 15-ADON to DON were observed in swine and chickens (Broekaert et al., 2015b). In contrast, the hydrolysis of D3G to DON was not observed in broiler chickens. As for swine plasma, an extensive pre-systemic hydrolysis of D3G to DON was observed, however, with greater absorption of DON. This hydrolysis demonstrates the need of investigating the levels of modified DON contained in foods, since, despite having a lower toxicity, it can increase the levels of DON in the organism and, consequently, its toxic effects. In addition, differences in metabolization and absorption among species must be taken into account (Broekaert et al., 2017).

The transformation of ZEN to α-ZEL can occur along the metabolism of animals and humans, considering that this derivative has an estrogenic activity three to four times greater than the parent mycotoxin (Wang et al., 2014). Furthermore, conjugated forms of ZEN and α-ZEN with Allyl isothiocyanate, a phytochemical found in vegetables, recognized for reducing mycotoxin levels by the formation of adducts between the nucleophile groups of the mycotoxin with the central carbon of isothiocyanates, demonstrated less bioaccessibility when present at higher levels (30 μM), however, at lower levels (15 μM) the bioaccessibility of the parent mycotoxin and its modified mycotoxin proved to be similar. The bioavailability of parents mycotoxins, on the other hand, was lower than their respective conjugated. Therefore, studies on the toxic effects of these conjugated forms have yet to be carried out (Bordin et al., 2017).

For an effective monitoring of total levels of mycotoxins and their effects on health, detection and quantification of the sum of free mycotoxins and modified mycotoxins in fluids like urine and feces are necessary (Ediagea et al., 2012).

DON, deoxynivalenol-glucuronide (DON-GlcA) and de-epoxy deoxynivalenol (DOM-1) were detected in the urine of rats treated with DON. DON-GlcA was the main excreted metabolite. While, when administering D3G, DON, DON-GlcA, DOM-1, and D3G were detected in the urine. These findings demonstrate that DON is formed from the hydrolysis of D3G, absorbed, and then metabolized to DON-GlcA. However, in feces, DON-GlcA was not detected, probably due to the action of bacterial glucuronidase in intestine, acting in the cleaving of the molecule. After treatment with DON, only DON and DOM-1 were detected in feces. The formation of DOM-1 is due to the action of bacteria of the intestinal microbiota. The feces of animals treated with D3G contained these same metabolites, whereas only traces of D3G were detected. Therefore, this metabolite presents lesser equivalent toxicity when compared with DON, since most of this derivative is cleaved to DON and excreted in feces (Nagl et al., 2012).

This same study was conducted with pigs. By treating the animals with DON; DON and the derivatives deoxynivalenol-3-glucuronide (DON-3-GlcA) and deoxynivalenol-15-glucuronide (DON-15-GlcA) were detected in the urine. While, by administering D3G; DON, DON-3-GlcA, DON-15-GlcA, and DOM-1 were found. In the feces, only DOM-1 was found when D3G was administered (Nagl et al., 2014).

In samples of rat feces and urine, previously treated with DON and D3G, sulfonates of DON, DOM, and D3G detected in feces accounted for approximately 50% of the total quantity of DON or D3G administered, while in the urine the concentration of sulfonates derivatives was < 1%. These results indicate a low absorption of these metabolites in the digestive tract of rats (Schwartz-Zimmermann et al., 2014).

ZEN-14G, ZEN-16G, and ZEN-14-sulfate were hydrolyzed to ZEN and converted to other unidentified metabolites along the digestion of pigs and excreted in the urine and feces. In addition to the species' influence and route of administration of the mycotoxin, variations between the individual's state and its digestion shall affect the metabolism of the mycotoxin. These data indicate the contribution of modified ZEN to the general toxicity of ZEN, demonstrating the need for inclusion of these derivatives in risk analysis and determination of a tolerable limit of total ZEN, including free and modified mycotoxin (Binder et al., 2017).

Modified trichothecenes and modified ZEN were also not efficiently transported through the intestinal epithelial monolayers in an *in vitro* study, showing low absorption of these compounds. Therefore, the greatest risk from the presence of these compounds is due to their reconversion to the parent mycotoxin by intestinal microbiota (Gratz et al., 2017).

Free mycotoxin DON, OTA, ZEN and citrinin and the metabolized products: ochratoxin α, 4-hydroxyochratoxin A, and β-ZEL were identified in urine samples. These metabolites co-occurred with the free mycotoxin, being higher in some cases, demonstrating they can be possible forms of detoxification and used as biomarkers (Ediagea et al., 2012).

Reactions between patulin and proteins (Fliege and Metzler, 1999) or glutathione (Fliege and Metzler, 2000), are considered the most likely form of detoxification in humans or animals after consumption of contaminated apples. OTA conjugation with glutathione (Dai et al., 2002) and cysteine (Brown et al., 2002) have also been demonstrated.

Although not considered modified mycotoxins, since these forms will hardly be present in food, these conjugates formed along the digestion can be used as biomarkers in risk assessments of mycotoxins intake, regardless of their source and way of consumption (Berthiller et al., 2009a,b).

The biotransformation of T2 to modified T2, thereby increasing its solubility, facilitating its excretion, and, therefore, reducing its toxicity, was observed in animal tissues. However, although modified T-2 showed a reduction in immune toxicity, it is possible that these compounds are reconverted into the parent mycotoxin, thus returning their toxicity. Besides, there is a hypothesis that, if these tissues, containing modified T2, are consumed by other animals, the parent mycotoxin would be freed through hydrolysis along digestion. These data,

Table 3
Cytotoxic effects of modified mycotoxins.

Modified mycotoxin	Cell line/system	Exposure dose	Effects	References
norDON A, B, and C	IHKE	100 μM	no toxic effects	Bretz et al. (2006)
15-ADON	GES-1 cells	0.5 ppm	reduced the viability of the cell	Yang et al. (2017)
3-ADON	GES-1 cells	12 ppm	low reduction of cell viability	Yang et al. (2017)
D3G	GES-1 cells	12 ppm	not reduce cell viability	Yang et al. (2017)
DOM-3-sulfate	animal ribosomes	100 μM	not inhibit an *in vitro* translation assay	Schwartz-Zimmermann et al. (2015)
DOM-15-sulfate	animal ribosomes	100 μM	not inhibit an *in vitro* translation assay	Schwartz-Zimmermann et al. (2015)
14-(R)-ochratoxin	IHKE	50 nM	cytotoxic and apoptotic effects	Cramer et al. (2008)
14-decarboxy-ochratoxin A	IHKE	10 μM	not cytotoxic effects	Cramer et al. (2008)
14-(R)-ochratoxin	IHKE	50 μM	cytotoxic effects	Bittner et al. (2015)
ochratoxin α amide	IHKE	50 μM	not reduce cell viability	Bittner et al. (2015)
ochratoxin α	IHKE	50 μM	not reduce cell viability	Bittner et al. (2015)

however, have not been published by the authors (Wang et al., 2017).

Despite the low toxicity of some substances that have been investigated so far, their destination along the metabolism should be investigated more thoroughly to assess the impact of modified mycotoxins in risk assessment of mycotoxins and aid in the definition of tolerable limits of modified mycotoxins in food by government agencies.

2.4.2. Cytotoxic effects of modified mycotoxins

The formation of degradation compounds of mycotoxins, still in the plant, throughout processing or during digestion can lead to a reduction of the general toxicity of the food (Table 3). The lack of cytotoxicity of DON degradation products may be related to the absence of the epoxy group in derived compounds, since it exercises a major role in the toxicity of DON (Bretz et al., 2006). This group allows the toxin to connect to ribosomes, preventing them from forming peptide bonds, thus affecting protein synthesis (Broekaert et al., 2017; Pestka, 2008).

Another derivative, DOM-1 can be formed from de-epoxidation of DON by bacteria from the intestinal microbiota. However, de-epoxidation of DON-3-sulfate or sulfation of DOM can also occur. The reduction of toxicity of these molecules relative to parent mycotoxin indicate the de-epoxidation of DON as a strategy for reducing the toxicity of DON (Schwartz-Zimmermann et al., 2015). The absence of cytotoxic effects of DOM was confirmed by Springler et al. (2017).

Human Caco-2 cells showed to have no capacity for absorbing D3G. Therefore, their bioavailability is probably low when compared with the free mycotoxin (DON) (De Nijs et al., 2012). The same modified mycotoxin showed no interaction with the ribosomes and did not activate MAPK (mitogen-activated protein kinases) due to the presence of glucoside in the molecule. They also did not reduce cell viability and the barrier function of human intestinal cells, as well as not causing histological and functional changes in intestinal explants. The profile of gene expression was also not changed. These events are considered as toxicity factors of DON. Most likely D3G has less ability to enter cells by diffusion or by carriers due to the higher molecular weight and polarity of its molecule (Pierron et al., 2016).

Numerous toxicity studies have shown a considerable reduction of the toxic effects caused by modified DON when compared with the parent mycotoxin (DON) (Gratz, 2017). Therefore, the biggest problem of the incidence of these derivatives in food is their re-conversion in free mycotoxin (DON) along digestion (Pierron et al., 2016).

OTA thermal degradation products may also be considered possible forms of detoxification of the parent mycotoxin due to the reduction of the cytotoxic and apoptotic effects observed in epithelial cells of the human kidney. The degradation product 14-(R)-ochratoxin A was toxic to IHKE cells, however, such toxicity is lower when compared to the effects caused by 14-(S)-ochratoxin A. Levels of 14-(R)-ochratoxin ten times higher are necessary for it to have toxic effects similar to those caused by 14-(S)-ochratoxin A. As for, 14-decarboxy-ochratoxin A, it did not present cytotoxic effects. Also, for 14-(R)-ochratoxin A to have an apoptosis potential comparable to 14-(S)-ochratoxin A, it is necessary for it to be present at concentrations 200 times higher. For 14-decarboxy-ochratoxin A, a 1000 times larger quantity is needed to produce comparable effects (Cramer et al., 2008).

The degradation of OTA to ochratoxin α amide and ochratoxin α may be favorable to the process of detoxification (Bittner et al., 2015). The absence of phenylalanine in these molecules may be responsible for the lack of toxicity (Cramer et al., 2010). However, the natural occurrence of the modified ochratoxin A in foods is not yet determined. Studies on its reconversion to OTA along digestion are needed since, if there is such a phenomenon, an increase in toxic effects caused by ingestion of contaminated food must occur (Berthiller et al., 2013).

The total toxicity of the compound will depend on the rate of release of the matrix after ingestion if it is reconverted to the parent mycotoxin or transformed into other compounds along digestion, and what is its rate of absorption by the organisms of different species. Furthermore, toxicity seems to be related to the type of effect assessed and to the cell or tissue used in the experiment. Besides, studies of toxicity in cells are not able to predict the biotransformations that can occur after ingestion of the compound (Yang et al., 2017; Rychlik et al., 2014; Berthiller et al., 2013).

However, a precise risk assessment for the modified mycotoxins present in foods is not yet possible due to the lack of data on their exposure and toxic properties (McCormick et al., 2015). Also, the lack of analytical standards also hinders the development of toxicity studies. Further studies are needed to assess the toxicokinetic and toxicodynamics of these compounds, as well as to evaluate the possible synergistic effects of the co-occurrence of free in modified mycotoxin.

2.5. Analytical challenges

Mycotoxins can bind to components of the food or have their structure modified by the process, which hinders their detection by conventional methods used for the quantification of free mycotoxin, thus causing under-reporting and low recovery of the mycotoxin (Dall'Asta et al., 2008; De Girolamo et al., 2001).

When binding covalently to a component of the food matrix, the modified mycotoxins are usually stable under the conditions of extraction used to detect the parent mycotoxin and can be detected by direct analysis. However, they need to be monitored properly (retention time, molecular mass, m/z ratio) not to escape the analysis, since structural change involves changing the chromatographic parameters and even the extraction efficiency. However, some authors indicate that these forms can be lost along the analysis if a proper cleavage through chemical or enzymatic hydrolysis is not carried out. Besides, various toxins can bind to the food's macromolecules through non-covalent bonds. These, on their turn, can be disrupted in the extraction process originating the parent mycotoxin again, thus requiring greater attention in the steps of extraction and cleanup. Besides, insoluble structures cannot be detected without a treatment that makes them soluble and facilitates their extraction. Therefore, different extraction methods can influence the recovery of the analyte and detected contamination levels,

including underreporting the total mycotoxins or overestimation of the parent mycotoxin (Berthiller et al., 2009a,b, 2013; Cirlini et al., 2012; Dall'Asta et al., 2009; Momany et al., 2006).

The technique of liquid chromatography coupled to mass spectrometry (LC-MS/MS) is currently the most used in the detection of modified mycotoxins, due to its high selectivity, sensitivity, and the possibility of structural elucidation, although the matrix effect is still very relevant since normally modified mycotoxins are found in low concentrations. The identification of these compounds occurs through their molecular weight and ions from molecule fragmentation formed by collision-induced dissociation in tandem mass spectrometry (MS/MS), which differ from ions of the parent mycotoxins (Vendl et al., 2009; Berthiller et al., 2005).

The main ionization sources used in high resolution mass spectrometry are electrospray and atmospheric pressure chemical ionization, since the mass analyzers that provide high-resolution data are the combinations (MS/MS) quadrupole-linear ion trap, quadrupole-time-of-flight, and linear ion trap-Fourier transform ion-cyclotron resonance, followed by triple quadrupoles and ion traps (Cirlini et al., 2012).

Allied to this technique, the use of isotopic patterns allowed us to track the fate of the toxin and formation of modified mycotoxins over processing and metabolism of plants, human and animal (Kluger et al., 2015; Baillie, 1981).

Studies have employed indirect methods to detect and quantify modified mycotoxins. In these, extractable and non-extractable forms were detected by conversion of non-extractable forms in the original toxin, through chemical or enzymatic hydrolysis. Quantification was made by the difference between total mycotoxin and free mycotoxin (Malachová et al., 2015). To this end, it is not necessary to use analytical standards, being possible to quantify the total of mycotoxins present (Berthiller et al., 2009a,b). However, it is not feasible to know the contribution of each modified mycotoxin, although such information is quite relevant as these metabolites have different toxic effects.

Shier et al. (1997), when assessing corn flour contaminated by radiolabeled, detected only 37% of the added toxin. When using a detergent solution (sodium dodecyl sulfate) to assist in the extraction, additional 46% of the mycotoxin was recovered. Probably, the fumonisin was bound to proteins or polysaccharides of the food matrix, or its structure was modified, and thus could not be recovered by the traditional method, being necessary to employ detergent in the extraction, due to its properties of solubilizing proteins (Park et al., 2004; Seefelder et al., 2003).

The stability of fumonisins B1 and B2 and formation of hidden fumonisins in corn during the ensiling process were also assessed by alkaline hydrolysis and high-performance liquid chromatography coupled to a mass spectrometer with ionization source heated electrospray ionization and triple quadrupole analyzer. All samples contained higher levels of hidden fumonisins than free fumonisins. As the corn did not undergo any processing, the modified mycotoxins were possibly associated with macromolecular components of the matrix and not covalently bound, thus being possible to determine them after alkaline hydrolysis (Latorre et al., 2015).

A predictive model to estimate levels of total fumonisins (free fumonisins and hidden fumonisins) in corn was proposed by Oliveira et al. (2015). The samples were subjected to alkaline hydrolysis for detection of HFB1 and HFB2 through LC-MS/MS. Hidden fumonisins levels were calculated by the difference between total hydrolyzed fumonisins (HFB1 + HFB2) and free fumonisins. All samples showed higher levels of total fumonisins when compared with free fumonisins. Additionally, a positive correlation between the concentrations of free fumonisins and total fumonisins was found, i.e., the higher the levels of free fumonisins, the greater the levels of total fumonisins.

Dall'Asta et al. (2008) used alkaline hydrolysis (NaOH) to assist in the detection of hydrolyzed fumonisins in corn-based products. Fumonisins B1, B2, B3, and free and bound hydrolyzed fumonisins (HFB1, HFB2, HFB3) were analyzed by LC-MS/MS equipped with a triple quadrupole analyzer and electrospray ionization source, without purification and pre-concentration steps. The levels of bound fumonisins were close to or higher than the levels of free toxins in all analyzed samples. Also, in samples in which the levels of free fumonisins were lower than the legal limits (800 μg/kg), the sum of the free and conjugated forms was higher, suggesting the underreporting present in the determination of mycotoxins in foods. To carry out the alkaline hydrolysis, the fumonisin molecule loses its tricarballylic acid side chain and, along with it, the conjugate (sugar, protein), releasing HFB1 (Bertillher et al., 2009a,b).

Acid hydrolysis and ELISA method were used in the detection of conjugated DON in corn. Conjugated DON levels were determined by subtracting the quantified DON after hydrolysis by free quantified DON before hydrolysis. After hydrolysis, the average increase in the recovery of DON was of 14% for all samples (Tran et al., 2012).

Dall'Asta et al. (2008) also demonstrated that in an analysis method using immunoassays (ELISA), antibodies against FB1 could also be able to recognize the fumonisins bound to proteins. These data justify the overestimation of the mycotoxin levels, which is usually attributed to the cross reaction of the antibody to the components of the food matrix. The detection of modified mycotoxin by this method occurs only if the epitope of the compound is not involved in conjugation bond of the food molecule (Köppen et al., 2010; Berthiller et al., 2009a,b).

However, the use of immunochemical methods does not allow the proper quantification of the parent mycotoxin and modified mycotoxins, since they typically co-occur in food (Berthiller et al., 2009a,b; Dall'asta et al., 2008). Furthermore, this method can overestimate the parent mycotoxin levels when detecting the modified mycotoxin as the parent mycotoxin. Therefore, it should be used only as a method of initial screening, needing confirmation of positive results by other more selective techniques (Berthiller et al., 2013).

Despite the chemical hydrolysis showing to be effective in the determination of modified fumonisins, this extraction technique does not seem to be effective for detecting other modified mycotoxins. Furthermore, it is not possible to determine hydrolysis efficiency, which can also lead to an underestimation of the total mycotoxin levels. Besides, this technique also makes the structural elucidation of modified mycotoxin impossible.

The use of indirect methods for the detection of modified mycotoxins through acidic and basic hydrolysis was assessed in wheat samples doped with the standard solution 3-ADON, 15-ADON, and D3G. To acid hydrolysis, trichloroacetic acid (TCA), trifluoroacetic acid (TFA), and trifluoromethanesulfonic acid (TFMSA) were used, while for basic hydrolysis, potassium hydroxide (KOH) was used. After hydrolysis of 3-ADON, 15-ADON, and D3G with TCA and TFA, DON was not detected under the tested conditions, indicating that, if these modified mycotoxins were degraded by acid, they were not hydrolyzed to DON. Also, 3-ADON and 15-ADON also showed to be stable to acid hydrolysis, although they were hydrolyzed under basic conditions. Most likely, the increase in the levels of DON after the acid hydrolysis detected in other studies may have been caused by matrix effect and, therefore, causing a difference in the recovery of DON in hydrolyzed and not-hydrolyzed matrices. These results discourage the use of acid and alkaline hydrolysis on the indirect determination of DON. Enzymatic hydrolysis is considered the method with the most promising results. However, direct methods using LC-MS/MS seems the best choice to determine modified mycotoxins (Malachová et al., 2015).

Evidence of the presence of phase II metabolites was observed in the urine of adults and children. However, the direct analysis of these metabolites showed low detection, most likely due to the difficulty of ionization of these molecules and matrix interference, as it is quite complex. The evidence of these phase II metabolites was ensured by the increase in OTA levels detected after enzymatic hydrolysis, with the use of β-glucuronidase. However, in this method, it is not possible to identify the metabolites formed (Muñoz et al., 2017). In contrast, Rodríguez-Carrasco et al. (2014a,b) developed a method using the

solvent extraction at high ionic strength followed by dispersive solid phase extraction for simultaneous determination of 15 mycotoxins and metabolites in human urine, among them, DOM-1, α-ZEL and β-ZEL by GC-MS/MS (gas chromatography tandem mass spectrometry).

The use of enzymes to aid in the stage of extraction of modified ochratoxin A in coffee proved to be suitable and advantageous. Detection of saccharide esters of OTA was carried out through LC-MS/MS. Due to the complexity of the sample, carbohydrolases were used to enhance the cleavage of the glycosidic bonds of present polysaccharides (mannan, arabinogalactan, cellulose, and xylan). Immunoaffinity column was also used for cleanup. Ochratoxin A mono- and disaccharide esters were detected, thus proving the effectiveness of the developed technique (Bittner et al., 2013). However, it is necessary to assess which enzyme can act on the bond in question, being often necessary to use a mix of enzymes so that a greater number of modified mycotoxins is reached and the parent mycotoxin is released (Berthiller et al., 2009a,b).

Simultaneous detection of DON, D3G, 3-ADON, ZEN, ZEN-4-glucoside, α-ZEL, β-ZEL, α-zearalenol-4-glucoside, β-zearalenol-4-glucoside, and ZEN-4-sulfate in cereals and cereal products was performed using LC-MS/MS. For such, SPE cartridges and immunoaffinity SPE columns were tested as cleanup methods. All cleanup methods tested were unsuitable for simultaneous detection of derivatives of DON and ZEN, most likely due to the wide range of polarity of the analytes in question. Therefore, more than one cleanup strategy is needed to remove matrix interference and concentration of the analyte, or the performance of a direct analysis of the extract (Vendl et al., 2009).

Kostelanska et al. (2011b) assessed the DON fate in samples obtained during the main technological steps in the beer production, using ultra high-performance liquid chromatography coupled to time-of-flight mass spectrometry. In this case, the cleanup technique used (immunoaffinity solid phase extraction cleanup) was shown to be suitable for quantification of DON and its derivatives. Rodríguez-Carrasco et al. (2014a,b) also demonstrated the suitability of a QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction and cleanup method for the silmutanea detection of DON and 3-ADON in breadsticks by GC-QqQ-MS/MS (gas chromatography-triple quadrupole tandem mass spectrometry).

Detection in LC-MS/MS of masked mycotoxins (D3G and ZEN-14-sulfate) present in grains were tested using extraction by cleanup column with different solvents. With the utilization of a PEG-based solvent, a recovery rate of 91% passing by the column and 128% not passing by the column was obtained for D3G. Using the solution of water/acetonitrile (20/80; v/v), we obtained recovery rates of 49% and 30% of D3G and ZEN-14-sulfate, respectively. However, for the same solution, but in inverted concentrations (80/20; v/v), only traces of D3G were detected. It is possible that the D3G molecule was retained in the column due to its sugar molecule, as this column has the function of retaining potential interferers to the matrix, such as sugars. Most of the ZEN-14-sulfate was also retained in the column (Maul et al., 2014).

Corroborating to such a finding, the need for direct analysis of ochratoxin α amide was also demonstrated using LC-MS/MS. When using immunoaffinity columns to cleanup, this compound was not detected, while the "dilute and shoot" approach proved satisfactory in the detection of ochratoxin α amide in cereals artificially contaminated with this toxin standard (Bittner et al., 2015).

These results indicate that such cleanup and extraction procedures, even though efficient for the parent mycotoxin, do not work for their derivatives, reinforcing the need for direct analysis or choice of reliable methods for detecting modified mycotoxins. Also, the use of indirect methods (hydrolysis to free mycotoxin) hinders the identification and structural elucidation of the modified mycotoxins.

3. Conclusions

Currently, an ongoing effort by the scientific community to identify and quantify modified mycotoxins in foods can be observed, elucidate their destination in the plant, throughout processing, or by metabolism of microorganisms and animals, in addition to possible effects on health. However, data on the natural occurrence of these compounds in various commodities in different regions of the world are still scarce and make a final risk assessment impossible.

The various processing steps greatly affect mycotoxin levels present in the final product (free and modified), although the results are still controversial regarding the increase or reduction of these levels, being strongly related to the type of process and the composition of the food in question. These changes are linked and are dependent on the initial level of contamination, temperature rise, process time, fermentation steps, use of additives and enzymes, physical and chemical processes (using acids or bases), and composition of the raw material. A raw material recognized as safe can become a final product with high mycotoxin levels (free and modified) due to the possibility of release of these compounds over processing. Therefore, only controlling raw materials is not enough to ensure the toxicological safety of the final product. Studies on the stability and fate of these modified mycotoxins along processing are necessary to estimate the total mycotoxin levels.

Although most of these metabolites are generated from a defense mechanism of the plants or of the metabolism of microorganisms and even animals as an attempt of detoxification of the parent mycotoxin, some metabolites still present toxicity, even if reduced. However, the reconversion to the parent mycotoxin in the gastrointestinal system of animals seems to be the major concern about the presence of modified mycotoxin in foods. Besides, some of these formed compounds can be even more toxic, in case they have higher bioaccessibility and bioavailability than the parent mycotoxin.

The use of the technique of high-performance liquid chromatography coupled to mass spectrometry has been essential to identify new metabolites and quantify the total mycotoxins in foods. New strategies of extraction and cleanup must be developed to detect and identify as many derivatives as possible for a more precise determination of the risk that food can represent. Direct injection techniques and use of enzyme in more complex matrices seem to be the most promising options. In addition, it is necessary to manufacture standards of these newly discovered metabolites, in such a way that, in addition to being identified, they can also be quantified and included in risk analysis. The lack of conclusive toxicokinetic and toxicodynamic studies, in addition to the limited understanding of the transformation mechanisms of mycotoxins also hinder the determination of the maximum tolerable level of these metabolites in food, being a major future challenge.

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CAPÍTULO 2

A quantitative study on growth variability and production of ochratoxin A and its derivatives by *A. carbonarius* and *A. niger* in grape-based medium.

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A quantitative study on growth variability and production of ochratoxin A and its derivatives by *A. carbonarius* and *A. niger* in grape-based medium

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Aspergillus carbonarius **and** ***Aspergillus niger*** **are the main responsible fungi for the accumulation of ochratoxin A (OTA) in wine grapes. Some strains are able to convert the parent mycotoxin into other compounds by means of hydrolysis and/or conjugation reactions through their defense mechanisms and enzymatic activity, leading to the formation of a modified mycotoxin. Thus, the variability of growth and metabolite production are inherent to the strain, occurring distinctively even when submitted to similar conditions. In this sense, this contribution aimed at determining the variability in multiplication and production of OTA by strains of *A. carbonarius* and *A. niger* isolated from grapes, as well as investigating the formation of modified mycotoxins. Strains were incubated in grape-based medium, and the diameter of the colonies measured daily. The determination of OTA was performed by high-performance liquid chromatography and the identification of modified mycotoxins was carried out using high-resolution mass spectrometry. Variabilities in terms of growth and OTA production were assessed across five different strains. Peak production of OTA was detected on day 15, and a decline on day 21 was observed, indicating that the observed reduction may be associated with the degradation or modification of the OTA over time by the fungus. Ethylamide ochratoxin A, a modified mycotoxin identified in this study, provides evidence that there may be underreporting of total mycotoxin levels in food, increasing uncertainty concerning health risks to the population.**

Grapes can be easily contaminated with filamentous fungi in any of the many processes across the production chain. The mycotoxigenic fungi most often associated with this context are from the *Aspergillus* genus, with *A. niger* and *A. carbonarius* figuring as the most relevant species. In addition to deteriorating the food product, these species are also responsible for the production of ochratoxin A (OTA), the main mycotoxin detected in grapes and derivatives (wine and juice)[1,2]. *A. carbonarius* is less common in grapes when compared to *A. niger* species; nonetheless, they are more relevant due to the increased proportion of high-level OTA-producing isolates, which are the ones responsible for the accumulation of OTA in grapes and derivatives[3,4]. OTA is a major clinically relevant mycotoxin, as it may accumulate in the circulatory system, liver, kidneys, and other tissues, such as adipose and muscle, potentially causing immunosuppressive[5], teratogenic[6], neurotoxic[7], genotoxic[8], mutagenic[9] and carcinogenic effects[10].

In addition to the production of secondary metabolites, some *Aspergillus* strains are able to convert the parent mycotoxin into OTA-related compounds through hydrolysis (degradation) and/or conjugation reactions, relying on the fungi's defense mechanisms and enzymatic complexes[11]. The formed metabolites present alterations in their structure when compared to parent mycotoxin, and are therefore named modified mycotoxins (MMs)[12,13]. Additionally, some strains are also capable of reconverting the modified mycotoxin into the parent mycotoxin, albeit the exact mechanisms are yet to be unraveled[13]. Within this context, it is possible to infer that there may be underreporting of the total levels of mycotoxins in a given sample, as their modified counterparts may not be

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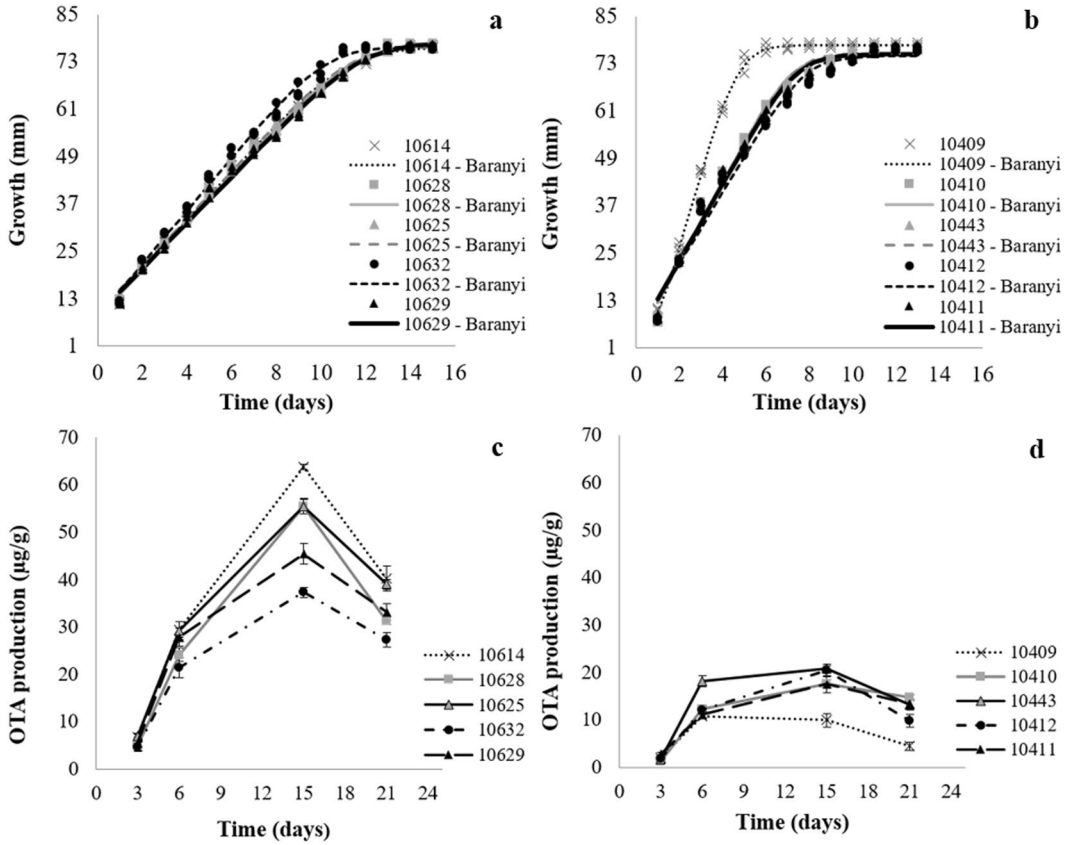

Figure 1. Growth kinetics of *A. carbonarius* strains (10628, 10632, 10614, 10625, 10629) (**a**) and *A. niger* strains (10409, 10410, 10443, 10412, 10411) (**b**) after Baranyi model fitting; and Kinetics of OTA production by *A. carbonarius* (**c**) and *A. niger* (**d**) strains in grape-based culture medium on days 3, 6, 15 and 21.

detected by traditional methods. Moreover, considering that some MMs may be as toxic as the parent species due to structural similarities, there may also be an increase in the total mycotoxin intake through the diet[14].

The detection of modified mycotoxins remains a major challenge today, as these compounds may contribute to overall toxicity associated with food and have been neglected by legislation. In this scenario, inaccurate risk estimates may result in noneffective risk management measures to protect public health[15].

Predictions of the fungal growth and metabolite production can be carried out through mathematical models; nonetheless, differences between predicted and actual values are often detected due to intrinsic variability, thereby impacting the Hazard Analysis[16]. Although the variability in the multiplication and production capacity of mycotoxins across different strains of the same fungal species have already been reported in the literature[17–19], they are yet to be systematically quantified, characterized, and compared. This is relevant to provide more realistic safety margins for both product and process designs, since these parameters may have different impact combined than when evaluated individually[20]. Given that, this study aims at determining the variability of multiplication and OTA production by strains of *A. carbonarius* and *A. niger* isolated from grapes, as well as investigating the formation of modified mycotoxins by these fungi.

Results and Discussion

Evaluation of growth and mathematical modeling of strains of *A. carbonarius* e *A. niger*.

Growth curves were obtained from diameters of the colonies (mm) vs. time (days) (Fig. 1a,b). The kinetic parameters of growth: growth rate, lag phase and maximum diameter were estimated after the fit of the Baranyi model to the data (Table 1). An excellent Baranyi model fit is observed for both *A. carbonarius* and *A. niger* strains, with low standard deviation, indicating good reproducibility. This model has become the most used and also the most suitable for evaluation of fungi growth due to its good data fit[21–23].

All curves showed an upper asymptote due to the limitation caused by the plate diameter (80 mm), which prevents multiplication, thus generating the maximum diameter (mm) for all strains (between 76.55–77.95 mm for *A carbonarius* and 74.87–77.65 mm for *A. niger*). Most strains of *A. niger* reached the maximum diameter (mm) before the strains of *A. carbonarius*, taking 8 to 13 days and 12 to 15 days of incubation, respectively.

Normally, under unrestricted growth conditions, fungal growth is described through a lag phase and a linear phase, and in some cases the lag phase is not observed. The stationary phase (upper asymptote) is observed only when the fungus is under sub-optimal conditions, such as unfavorable water activity, temperature or pH[24]. This was not the case, as there are no limitations in these factors, only the restriction of growth by reaching the diameter of the Petri dish.

Species	Strains	Growth rate (μ; mm.day^{-1})	Lag phase (λ; days)	Maximum diameter (mm)	R^2
Aspergillus carbonarius	10629	$5.84^{a} \pm 0.14$	NA	$77.95^{d} \pm 0.63$	0.99
	10628	$6.04^{a} \pm 0.17$	NA	$77.70^{d} \pm 0.63$	0.99
	10625	$6.05^{a} \pm 0.20$	NA	$77.07^{cd} \pm 1.49$	0.99
	10614	$6.05^{a} \pm 0.10$	NA	$76.55^{bcd} \pm 0.79$	0.99
	10632	$6.75^{a} \pm 0.52$	NA	$77.06^{cd} \pm 0.42$	0.99
Aspergillus niger	10412	$9.11^{b} \pm 0.20$	NA	$74.87^{a} \pm 0.44$	0.98
	10411	$9.74^{bc} \pm 0.60$	NA	$75.27^{ab} \pm 0.86$	0.98
	10443	$10.14^{c} \pm 0.54$	NA	$75.67^{abc} \pm 1.06$	0.98
	10410	$10.23^{c} \pm 0.66$	NA	$75.18^{ab} \pm 0.90$	0.98
	10409	$19.32^{d} \pm 1.03$	1.12 ± 0.07	$77.65^{d} \pm 0.73$	0.99

Table 1. Growth parameters of *A. carbonarius* and *A. niger*, obtained after fit the Baranyi model. *Different letters show statistically significant difference at $p < 0.05$. NA: no lag was obtained in this case.

We did not observe lag phase for any of the strains studied after model adjustment, except for strain 10409 of *A. niger*, which presented a lag phase of $\lambda = 1.12$ days. We hypothesize that the lag phase of most strains occurs in the 0 to 24-hour period and therefore could not be obtained. Therefore, the sooner the measurements of the colony diameter are performed, the better the precision in determining the lag phase. However, this parameter has no biological significance for fungi, since it is calculated from macroscopic observations of the colony. Despite that, in a study with *Mucor racemosus*, in which the initial inoculum was controlled, lag phase coincided with the end of germination[25]. In addition, lag phase is also dependent on the number of conidia inoculated. The higher the inoculum, the lower the lag phase, so it is of fundamental importance to take into account the size of the inoculum in the model, although this correlation is not observed across inoculum sizes and growth rates[26,27].

Growth rate values ranged from 5.84–6.75 mm.day^{-1} for *A. carbonarius* strains, but there was no statistical difference between strains ($p < 0.05$). For *A. niger*, all strains presented a higher comparative growth rate, varying between 9.11–19.32 mm.day^{-1}, with strain 10409 presenting the highest growth rate among all others (19.32 mm.day^{-1}).

Models developed for bacteria, such as the Baranyi model, can be used to evaluate the kinetics of fungal growth[27]. Furthermore, models generated from experiments carried out in culture medium may also be used to extrapolate the behavior and physiology of the microorganisms in a food to improve quality and safety[27,28]. However, the specificities of these microorganisms must be considered.

OTA production by *A. carbonarius* and *A. niger* strains. The strains of *A. carbonarius* and *A. niger* were previously evaluated for OTA production capacity by the the Plug Agar method in thin layer chromatography (data not shown). All strains showed the characteristic retention factor and fluorescence spot similar to OTA pattern and, therefore, were considered potentially ochratoxigenic.

In the assay evaluating OTA production over a 21-day incubation in grape-based culture medium, all strains of *A. carbonarius* and *A. niger* were OTA producers on each day of experiment. The lowest levels of OTA were observed at day 3 for all strains. On the other hand, the highest production of this mycotoxin was detected on day 15, when all the strains had already reached the maximum diameter. Thereafter, a reduction in mycotoxin levels was observed (Fig. 1c,d). Lappa, *et al.*[29] also observed the production of OTA by strains of *A. carbonarius* beginning at the third day of experiment. Maximum levels, nonetheless, were observed between the ninth and eleventh day, although this was the last day of the experiment. Astoreca, *et al.*[30] also detected differences on the day of maximum production for two strains of *A. niger* aggregate; strain RCP176 had its peak production at day 7 for all conditions, and strain RCP42 at day 14 with 0.995 water activity and a range between 25 and 30 °C. Additionally, strain RCP42 was also considered the largest OTA producer.

In all days of experiments, *A. carbonarius* strains produced the highest levels of OTA when compared to strains of *A. niger*. On day 6, *A. carbonarius* strains: 10614, 10625, and 10629 were the largest producers of OTA, with levels of 29.52, 29.23 and 27.9 μg/g, respectively. On day 15, the highest concentration of OTA was produced only by strain 10614 (*A. carbonarius*), 63.79 μg/g. On day 21, strains 10614 and 10625 (*A. carbonarius*) were the largest producers, 40.32 μg/g and 39.04 μg/g, respectively (Table 2).

Among the filamentous fungi detected in grapes, these species are the most commonly found, accounting for up to 98.5% of fungi of the *Aspergillus* genus isolated from grapes[1]. Although the detection of *A. carbonarius* has a lower incidence in grapes, this species is highly relevant for risk analysis, since it produces higher levels of OTA when compared to *A. niger*[2–4,31]. Moreover, it is possible that strains of the *A. carbonarius* species were adapted to the grape-based culture medium differently than strains of *A. niger*, and therefore, higher levels of OTA were detected. Requirements for OTA production vary across species, and are more restricted when compared to the requirements for their growth[32].

According to Passamani, *et al.*[31], both *A. niger* and *A. carbonarius* produced higher levels of OTA in semi-synthetic grape medium under conditions: 15 °C, 0.99 aw and pH 5.35. Optimal growing conditions were: 24 to 37 °C, water activity greater than 0.95 and pH between 4 and 6.5 for *A. niger* and 20 to 33 °C, 0.95 to 0.99 aw and pH between 5 and 6.5 for *A. carbonarius*. Although the optimal conditions for OTA production were the same for both *A. carbonarius* and *A. niger*, the levels of mycotoxin produced by *A. carbonarius* were higher. However, in this study only one strain of each species was used, which does not allow to evaluate the variability between the strains.

Species	Strains	3 (days)	6 (days)	15 (days)	21 (days)
Aspergillus niger	10409	$2.10^{Aa} \pm 0.44$	$10.89^{A} \pm 0.51^{c}$	$9.98^{Ac} \pm 1.37$	$4.49^{Ab} \pm 0.82$
	10410	$1.34^{Aa} \pm 0.49$	$12.24^{Ab} \pm 0.72$	$17.73^{Bd} \pm 0.87$	$14.69^{Cc} \pm 0.63$
	10443	$1.73^{Aa} \pm 0.29$	$18.16^{Bc} \pm 1.16$	$20.82^{Cd} \pm 0.67$	$13.31^{Cb} \pm 1.18$
	10412	$1.92^{Aa} \pm 0.26$	$12.22^{Ac} \pm 0.91$	$20.52^{Cd} \pm 1.38$	$9.87^{Bb} \pm 1.35$
	10411	$2.86^{Aba} \pm 0.36$	$11.14^{Ab} \pm 0.67$	$17.58^{Bd} \pm 1.75$	$13.45^{Cc} \pm 0.72$
Aspergillus carbonarius	10628	$4.95^{Bca} \pm 0.90$	$24.19^{Db} \pm 1.47$	$55.49^{Fd} \pm 1.64$	$31.29^{Ec} \pm 0.80$
	10632	$4.80^{BCa} \pm 0.92$	$21.46^{Cb} \pm 2.07$	$37.33^{Dd} \pm 1.02$	$27.37^{Dc} \pm 1.49$
	10614	$6.90^{Ca} \pm 0.68$	$29.52^{Eb} \pm 1.61$	$63.79^{Gd} \pm 0.58$	$40.32^{Fc} \pm 2.66$
	10625	$7.02^{Ca} \pm 0.43$	$29.23^{Eb} \pm 1.83$	$55.45^{Fd} \pm 1.60$	$39.04^{Fc} \pm 1.02$
	10629	$5.64^{Ca} \pm 1.14$	$27.90^{Eb} \pm 1.82$	$45.46^{Ed} \pm 2.20$	$33.20^{Ec} \pm 1.64$

Table 2. OTA production (μg/g) by *A. carbonarius* and *A. niger* strains along 21 days of incubation in grape-based culture medium. *Mean followed by lowercase letters compare the OTA production by the strain in the line at different times, and uppercase letters compare the OTA production by the strains in the column. Different letters show statistically significant difference at $p < 0.05$.

Between days 15 and 21, reductions in OTA levels were observed for both *A. carbonarius* and *A. niger*, from 26.67 to 43.61%, and 17.12 to 54.98%, respectively. For *A. carbonarius*, the greatest reduction was observed in strain 10628, and for *A. niger*, the greatest reduction was observed in strain 10409. We hypothesize that such reductions in OTA levels may be related to the formation of modified mycotoxins. OTA, after being produced by the fungus, may either have been transformed into derived metabolites by the action of the enzyme complex of the fungus itself, or became strongly adsorbed by the culture medium matrix over the days of incubation (15 to 21) and was not extracted by the employed method.

Seefelder *et al.*[33] showed that fumonisins are able to bind to polysaccharides and proteins. Therefore, it is possible that OTA and any related compound may have bound with polysaccharides present in the grape-based culture medium, and were ultimately not detected. Brodehl, *et al.*[11] indicate that up to 50% of the parent mycotoxin may be strongly adsorbed to the culture medium or fungi mycelium through the formation of adducts and thus not detected. The adsorption of zearalenone in *A. niger* mycelium was also observed as one of the main forms of detoxification of this mycotoxin, in addition to the metabolism of mycotoxin by the fungus generating degraded compounds of lower toxicity[34]. Conidial suspension of *A. niger*, *A. carbonarius* and *A. japonicus* were also able to adsorb OTA, probably through hydrophobic interactions, apart from converting it into α-OTA after germination and growth[35].

Astoreca, *et al.*[36], when evaluating OTA production by two strains of *A. niger* aggregate, also observed a reduction in toxin levels on day 21 in almost all trials. This reduction was justified by a possible degradation of the mycotoxin by the strain itself, which would use OTA as an alternative carbon source to continue maintaining its metabolic rate. Lappa, *et al.*[29] also observed a reduction in OTA levels for some strains of *A. carbonarius* evaluated in culture medium. In studies with orange juice, the reduction in OTA levels, produced by an *A. niger* strain, was observed only on the twenty-eighth day. However, another tested strain showed an intense reduction from the seventh day[37]. Romero, *et al.*[38] also detected a reduction in OTA levels produced by two strains of *A. carbonarius* on the twenty-eighth day, although for the other two strains this reduction was not observed. Other findings also corroborate this hypothesis, in which *Aspergillus* section *Nigri* strains demonstrate the ability to degrade OTA by generating α-OTA through its enzymatic complex. It is possible that the fungus removes and assimilates phenylalanine from OTA to be used as a source of nitrogen, since nutrients may be scarce at the end of the incubation period[39,40]. Nevertheless, OTA-producing *A. carbonarius* strains were also producing α-OTA[41], which renders inconclusive the assertion that the formation of α-OTA is only due to the degradation of OTA, and may also be related to the metabolism of the fungus itself.

OTA levels did not correlate with growth rate. Although *A. niger* strains had the highest growth rates, they had lower OTA concentrations when compared to *A. carbonarius* strains. However, among strains of the same species (*A. niger*), a positive correlation was observed: strain 10409 showed the highest growth rate and the highest OTA level on days 15 and 21. According to Astoreca, *et al.*[36], higher growth rates are associated with lower levels of OTA production, and may even be related to a restriction in the production of this metabolite. In contrast, Lappa, *et al.*[29] found a positive correlation between growth parameters and OTA production levels.

Variability of multiplication and OTA production by *A. niger* and *A. carbonarius*. When evaluating daily multiplication data, strain variability was greater than both biological and experimental variabilities for *A. niger*, on most days. As for *A. carbonarius*, the strain and biological variabilities did not differ, but were greater than the experimental variability, on most days by F-test. These results corroborate the variability found for strains of *Lactobacillus plantarum* and *Listeria monocytogenes*[16,42]. At the beginning and at the end of the evaluated period, *A. carbonarius* and *A. niger* appear to have greater biological variability (Fig. 2a,b). This may be related both to the initial phase of the experiment, in which the fungus is in the stage of adaptation and intense metabolic activity, and to the stationary phase (end of the experiment) in which the fungus may undergo changes in its mechanism, due to the scarcity of nutrients, which may not be reproducible in new experiments, even under similar conditions.

Strain variability is inherent to the microorganism, as it occurs even when they are submitted to the same conditions[43]. A higher coefficient of variation in the growth data of *A. carbonarius* strains in culture medium was also observed on the third day of incubation by Lappa, *et al.*[29]. Gougouli and Koutsoumanis[28] observed a similar phenomenon in which, although the variation between the replicates was low in the optimal conditions,

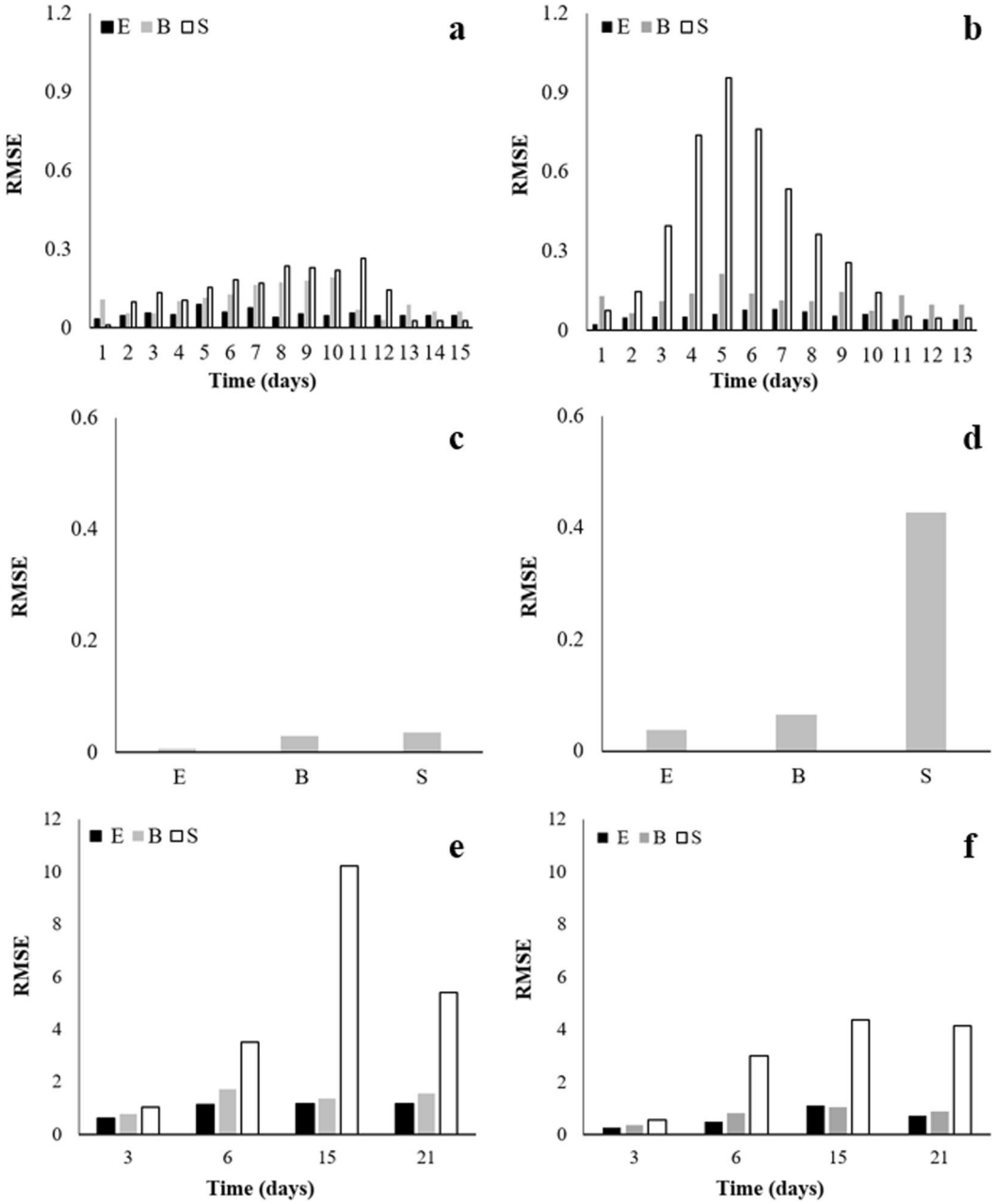

Figure 2. Experimental (E), Biological (B) and Strain (S) variabilities of daily growth of *A. carbonarius* (**a**) (15 days) and *A. niger* (**b**) (13 days); experimental (E), Biological (B) and Strain (S) variabilities of growth rate of *A. carbonarius* (**c**) and *A. niger* (**d**) and; experimental (E), Biological (B) and Strain (S) variabilities of OTA production by *A. carbonarius* (**e**) and *A. niger* (**f**) on days 3, 6, 15 and 21. Variability is expressed as root mean square error (RMSE).

under conditions close to the growth limit, the experimental variability increased significantly. Baert, *et al.*[44] also reported an increase in the variability of kinetic parameters of fungi under stress conditions; the highest variabilities were observed in the central period of the experiment. Typically, most filamentous fungal assays are conducted over 7 days; therefore, due to the great variability observed during this period, the use of a greater number of strains is important, so that predictions and results approach the reality.

By evaluating growth rate variability, we found that strain variability was higher than the biological and experimental (S>B=E) for *A. niger* species and, for *A. carbonarius*, the strain and biological variabilities were similar and greater than experimental variability (S=B>E) (Fig. 2c,d). When comparing the variabilities across species, greater variability of multiplication, both daily and in growth rate, is observed for *A. niger* (approximately ten times greater).

As far as OTA production is concerned, there is greater strain variability throughout the evaluated days, for both species, except on day 3 (E=B=S). However, *A. carbonarius* presented higher variability (Fig. 2e,f). On day

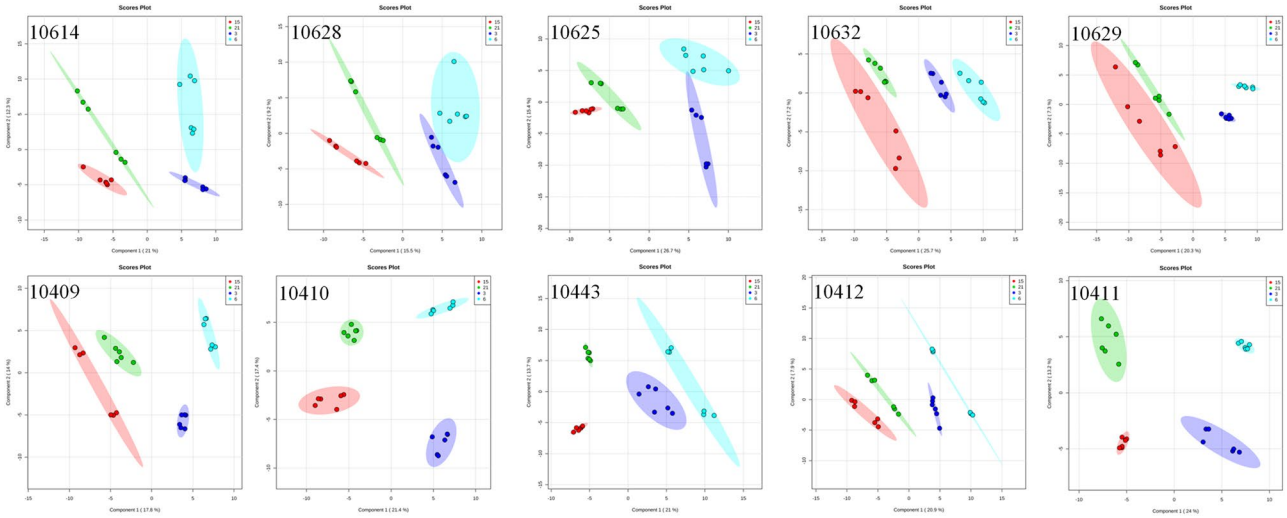

Figure 3. Scores plot of resulting from PLS-DA analysis for *A. carbonarius* (10614; 10628; 10625; 10632; 10629) and *A. niger* (10409; 10410; 10443; 10412; 10411) strains along 21 days of incubation in grape-based culture medium (Day 3: dark blue; Day 6: light blue; Day 15: red; Day 21: green).

15, in which the highest levels of OTA were observed, greater variability was also observed. Therefore, in addition to the existing intra and inter-species variability, it is observed that such variability is dependent on time and response to be studied (growth/production of secondary metabolites). Lappa, *et al.*[29] observed higher coefficients of variation between strains than between times of analysis of OTA production by strains of *A. carbonarius*, which reinforces the existence of inter-specificity across species. Although all strains were isolated from wine grapes cultivated in the tropical semi-arid region of Brazil, strains 10614 and 10625, the largest producers of OTA, were the only strains isolated from the region of Casa Nova-BA, Brazil. These data demonstrate that, in addition to geographic influence over the strain variability and variation of species isolated from grapes, there is also the influence of the microclimate of each region[2]. Moreover, genetic and phenotypic factors will also influence the production of secondary metabolites by strains within the same species[20,29].

The use of a greater number of strains makes it possible to obtain information on the individual responses of the strains, which allows extreme conditions of growth and production of mycotoxin for a species to be known. Likewise, the difference in OTA production capacity and growth among strains indicates that the extrapolation of models obtained from data from only one individual strain may not be representative of most strains in a given species, leading to overestimation or underestimation of the predicted data[38]. Notwithstanding, it is important to emphasize that along with microbiological variability, there are also inherent variabilities to the process to which the food is submitted, although in some cases these are easier to control and the variability microbiology becomes the most determining factor in the final contamination[20].

Modified ochratoxin identification by HRMS. The reduction of OTA observed between days 15 and 21 of incubation may be related to the formation of modified mycotoxins. To detect the presence of these metabolites, high resolution mass spectrometry and Partial Least Squares-Discriminant Analysis (PLS-DA) were used to assess the obtained mass spectral data. A discrimination of produced metabolites is observed, with the formation of 4 clusters, grouping samples with similar ion content, at times 3, 6, 15 and 21 days, for all studied strains (Fig. 3). Interestingly, biological variability is also observed within each cluster.

The loadings plot of the statistical model formed by features selected by PLS-DA indicated the 65 main candidate biomarkers of each strain over the assessed period (21 days). From this list of ions, it was possible to perform the search for modified mycotoxin. Among the targets sought, we identified only ethylamide-ochratoxin A ($[M + Na]^+$: 453.1202) as a biomarker produced by strain 10443 (*A. niger*) on day 21. The model used by PLS-DA only shows the differences between the analyzed groups, and therefore ethylamide-ochratoxin A was the only molecule exclusive of one of the strains. It is possible, however, that other mycotoxins are also present, but have been produced by more than one strain at different times, which makes it impossible to identify these compounds as biomarkers. In addition, the reduction in OTA levels may also be related to a strong adsorption of mycotoxin by the matrix, making it impossible to extract it.

A series of isomeric candidate biomarkers within the same *m/z* range, 1-dodecanoyl-2-eicosapentaenoyl-glycero-3-phosphoethanolamine and/or 1-tetradecenoyl-2-octadecatetraenoyl-glycero-3-phosphoethanolamine and/or 1-octadecatetraenoyl-tetradecenoyl-glycero-3-phosphoethanolamine and/or 1-eicosapentaenoyl-2-dodecanoyl-glycero-3-phosphoethanolamine ($[M + Na]^+$: 704.4248) was also identified among the metabolites produced by strain 10443 (*A. niger*) on day 15. Since this biomarker belongs to the phosphatidylethanolamine class, it may be a possible donor of the ethylamine group for the OTA molecule forming ethylamide-ochratoxin A. Such reaction may occur due to the enzymatic complex of the fungus capable of converting phosphatidylethanolamines into 2-hydroxyethylamine through the glycerophospholipid metabolism, reacting with OTA (Fig. 4).

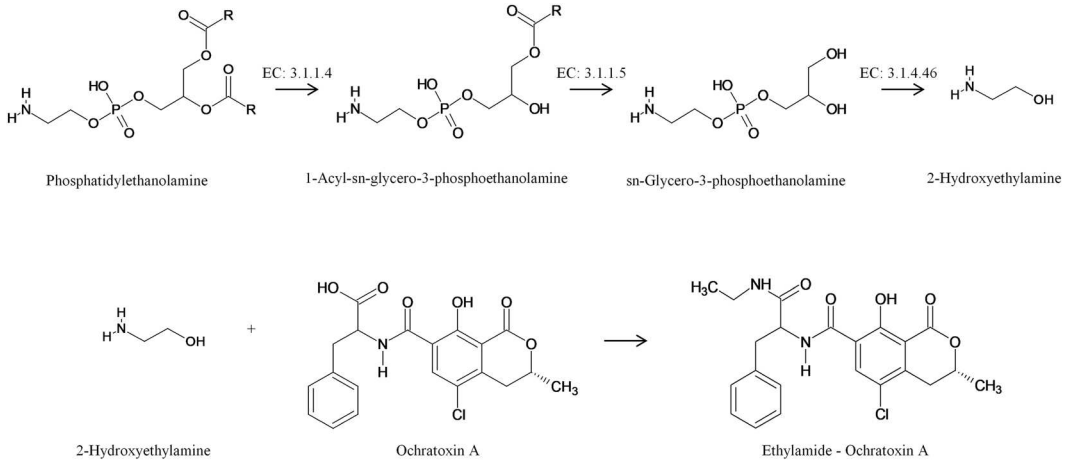

Figure 4. Glycerophospholipid metabolism of *A. niger* and proposal formation of ethylamide-OTA.

The membrane of *A. niger* is mainly composed of phospholipids (85–90%), sterols (10–15%) and sphingolipids (1–2%). In the phospholipid fraction, the main representatives are: phosphatidylethanolamines, phosphatidylcholines, cardiolipins, and phosphatidic acids[45]. Phosphatidic acid, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine were also detected as the major phospholipid species of the *Aspergillus* genus[46]. Under stress conditions such as thermal, osmotic, and oxidative shock, *A. niger* showed a reduction in the levels of phosphatidylethanolamines[45]. It is possible, therefore, that at the end of the incubation period, between 15 and 21 days, the metabolism of the fungus also changes due to the stress caused by nutrient shortages, reflecting the levels of phosphatidylethanolamine. These alterations may also be related to metabolic transformations and use of specific moieties of these lipids in the formation of modified mycotoxin. However, more studies are needed to elucidate which enzymes are associated with these reactions.

Some microorganisms are able to modify mycotoxins, mainly by degradation and conjugation reactions through their enzymatic complex[11]. A strain of *Saccharomyces cerevisiae* was shown to be able to convert mycotoxins, converting zearalenone to α-zearalenol, β-zearalenol, zearalenone-14-glucoside and zearalenone-16-glucoside[47], and *Saccharomyces pastorianus* was also able to modify deoxynivalenol to deoxynivalenol-3-glucoside during fermentation[48]. The filamentous fungi belonging to the genus *Rhizopus* and *Thamnidium* were responsible for the metabolization of zearalenone to zearalenone glycosides, and *Fusarium* and *Aspergillus* species for zearalenone sulfates[49–52].

Hydrolysis, hydroxylation, lactone opening, and conjugation reactions are the main biotransformation pathway of OTA[53]. The following biotransformation products: ochratoxin α, ochratoxin β, 4-R-OH-ochratoxin A, 4-R-OH-ochratoxin B, and 10-OH- ochratoxin A were detected in cultures of *Aspergillus ochraceus* incubated with OTA and ochratoxin B[54]. Ochratoxin α and L-β-phenylalanine were the metabolites generated by the degradation of OTA by *Aureobasidium pullulans*[55].

The presence of these formed metabolites is another strong evidence that there may be underreporting of total mycotoxin levels, especially in fermented food products that are manufactured using microorganisms, as well as in products made from contaminated raw material[11,13].

Our findings demonstrate the existence of both interspecies and intra-species variability, which confirms the need for selection of representative strains that can cover a wider range of conditions, considering their different kinetic behaviors. Such selection allows models to increasingly reflect reality and can be used in risk analysis, predicting product deterioration and/or the presence of mycotoxins. Whereas mycotoxins levels do not always correlate directly with growth, as demonstrated by the results presented here, the most effective control against OTA presence in food may be performed with fungus growth control. By avoiding the development of these species in food, mycotoxin production will not occur, thereby justifying the importance of modeling the growth of potentially mycotoxigenic fungi, and not only the factors that will influence mycotoxin production evidenced in secondary models. The description of strain growth over time is the basis for adopting preventive practices, from the field to the consumer, assisting in food quality and safety[21,56]. Moreover, the use of quantitative modeling is a crucial tool in Hazard Analysis and Critical Control Points, and also helps determining the maximum tolerable limits of mycotoxins.

In addition, the reduction of OTA levels produced by *A. carbonarius* and *A. niger* in the final incubation period, may be related to the formation of modified mycotoxins by the fungus itself or by adsorption of the compound in the matrix. In both cases, the results demonstrate the possibility of underreporting of the total levels of mycotoxins present and a potential health risk. Furthermore, modified mycotoxins may be reconverted into the parent mycotoxin, either by the industrial process itself, or in the digestive system after ingestion of the contaminated food, recovering the toxicity potential responsible for health effects in humans and animals[13,51].

Material and Methods

***Aspergillus carbonarius* and *A. niger* strains.** Five strains of *Aspergillus carbonarius* and five of *A. niger*, isolated from grapes, were obtained from the Culture Collection of the Department of Food Science/CCDCA-UFLA. *A. niger* strains: 10409, 1410, 10443, 10412 and 10411 were isolated from grapes collected in Petrolina - PE - Brazil. *A. carbonarius* strains: 10628, 10632 and 10629 were isolated from grapes collected in Lagoa Grande - PE – Brazil, and strains: 10614 and 10625 from Casa Nova - BA - Brazil. Strains were previously characterized as potentially ochratoxigenic by the Plug Agar method in thin layer chromatography, according to Filtenborg and Frisvad (1980)[57].

Preparation of the conidial suspensions. Conidial suspensions of each strain were individually prepared and had their concentration standardized at 10^6 conidia/mL according to Wigmann, *et al.*[58]. *A. carbonarius* and *A. niger* strains were inoculated in MEA- Malt Extract Agar medium (Acumedia) (Malt Extract: 20.0 g, Peptone: 1.0 g, Glucose: 30.0 g, Agar: 20 g, Distilled Water: 1 L) and incubated at 25 °C for 7 days. Conidia were collected by scraping the mycelium from each plate with sterile distilled water and 0.1% Tween 80 (Labsynth). Subsequently, they were filtered and then centrifuged at 11962.6 × g three consecutive times for 15 minutes at 5 °C (Sorvall Legend XTR, Thermo Scientific, Hampton, USA). The final concentration of conidia in each fungal suspension was determined in Neubauer chamber (Sigma-Aldrich).

Preparation of the grape-based culture medium and inoculation of strains. The experimental, biological and strain variabilities between strains of multiplication and OTA production were evaluated in a culture medium based on Syrah grapes, according to Passamani, *et al.*[31]. The medium was prepared by adding 175 mL of juice obtained from crushed grapes in 825 mL of distilled water and 20 g of agar (Inlab). A 10 μL aliquot of the conidial suspension of each strain was inoculated individually, and incubated at 25 °C, over 21 days. The control plate was inoculated with 10 μL of sterile distilled water without spore suspension.

Growth evaluation and mathematical modeling. The mycelial radial growth of the strains was evaluated by daily measurement (one measure/day) of the colony diameter (mm) in two perpendicular directions during 21 days, using a digital caliper with a 0.01 mm resolution (Digimess -100.175BL, Brazil). The Baranyi and Roberts model[59] (Equations 1 and 2) were fitted to data on colony diameter as a function of time, using DMFit software, an Excel add-in. The multiplication rate (μ_{max}, mm.day^{-1}) and lag phase time (λ, days) were estimated after model fitted.

$$D(t) = \mu_{max}A - \ln\left[1 + \frac{\exp(\mu_{max}A) - 1}{\exp(D_{max})}\right] \quad (1)$$

$$A = t + \left(\frac{1}{\mu_{max}}\right)\ln[\exp(-\mu_{max}\, t) + \exp(-\mu_{max}\, \lambda) - \exp(-\mu_{max}\, t - \mu_{max}\, \lambda)] \quad (2)$$

where: D(t) is the colony diameter (mm) as a function of time, μ_{max} is the maximum multiplication rate (mm. day^{-1}), λ is the time obtained by the intersection (days), and D_{max} is the maximum diameter of colony.

Determination of OTA production. *Sample preparation and extraction.* In order to determine the levels of OTA produced by the strains, three pieces of the grape-based medium were removed from the internal, middle and external areas of each colony during 21 days of incubation at 25 °C (day 3, 6, 15 and 21). The reagents used were all of high purity or HPLC grade (99.9%). For extraction, 1 mL of methanol (Sigma-Aldrich) was added, followed by vortex homogenization for 5 seconds and incubation at room temperature for 60 minutes. The extracts were filtered in PVDF - Polyvinylidene Fluoride (0.22 μm) filter units (Millipore), according to the methodology proposed by Bragulat, Abarca, and Cabañes[60] and then submitted for quantification. The standard curve was prepared using a stock solution previously prepared by dissolving the commercial OTA standard (Sigma-Aldrich) in methanol (1 μg/mL). Subsequently, standard solutions with concentrations of 3.75; 15.0; 30.0; 105.0 and 135.0 ng/mL were prepared by dilution. For the recovery assay, the grape-based culture medium was spiked at three levels with concentrations equal to 1.0 μg/g; 3.0 μg/g and 6.0 μg/g, in triplicates. The results of the recovery trials were 98.32% (±12.29), 97.23% (±7.07), and 97.47 % (±7.82), respectively.

OTA quantification by HPLC. OTA quantification was performed on an Agilent Techonlogies 1290 infinity HPLC with a DAD (diode array) detector at the wavelength of 330 nm. The Agilent-Zorbax Eclipse XDB-C18 column (4.6 × 250 mm, 5 μm) was used, with a column temperature of 25 °C, flow of 0.5 mL.min^{-1} and injection volume of 20 μL. Elution was performed in an isocratic system of acetonitrile: methanol: aqueous acetic acid (35: 35: 29: 1) (J. T. Baker). The mean retention time obtained for OTA was 4.7 ± 0.1 min. OTA quantification was performed by building an analytical curve obtained by linear regression (y = 4987.8× + 86.735), with determination coefficient (R^2) of 0.997. The limits of detection (LoD) and quantification (LoQ) were 0.001 and 0.004 μg/g, respectively. All samples were analyzed in duplicates, and standard OTA solutions were injected in triplicates.

***A. niger* and *A. carbonarius* variability of multiplication and OTA production.** Experimental variability was quantified by conducting the experiment in duplicate at the same time using the same conidial suspension. To quantify biological variability, the experiment was also replicated two more times on different days using fresh suspensions. This procedure resulted in six colony diameter values (mm) and OTA concentrations

(μg/g). Subsequently, the experimental variability (E), biological (B), and strains (S) indexes, in relation to the multiplication and production of OTA, were determined[16]. (Equation 3: Experimental variability; 4: Biological variability; 5: Strain variability):

$$MSE = \frac{RSS}{DF} = \frac{\sum_{S=1}^{5}\sum_{B=1}^{3}\sum_{E=1}^{2}(X_{EBS} - X_{BS})^2}{n - p} \quad (3)$$

$$MSE = \frac{RSS}{DF} = \frac{\sum_{S=1}^{5}\sum_{B=1}^{3}(X_{BS} - X_S)^2}{n - p} \quad (4)$$

$$MSE = \frac{RSS}{DF} = \frac{\sum_{S=1}^{5}(X_s - X)^2}{n - p} \quad (5)$$

Where: MSE is the mean square error calculated from the sum of the squares of the residuals (RSS) divided by the degree of freedom (DF); X_{EBS} is the diameter (mm) or concentration of mycotoxin (μg/g) obtained from each replicate of the duplicate experiment performed at the same time "E", biological reproducibility "B", and strain "S" (E = 1, 2; B = 1.2, S = 1, 2, 3, 4, 5); X_{BS} is the mean of X_{EBS} for the "S" strain obtained on the same day; X_S is the mean of X_{BS} of three experiments performed on different days for strain "S", X is the mean of X_S for the five strains; (n-p) is the number of data minus the number of parameters. F-test was used to compare the experimental, biological and strain variability with an alpha of 0.05. The variabilities were expressed as root mean square error (RMSE).

Modified ochratoxin identification by HRMS. For search and identification of OTA derivatives: ochratoxin β (222.0528 g/mol), α-ochratoxin (256.0139 g/mol), α-ochratoxin amide (255.0298 g/mol), 14-decarboxy-ochratoxin A (359.0924 g/mol), ochratoxin B (369.1212 g/mol), ochratoxin B methyl ester (383.1369 g/mol), ochratoxin B ethyl ester (397.1525 g/mol), ochratoxin A (403.0823 g/mol), 4-hydroxyochratoxin A (419.0772 g/mol), ethylamide ochratoxin A (430.1296 g/mol), ochratoxin A glucose ester (565.1351 g/mol), (22 → 6′) ochratoxin A-methyl-α-D-glucopyranoside ester (579.1507 g/mol), ochratoxin A cellobiose ester (727.1879g/mol), ochratoxin A quinone (383.1005 g/mol), ochratoxin A hydroquinone (385.1162 g/mol), ochratoxin C (431.1136 g/mol); 10 μL of the obtained extract (item 2.5.1) were diluted in 490 μL of methanol and homogenized under vortex for 30 seconds. Then, 0.1% formic acid was added and the sample was directly injected. An ESI-LTQ-XL Orbitrap Discovery (Thermo Scientific, Bremen, Germany) mass spectrometer with a nominal resolution of 30,000 (FWHM) was used to acquire the data in the survey scan mode. The parameters used were: flow rate at 10 μL.min^{-1}, capillary temperature at 280 °C, spray current at 5 kV, and sheath gas at 5 arbitrary units. Data were obtained in the positive mode using mass range of 250–750 *m/z* in triplicate.

Statistical analyses. Analysis of variance (ANOVA), with *a posteriori* Tukey test, was used to evaluate the difference in growth parameters and OTA levels by *Aspergillus carbonarius* and *A. niger* strains in a grape-based medium. To assist in the identification of OTA derivatives, the multivariate regression method, Partial Least Squares-Discriminant Analysis (PLS-DA) was used. The analyses were performed through the online platform MetaboAnalyst 3.0[61,62], using interquartile range for data filtering, and quantile normalization. The Lipid MAPS online database (University of California, San Diego, CA) and METLIN (Scripps Center for Metabolomics, La Jolla, CA), as well as bibliographic references were consulted to identify compounds of interest through their exact mass, with a maximum error of 2 ppm.

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Author Contributions

L.F.: execution of all analyzes and writing of the paper; T.M.G.: OTA quantification and identification of modified mycotoxins and assistance in writing the paper; A.K.R.P.: assistance in fit the data to the models; E.O.L.: OTA quantification and identification of modified mycotoxins; D.N.O.: OTA quantification and identification of modified mycotoxins and assistance in writing the paper; C.F.O.R.M.: OTA quantification and identification of modified mycotoxins; R.R.C.: OTA quantification and identification of modified mycotoxins; A.S.S.: work orientation and assistance in writing the paper.

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CAPÍTULO 3

Influence of maturation stages in different varieties of wine grapes (*Vitis vinifera*) on the production of ochratoxin A and its modifieds by *A. carbonarius* and *A. niger.*

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Influence of Maturation Stages in Different Varieties of Wine Grapes (*Vitis vinifera*) on the Production of Ochratoxin A and Its Modified Forms by *Aspergillus carbonarius* and *Aspergillus niger*

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Supporting Information

ABSTRACT: Ochratoxin A is the main contaminant mycotoxin of grapes produced mainly by *Aspergillus niger* and *Aspergillus carbonarius*. Besides, it is possible that the formation of modified mycotoxin occurs through the plant defense mechanism or also by fungus actions itself. The objective of this study was to evaluate the influence of grape variety and maturation stage on the formation of OTA and modified mycotoxin. The determination of OTA was performed by high-performance liquid chromatography, and a high-resolution mass spectrometry was used for the detection of modified ochratoxin. A positive correlation was observed between the following grapes physicochemical parameters: pH, total soluble solids, total glycosides in glucose, total anthocyanin, and OTA levels produced by *A. niger* and *A. carbonarius*. Therefore, the higher the concentrations of these parameters, the greater the production of mycotoxin in grapes. Among the elected targets, we identified the 14-decarboxy-ochratoxin A in Muscat Italia variety at veraison and 15 days after the beginning of veraison stages; and ethylamide-ochratoxin A as a biomarker in the Syrah variety at the ripeness stage.

KEYWORDS: *conjugated mycotoxin, masked mycotoxin, mass spectrometry, modified mycotoxin, Vitis vinífera, food safety*

INTRODUCTION

European *Vitis vinifera* grapes, which grow better in regions with a dry climate, high insolation, and low relative humidity, are the most used in wine production due to their specific organoleptic characteristics.[1] However, there are many grape varieties and cultivars with distinct physicochemical and sensorial features in different producing regions.[2] This variability, in addition to being responsible for the typicity of its derivatives, also influences the microbiological contamination of the grapes.[3]

The consumption of grapes and wines has been associated with beneficial health effects, due to the presence of phenolic compounds, which have antioxidant, anti-inflammatory, and bactericidal properties, and aid to prevent cardiovascular diseases.[4,5] However, microorganisms present in grapes, besides affecting grapevines health and being responsible for deterioration, may also produce toxic compounds to humans, such as mycotoxins.

Among mycotoxins, ochratoxin A (OTA) is the most commonly detected in grapes and their derivatives. OTA is produced mainly by *Aspergillus niger* and *Aspergillus carbonarius*.[3,6−8] Contamination of grapes by these species can occur since the beginning of maturation stage and becomes more prominent near harvest time, due to the increase of both relative humidity and temperature of the vineyard, besides changes in physicochemical characteristics: berry softening, sugars accumulation, and acidity reduction.[9,10] However, it is possible that, besides grape deterioration and OTA production by these fungi, the production of modified mycotoxins still occurs in the vineyard.

The occurrence of mycotoxicoses that did not correlate with total levels of mycotoxin present in foods led to the emergence of the term masked mycotoxin, nowadays also called modified mycotoxin, characterized as conjugates formed from the defense metabolism in plants, which cannot be detected by traditional analytical methods.[11]

It is possible that these conjugates are formed at different stages of grape development. The formation of these conjugates is possible because plants have mechanisms that neutralize microbial toxins through detoxification by conjugation of endogenous metabolites.[12] Such chemical modifications are obtained through hydrolysis, reduction, and oxidation reactions (Phase I metabolism) or by polar binding components (such as sugars) to the parent mycotoxin (Phase II metabolism). Such reactions facilitate sequestration of the compound formed in the vacuole or apoplast or its incorporation into cell wall components (Phase III metabolism), leading to a decrease in the toxicity of the metabolite formed.[13−15] However, after ingestion of the conjugate, enzymes and components of human or animal microbiota may act on the modified mycotoxin, causing its hydrolysis, the

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release of the parent mycotoxin, and its absorption, increasing the individual's total exposure to the toxic compound.[16]

Several changes in physicochemical features of grapes occur throughout maturation. These reactions include the increase in berry size, color development, anthocyanins accumulation (in red grapes), berries softening due to the transformation of pectins, accumulation of sugars and reduction of organic acids, synthesis of volatile aromatic compounds, and reduction and polymerization of tannins.[17] These changes have a direct influence on toxigenic fungi contamination. However, little is known about the effect of these parameters on OTA production by *A. carbonarius* and *A. niger* and the formation of modified mycotoxin. In this sense, this study aims to evaluate the influence of maturation stage and grape varieties on OTA production and modified mycotoxin formation.

MATERIAL AND METHODS

Safety Information. OTA is classified as a possible human carcinogen (group 2B). Therefore, it must be handled with care.

Wine Grapes. Different grape varieties (Syrah, Touriga Nacional, and Muscat Italia) were collected throughout maturation stage. The samples were collected at the beginning of the veraison (onset of maturation process); 15 days after the beginning of veraison; and ripeness (in which grapes reach optimum maturation point). Grapes were collected in a semiarid tropical region of Brazil located in the border between the states of Pernambuco and Bahia (Brazil) according to Freire et al.[3]

Physicochemical Characterization of Wine Grapes. For grape characterization, 15 g of each grape variety was weighed and mashed in a food processor (RI7761, Philips Walita, China) in triplicate for each analysis performed. Titratable acidity (g of tartaric acid per 100 g of grape), total soluble solids (% of soluble solids), and pH were determined according to AOAC.[18] A digital refractometer was used to determine the total soluble solids (Pocket PAL-1, Atago, Ribeirão Preto, SP, Brazil). For pH determination, a digital potentiometer (K39-2014b, Kasvi, São José do Pinhais, SP, Brazil) was used. Sugars were determined according to a methodology previously described by Instituto Adolfo Lutz,[19] and results were expressed as % of total glycosides in glucose. Pectins were determined by gravimetry, and results were expressed as % of pectic acid.[19] Total monomeric anthocyanin levels were measured using the differential pH method[20] and results expressed as mg equivalents of cyanidin-3-glycoside per 100 g of grape. Extraction used to determine phenolic compounds, and antioxidant activity was performed according to Paz et al.[21] Determination of antioxidant activity was performed by DPPH[22] and ORAC[23] methods, and results were expressed as DPPH value (μMol Trolox equivalents per 100 g of grape) and ORAC value (μMol Trolox equivalents per 100 g of grape), respectively. Total phenolic compounds were obtained using the Folin−Ciocalteu reagent.[24] Results were expressed as mg of gallic acid equivalents per 100 g of grape.

Inoculum Preparation. Strains of *Aspergillus carbonarius* (10614) and *A. niger* (10443), isolated from wine grapes and obtained from the Culture Collection of the Department of Food Science/CCDCA-UFLA, were used in the experiments. These strains were previously selected in grape-based culture medium.[25] Conidia suspensions of each species were prepared individually.[26] Once suspensions were obtained, the final concentration of the inoculum was standardized at 10^7 conidia/mL.

Grape Berry Inoculum. Twenty-five grams of grape berries from each sample were disinfected with 1% peracetic acid solution (Diversey, São Paulo, SP, Brazil) for 2 min and washed three times with sterile distilled water. After that, excessive water was withdrawn with the aid of a manual centrifuge. The inoculation was made by spraying (in laminar flow) 1.25 mL of each conidia suspension, individually, in triplicate. As a control, each grape variety at all maturation stage studied was inoculated with 1.25 mL of sterile distilled water without containing the conidial suspension. After inoculation, grapes were placed in plastic boxes previously disinfected with 1% peracetic acid solution for 30 min. Then, the grapes were kept at 25 °C for 7 days in the absence of light and under high relative humidity (95 ± 5%).[27] Relative humidity was controlled using a K_2SO_4[28] saturated solution and measured throughout the incubation period with a hygrometer (LOG32TH, Incoterm, Porto Alegre, RS, Brazil). After the incubation period, samples were used for extraction.

Determination of OTA Concentration. *Sample Preparation and Extraction.* After grape homogenization with the aid of a pistil, 5 g of the sample were added to 25 mL of acetonitrile/water/formic acid (79:20.9:0.1, v/v/v) (JT Baker, Xalostoc, Mexico), vortexed, and extracted using a rotary shaker at 200 rpm for 90 min at room temperature (Series 25 Shaker/Incubator, New Brunswick Scientific, Minnesota, USA). Following, the extracts were centrifuged at 10,000*g* for 10 min at 4 °C (Sorvall Legend Xtr, Thermo Scientific, Hampton, USA) according to Nathanail et al. with modifications.[29] Supernatants obtained from the extraction procedure were filtered in polyvinylidene fluoride (PVDF, 0.22 μm) filter membranes units (Jet Biofil, Guangzhou, China) and used for quantification.

OTA Quantification by HPLC. For OTA quantification in grapes, an Agilent 1290 Infinity HPLC system (Agilent Technologies, Palo Alto, CA, USA) with a DAD detector set at a wavelength of 330 nm and an Agilent-Zorbax Eclipse XDB-C18 column (4.6 × 250 mm, 5 μm) were used. Flow and injection volume used were, respectively, 0.5 mL/min and 20 μL. An isocratic system of acetonitrile/methanol/aqueous acetic acid (35:35:29:1) (J.T Baker, Xalostoc, Mexico) was used for elution. A standard curve for OTA (Sigma-Aldrich, Darmstadt, Germany) was obtained using a stock solution previously prepared in methanol (1 μg/mL). Subsequently, standard solutions were prepared by serial dilutions, and their concentrations were of 0.00375, 0.015, 0.03, 0.105, and 0.135 μg/mL. OTA quantification was performed after obtaining an analytical curve [$y = 4987.8x + 86.735$; coefficient of determination (R^2) = 0.997]. Limits of detection (LD) and quantification (LQ) were 0.001 and 0.004 μg/g, respectively. Standard OTA solutions were injected in triplicate, and all samples were analyzed in duplicate.

For the recovery test, grape berries were fortified, in triplicate, at two levels (1.0 μg/g and 3.0 μg/g) and extracted as described above. Results of the recovery tests were 87.61% (±5.38) and 112.64% (±6.06), respectively.

Identification of Modified Ochratoxin by HRMS. A mass spectrometer of the type ESI-LTQ-XL Orbitrap Discovery (Thermo Scientific, Bremen, Germany) with a nominal resolution of 30,000 (fwhm), a flow rate at 10 μL/min, capillary temperature at 280 °C, spray current set at 5 kV, and sheath gas at 5 arbitrary units was used for identification of the possible elected OTA derivatives (Table 1).

Table 1. Ochratoxin A and Its Derived Metabolites

metabolites	exact mass (g/mol)	refs
ochratoxin β	222.0528	52−54
α-ochratoxin	256.0139	52−55
α-ochratoxin amide	255.0298	47
14-decarboxy-ochratoxin A	359.0924	46−48,53
ochratoxin B	369.1212	52−55
ochratoxin B methyl ester	383.1369	52−54
ochratoxin B ethyl ester	397.1525	52,54
ochratoxin A	403.0823	52−54
4-hydroxyochratoxin A	419.0772	52−54
ethylamide ochratoxin A	430.1296	54−56
ochratoxin A glucose ester	565.1351	48,54
(22 → 6′) ochratoxin A-methyl-α-D-glucopyranoside ester	579.1507	48
ochratoxin A cellobiose ester	727.1879	48
ochratoxin A quinone	383.1005	54,57
ochratoxin A hydroquinone	385.1162	53,54,57
ochratoxin C	431.1136	52−55

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Table 2. Physicochemical Characterization of Grapes at Different Maturation Stages

physico-chemical characteristic	varieties	maturation stages[a] veraison	15 after veraison	ripeness
pH	Syrah	$3.4^{Aa} \pm 0.0$	$3.9^{Ab} \pm 0.0$	$4.4^{Bc} \pm 0.0$
	Touriga Nacional	$3.5^{Ba} \pm 0.0$	$3.9^{Ab} \pm 0.1$	$4.2^{Ac} \pm 0.0$
	Muscat Italia	$4.1^{Ca} \pm 0.0$	$4.3^{Bb} \pm 0.0$	$4.3^{Bc} \pm 0.0$
titratable acidity (g of tartaric acid/100 g)	Syrah	$1.5^{Bc} \pm 0.1$	$0.6^{Bb} \pm 0.0$	$0.4^{Aa} \pm 0.0$
	Touriga Nacional	$1.4^{Bc} \pm 0.1$	$0.8^{Cb} \pm 0.0$	$0.4^{Aa} \pm 0.0$
	Muscat Italia	$0.5^{Ab} \pm 0.0$	$0.4^{Aa} \pm 0.0$	$0.5^{Aab} \pm 0.0$
total soluble solids (%)	Syrah	$12.5^{Ba} \pm 0.0$	$17.5^{Cb} \pm 0.0$	$22.5^{Cc} \pm 0.0$
	Touriga Nacional	$7.5^{Aa} \pm 0.0$	$12.5^{Ab} \pm 0.0$	$17.5^{Ac} \pm 0.0$
	Muscat Italia	$15.0^{Ca} \pm 0.0$	$15.0^{Ba} \pm 0.0$	$20.0^{Bb} \pm 0.0$
total soluble solids/titratable acidity	Syrah	$8.3^{Aa} \pm 0.3$	$28.3^{Bb} \pm 1.2$	$57.2^{Bc} \pm 3.8$
	Touriga Nacional	$6.3^{Aa} \pm 1.6$	$16.3^{Ab} \pm 0.6$	$44.5^{Ac} \pm 3.0$
	Muscat Italia	$28.6^{Ba} \pm 0.0$	$41.2^{Cb} \pm 1.7$	$47.5^{Ab} \pm 3.9$
% pectic acid	Syrah	$0.5^{Aa} \pm 0.1$	$0.9^{ABb} \pm 0.0$	$0.9^{Ab} \pm 0.0$
	Touriga Nacional	$1.2^{Cb} \pm 0.0$	$1.1^{Bb} \pm 0.1$	$0.9^{Aa} \pm 0.1$
	Muscat Italia	$0.8^{Ba} \pm 0.1$	$0.8^{Aa} \pm 0.1$	$1.1^{Bb} \pm 0.0$
% total glycosides in glucose	Syrah	$9.4^{Ba} \pm 0.1$	$16.7^{Bb} \pm 0.0$	$21.0^{Bc} \pm 0.2$
	Touriga Nacional	$6.6^{Aa} \pm 0.0$	$12.8^{Ab} \pm 0.3$	$17.9^{Ac} \pm 0.4$
	Muscat Italia	$9.5^{Ba} \pm 0.0$	$13.2^{Ab} \pm 0.0$	$18.4^{Ac} \pm 0.2$
total anthocyanin (mg/100 g)	Syrah	$26.7^{Aa} \pm 2.3$	$69.7^{Bb} \pm 3.4$	$106.2^{Bc} \pm 5.4$
	Touriga Nacional	$19.3^{Aa} \pm 5.4$	$46.7^{Ab} \pm 0.6$	$82.2^{Ac} \pm 4.5$
	Muscat Italia	ND[b]	ND[b]	ND[b]
total phenolics (mg GAE/100 g)	Syrah	$87.9^{Ba} \pm 5.5$	$144.9^{Bb} \pm 14.8$	$134.4^{Bb} \pm 2.2$
	Touriga Nacional	$295.4^{Cc} \pm 2.7$	$145.7^{Ba} \pm 8.2$	$173.2^{Cb} \pm 5.5$
	Muscat Italia	$39.2^{Aa} \pm 1.8$	$56.4^{Aa} \pm 0.0$	$39.6^{Aa} \pm 2.0$
DPPH value (μM TE/100 g)	Syrah	$49.1^{Ba} \pm 6.2$	$64.8^{Bb} \pm 3.7$	$50.0^{Ba} \pm 4.9$
	Touriga Nacional	$157.1^{Cb} \pm 11.1$	$77.0^{Ba} \pm 3.7$	$86.6^{Ca} \pm 2.5$
	Muscat Italia	$27.3^{Aa} \pm 0.0$	$38.6^{Aa} \pm 1.2$	$27.3^{Aa} \pm 2.5$
ORAC value (μM TE/100 g)	Syrah	$5450.9^{Ba} \pm 87.0$	$9434.1^{Bc} \pm 279.6$	$7456.9^{Bb} \pm 883.2$
	Touriga Nacional	$27845.2^{Cb} \pm 672.8$	$8502.2^{Ba} \pm 116.0$	$9593.3^{Ca} \pm 27.0$
	Muscat Italia	$2468.6^{Aa} \pm 123.6$	$4014.0^{Ab} \pm 27.4$	$4889.6^{Ab} \pm 166.3$

[a]Average followed by lowercase letters compare the physicochemical characteristic in the line at maturation stages; and uppercase letters compare the physicochemical characteristic in the column at varieties. Different letters show a statistically significant difference at $p < 0.05$. [b]ND: not detected.

An aliquot of 10 μL of the obtained extract was diluted in 490 μL of methanol and homogenized in a vortex for 30 s. Then, a 1 μL formic acid 100% (Sigma-Aldrich, Darmstadt, Germany) was added, and direct injection of the extract was performed. Data were acquired in the survey scan mode and obtained in a positive mode in a mass range of 200−750 m/z, in five replicates.

Statistical Analysis. The evaluation of the influence of grape varieties at different maturation stages on OTA production by *A. carbonarius* and *A. niger* was done through the analysis of variance with a posthoc Tukey test. Principal component analysis (PCA) was used to demonstrate the correlation between OTA production and physicochemical characteristics of grapes. Partial Least Squares-Discriminant Analysis (PLS-DA), a multivariate regression method, was used as a tool to aid in the identification of modified mycotoxin. Online platform MetaboAnalyst 3.0, with the option Interquartile Range for data filtering and Normalization by a pooled sample from the group was used.[30,31] Identification of the compounds was made through the comparison of their exact mass available in databases: METLIN (Scripps Center for Metabolomics, La Jolla, CA, USA) and literature references. These compounds were identified in order that a maximum error of 2 ppm accuracy between the experimental and theoretical values was obtained.

RESULTS AND DISCUSSION

Physicochemical characteristics of the different grape varieties studied changed throughout maturation. An increase in pH (Syrah, 29.4%; Touriga Nacional, 20%; Muscat Italia, 4.9%), total soluble solids (Syrah, 80%; Touriga Nacional, 133.33%; Muscat Italia, 33.33%), and total glycosides in glucose (Syrah, 123.4%; Touriga Nacional, 171.2%; Muscat Italia, 93.7%) was observed. Total anthocyanin levels also increased throughout maturation (Syrah, 297.8%; Touriga Nacional, 325.9%); however, this was not observed for the white variety (Muscat Italia) at all stages, as expected. Percentage of pectic acid also increased for all varieties (Syrah, 80%; Muscat Italia, 37.5%), except for Touriga Nacional, which exhibited the highest amounts of the compound at veraison stage, whereas a remarkable reduction was observed throughout maturation (25%). Titratable acidity decreased for all varieties throughout maturation (Syrah, 73.3%; Touriga Nacional, 71.4%), except for Muscat Italia, which remains almost constant (Table 2).

Regarding the concentrations of total phenolic compounds and antioxidant activity (DPPH and ORAC), a different behavior was observed among varieties tested. In Syrah variety, an increase in the concentration of total phenolics compounds was found (52.9%). In Touriga Nacional variety, a reduction (50.7%) followed by an increase (18.9%) was observed, and it remained constant in Muscat Italy variety. For antioxidant capacity (DPPH value), despite a similar behavior to the one observed for the concentrations of phenolics compounds, a higher concentration was observed at 15 days after the beginning of veraison stage for Syrah variety (64.8 uM TE/100

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J. Agric. Food Chem. 2018, 66, 8824−8831

Table 3. Production of Ochratoxin A (μg/g) by *A. carbonarius* and *A. niger* in Grapes during Maturation

		maturation stages[a]		
species	varieties	veraison	15 after veraison	ripeness
A. niger	Syrah	$81.05^{Aa} \pm 0.82$	$188.97^{Ac} \pm 18.01$	$148.04^{Ab} \pm 18.26$
	Touriga Nacional	$73.44^{Aa} \pm 1.68$	$235.39^{Bb} \pm 18.06$	$235.52^{Bb} \pm 14.84$
	Muscat Italia	$106.83^{Aa} \pm 1.47$	$168.10^{Ab} \pm 2.53$	$246.74^{Bc} \pm 9.10$
A. carbonarius	Syrah	$96.87^{Ba} \pm 0.16$	$148.55^{Bb} \pm 4.14$	$93.93^{Aa} \pm 2.27$
	Touriga Nacional	$73.35^{Aa} \pm 4.53$	$144.39^{Bb} \pm 4.35$	$159.99^{Bb} \pm 3.78$
	Muscat Italia	$152.75^{Cb} \pm 3.17$	$105.36^{Aa} \pm 17.52$	$115.21^{Aa} \pm 11.39$

[a]Average followed by lowercase letters compare the OTA levels in the line at maturation stages; and uppercase letters compare the OTA levels in the column at varieties. Different letters show a statistically significant difference at $p < 0.05$.

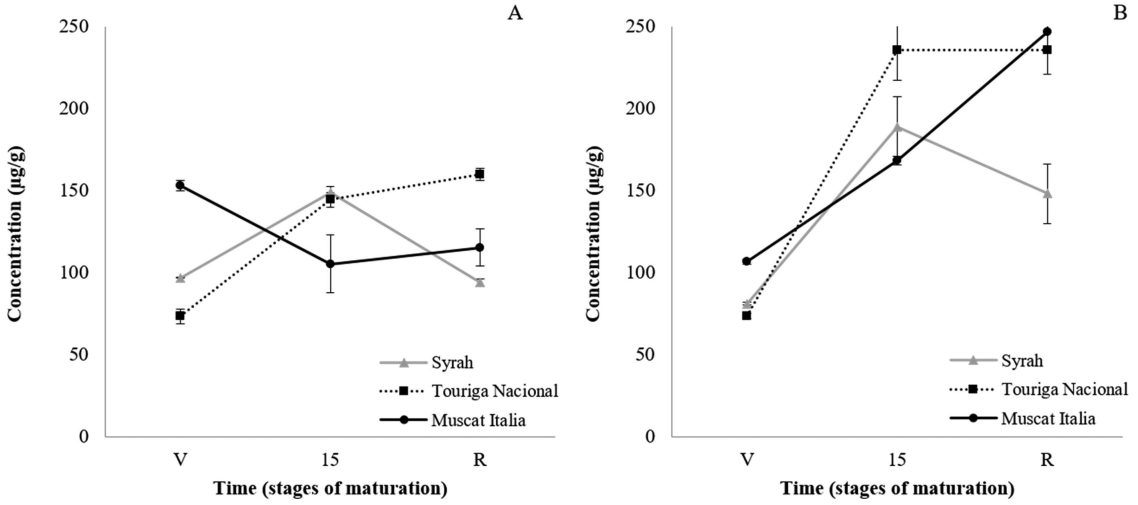

Figure 1. OTA production (μg/g) by *A. carbonarius* (A) and *A. niger* (B) strains in grapes over maturation.

g). A similar behavior between ORAC and DPPH was observed, except for Muscat Italy variety, which exhibited increased levels of ORAC over maturation (98.1%).

Levels of the physicochemical parameters obtained were also different among varieties considering the same maturation stage. Muscat Italia variety presented higher pH values (4.1 to 4.3) and, therefore, a lower percentage of titratable acidity (0.4 to 0.5 g of tartaric acid/100 g) at all maturation stages studied. Syrah variety showed a higher percentage of total soluble solids (12.5% to 22.5%) and total glycosides in glucose (9.4 to 21%) in most of the stages evaluated. A higher concentration of total anthocyanin was also observed in this variety (106.2 mg/100 g). Touriga Nacional variety showed the highest percentage of pectic acid in most stages (1.2 to 0.9%), except at ripeness stage, in which the highest percentage was observed for Muscat Italia (1.1%). Higher concentrations of total phenolics compounds and antioxidant capacity (DPPH value and ORAC value) were also observed in Touriga Nacional variety in most maturation stages evaluated, 295.4 to 145.7 mg GAE/100 g; 157.1 to 77.0 μM TE/100 g; 27,545.15 to 8,502.24 μM TE/100 g, respectively.

A higher OTA production by *A. niger* species was observed in all varieties and maturation stages, except in veraison stage, in which *A. carbonarius* produced higher levels of the mycotoxin (Table 3). Levels of OTA produced by *A. niger* ranged from 73.44 μg/g in Touriga Nacional variety at the veraison stage to 246.74 μg/g in Muscat Italia variety at ripeness stage. For *A. carbonarius*, the lowest OTA production was also observed at veraison stage for Touriga Nacional variety (73.35 μg/g); however, its highest production was detected in Touriga Nacional variety at ripeness stage (159.99 μg/g). Although most studies consider *A. carbonarius* the main OTA-producing species at higher levels, some strains of *A. niger* may be higher producers than *A. cabonarius*.[32] In addition to *A. niger* being a good competitor, the microorganism adapts extremely well to the environment present in vineyards and grapes.[33] Thus, the highest OTA production by this species in the grape varieties tested may be related to a better substrate adaptation. However, the best growth conditions are not always related to the higher production of secondary metabolites. These same isolates presented different behavior when evaluated in a grape-based culture medium in which *A. niger* produced lower amounts of OTA compared to *A. carbonarius*.[25] These results demonstrate the importance of experiments performed directly in the food.

Both *Aspergillus carbonarius* and *A. niger* species were able to produce OTA at all stages of maturation, although OTA levels observed were different. An increase in mycotoxin levels produced by *A. niger* throughout maturation was detected for all varieties (Touriga Nacional, 220.70%; Muscat Italia, 130.96%), except for Syrah, which exhibited the highest levels of OTA at the 15 days after the beginning of veraison stage (188.97 μg/g), whereas reduced levels of the compound were observed at ripeness stage (148.04 μg/g). Similar behavior was observed for OTA production by *A. carbonarius*, except for Muscat Italia variety, in which the highest levels of the mycotoxin were obtained at veraison stage (152.75 μg/g) (Figure 1).

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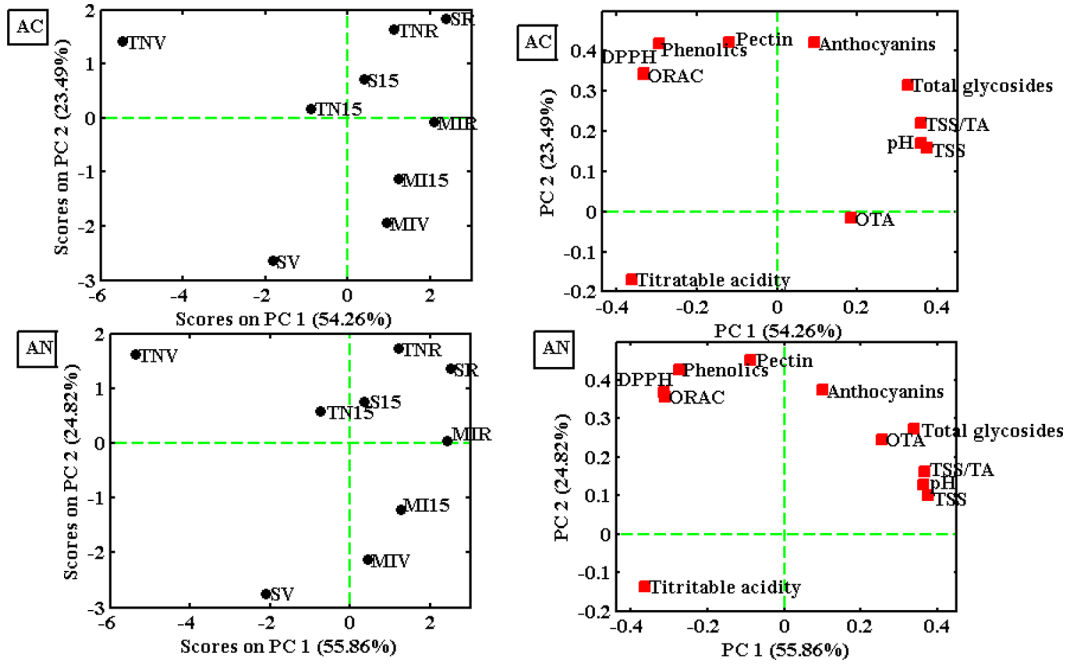

Figure 2. Principal components analysis for physicochemical characterization of grapes and OTA production by *A. carbonarius* (AC) and A. *niger* (AN), where TSS = total soluble solids; TSS-TA = total soluble solids/titratable acidity; V = veraison; 15 = 15 days after the beginning of veraison; R = ripeness; MI = Muscat Italia; S = Syrah; TN = Touriga Nacional.

Expression of strain biosynthetic genes responsible for mycotoxins production may be related to the modification of nutritional factors of the plant by the pathogen itself or natural chemical components present in the plant.[34] Complex interactions between intrinsic and extrinsic factors (geographic location, cultivation and management practices, the microclimate of the region, microbial competition, grape variety, and maturation stage) will determine both the growth of toxigenic fungi and OTA production.[35]

Due to changes that occur during maturation, the period between early veraison and harvest is critical for the growth of ochratoxigenic fungi and consequently for OTA production. Grape variety also has influence in this contamination.[36] Jiang et al.[37] detected different OTA levels in Thompson seedless (white, low seedless storability) (30.341 ng/mL), Kyoho (red, seeded, average storability) (13.807 ng/mL), and Red Earth (red, seeded, high storability) (0.5 ng/mL) grapes produced by *A. carbonarius*, artificially inoculated, and determined over 6 days at 25 °C. Presence of the fungus also led to changes in physicochemical features of the grapes due to nutritional requirements and action of its enzymatic complex in the hydrolysis of some compounds.

Lasram et al.[38] observed a higher OTA production by *A. carbonarius* in early veraison stage at Cabernet Sauvignon variety. At this stage, lower levels of sugar and higher levels of acidity were determined. Moreover, a reduction in mycotoxin levels throughout maturation was observed in which there was an increase in sugars and a reduction in acidity. However, an increase in OTA levels in the surmaturity stage was observed. In this stage, no significant change was observed for these physicochemical parameters, and therefore, the increase in mycotoxin levels may be related to facilitation of fungus penetration, probably due to the softening of the berries. Although we observed a similar result for Moscato Italia variety contaminated with *A. carbonarius* (24.58% reduction), an increase in OTA production was observed in the other varieties throughout maturation. This indicates an influence of grape variety on ochratoxin A production.

Even though higher levels of OTA have been detected in late maturation stages, this mycotoxin seems to be present since early stages. Therefore, although more advanced stages of maturation favor fungal growth, if the microorganism is present in early stages, the mycotoxin will most likely be produced.[9,38]

OTA levels were also different among grape varieties at the same maturation stage. Toxin levels produced by *A. niger* did not differ in veraison stage. After 15 days of the beginning of veraison stage, higher production of the mycotoxin was observed for Touriga Nacional variety (255.39 μg/g). At ripeness stage, the highest OTA levels were produced in Touriga Nacional (235.52 μg/g) and Muscat Italia (246.74 μg/g) varieties. For *A. carbonarius*, at veraison stage, the highest OTA production was observed in Muscat Italia variety (152.75 μg/g). After 15 days of the beginning of veraison stage, higher concentrations were observed for Syrah (148.55 μg/g) and Touriga Nacional (144.39 μg/g) varieties; and at ripeness stage for Touriga Nacional variety (159.99 μg/g).

The results indicate the influence of both variety and maturation stage of the grapes. Besides, fungal species also have a direct influence on the levels of mycotoxins produced. Therefore, principal component analysis (PCA) was performed to demonstrate a possible correlation between physicochemical parameters of the grapes and OTA production by *A. niger* and *A. carbonarius* (Figure 2). The data were autoscaled. The first four PC (principal components) explained 96.46% of the variability in data obtained for *A. carbonarius*, and the first two PC explained 80.68% of the variability in data obtained for *A. niger*. Through the principal component 1, a positive correlation was observed between the following physicochemical parameters: pH, total soluble solids, total glycosides in glucose and total anthocyanin, and OTA levels. Therefore, the higher the concentrations of these parameters, the greater the production of mycotoxin by *A. niger*. In contrast, a negative

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correlation was observed between the following physicochemical parameters: titratable acidity, pectic acid, total phenolic compounds, and antioxidant activity (DPPH value and ORAC value). Thus, the higher the concentration of these parameters, the lower the OTA production. According to PC1 evaluation, similar correlations were observed for OTA production by *A. carbonarius*, although such correlation appears to be stronger for *A. niger*.

Growth and production of metabolites by filamentous fungi are closely linked to substrate composition. Different carbon sources and levels of sugars available are closely related to induction of the production of enzymes responsible for hydrolysis and nutrients utilization.[39] OTA production by *A. carbonarius* was variable in the presence of different carbon sources.[40] A positive correlation between total pectin content of the grapes and *A. carbonarius* presence was evidenced.[3] In contrast, inhibition of pectinases production by *Aspergillus japonicus* was observed when glucose, sucrose, and pectin concentrations were excessive.[39] As observed in the present study, an increase in OTA production by *A. carbonarius* was directly proportional to an increase in pH up to 4.0.[40] OTA production by this species was also affected by pH and chemical parameters of grape at the different maturation stages.[38] Therefore, stimulation or inhibition of fungal growth and production of primary and secondary metabolites is directly related to the levels that these compounds are found in the substrate.

Some antioxidant compounds had a positive correlation with OTA production by several species belonging to the genus *Aspergillus*, whereas other species showed a negative correlation, suggesting the role of structure-dependent signals.[41] The concentration of these compounds will also influence mycotoxin production.[42]

Although a positive correlation has been previously demonstrated between *A. niger* and phenolic compounds [(−)-epicatechin, procyanidin A2, procyanidin B2, rutin, isohamnetin-3-*O*-glycoside, and phenolic and gallic acids] and between *A. carbonarius* and (−)-gallate epicatechin present in grapes,[3] in our work, a negative correlation between OTA production and phenolic compounds was observed, which indicates a possible antagonistic effect of the some grapes phenolic compounds against the production of fungal metabolites. Even though, mycotoxin production was not inhibited. However, the contamination of grapes by fungi of the genus *Aspergillus* is very common. This paradox indicates that the relationship between the physicochemical composition of the grapes and their contamination by toxigenic fungi seems to be a complex issue.[41]

In addition to OTA production, some toxigenic fungi are also able to produce modified mycotoxin through their enzymatic complex, which acts on the metabolism of the parent mycotoxin.[25,43] Moreover, plants are also able to modify parent mycotoxins through their defense mechanisms (phase I and II metabolism).[12] To search for these metabolites we used high-resolution mass spectrometry (HRMS) and PLS-DA.

The loadings plot of the statistical model formed by features selected by PLS-DA indicated the 65 main candidate biomarkers. From this list of ions, a search for modified mycotoxins was performed. Only two candidate OTA derivatives were identified among the elected targets (Table 1). Modified ochratoxins were identified only in the tests performed with *A. niger*. Candidate biomarkers identified were 14-decarboxy-ochratoxin A ($[M + Na]^+$: 382.0815) in Muscat Italia variety at maturation stages: veraison and 15 days after the beginning of veraison and ethylamide-ochratoxin A ($[M + K]^+$: 469.0939) in the Syrah variety at ripeness stage.

Candidate ethylamide-ochratoxin A was previously identified in the grape-based medium after *A. niger* inoculation throughout 21 days. It is possible that such a molecule may have been formed from the metabolism of the fungus itself.[25] *Fusarium* spp. has already demonstrated the ability to form ZEN sulfates through the metabolism of parent mycotoxin (ZEN).[44]

However, some metabolites can also be formed from the interaction between mycotoxigenic fungus and host, due to an effort of the plant for detoxification.[13] Seven putative phase I and 18 putative phase II metabolites of ZEN were identified by Rolli et al. in durum wheat.[45] The reductive and oxidative hydroxylation, followed by glycosylation and malonyl-conjugation, are major biotransformation pathways of ZEN as a response to wheat detoxification.[45]

Some molecules searched in this work were initially described as OTA derivatives formed from thermal processes: ochratoxin α, ochratoxin α-amide, 14-decarboxy-ochratoxin A, and 14-(*R*)-ochratoxin A.[46,47] Moreover, it is possible that the OTA binds to food components. The conjugating compounds, ochratoxin A disaccharide esters and ochratoxin A monosaccharide esters, were identified during coffee roasting.[48] Therefore, the formation of modified OTA can occur throughout the production chain: still in the field, by microorganisms or by processing.[49] However, based on our findings, it is not possible to ensure that the metabolites identified (ethylamide-ochratoxin A and 14-decarboxy-ochratoxin A) have been produced by the fungus metabolism or as a plant defense mechanism.

OTA derivatives including ochratoxin α, 4*S*-hydroxyochratoxin A, 4*R*-hydroxychratoxin A, hydroxychratoxin A-β-glucoside, ochratoxin A methyl ester, and other unidentified polar metabolites have been previously detected in plant-cell suspension cultures (carrots, tomatoes, cotton, soybeans, wheat, barley, and potatoes) contaminated with OTA. The metabolism of OTA occurred at different times in the cultures evaluated. Production of enzymes specifically capable of metabolizing the ochratoxin may be responsible for this difference.[50] Therefore, it is possible that the detoxification process of OTA in plants is related to the performance of enzymes in the conjugation or cleavage of the ochratoxin forming modified ochratoxin.[25]

Although the formation of these compounds, still in the plant, has the main purpose of detoxification, some of them, such as hydroxyochratoxin A-β-glucoside, can be cleaved by humans and animal metabolism or through food processing, releasing the parent mycotoxin and increasing the overall toxicity of food.[50]

It is interesting to highlight that 14-decarboxy-ochratoxin A, a degradation compound, appeared at the initial stages of maturation in which OTA levels were lower (106.83 μg/g in veraison and 168.10 μg/g in 15 days after the begging of veraison) when compared to others. However, ethylamide-ochratoxin A, a conjugation compound, appeared in the late stage of maturation with an intermediate OTA level (148.04 μg/g). A correlation between OTA levels and the presence of modified ochratoxin cannot be made yet. However, some studies indicate that the correlation between parent mycotoxin and modified mycotoxin may exist up to a threshold concentration of parent mycotoxin, and above that, parent

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J. Agric. Food Chem. 2018, 66, 8824−8831

mycotoxin conversion might be reduced. Therefore, it is possible the there is a limitation in plant defense metabolism when it is highly contaminated by mycotoxin.[51] It is possible that other physicochemical parameters not evaluated in this study may affect grapes contamination by toxigenic fungi and, consequently, OTA production. Moreover, the species also behave differently in the same substrate.

According to our findings, the production of OTA can occur from the initial stages of maturation, which indicates the importance of adoption of good agricultural and production practices throughout all stages of production and not only near the harvest, as occurs in some vineyards. In addition, it is of fundamental importance that predictive analyzes and risk assessments cover a higher number of species and different grape varieties to assist in a better understanding of the ecosystem related to the growth and production of secondary metabolites by fungi in the wine environment, thus ensuring safety and quality of grapes and their derivatives.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b02251.

Spectra containing the precursor ion and their respective products ions (PDF)

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Notes

The authors declare no competing financial interest.

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Influence of maturation stages in different varieties of wine grapes (*Vitis vinifera*) on the production of Ochratoxin A and its modified forms by *Aspergillus carbonarius* and *Aspergillus niger*

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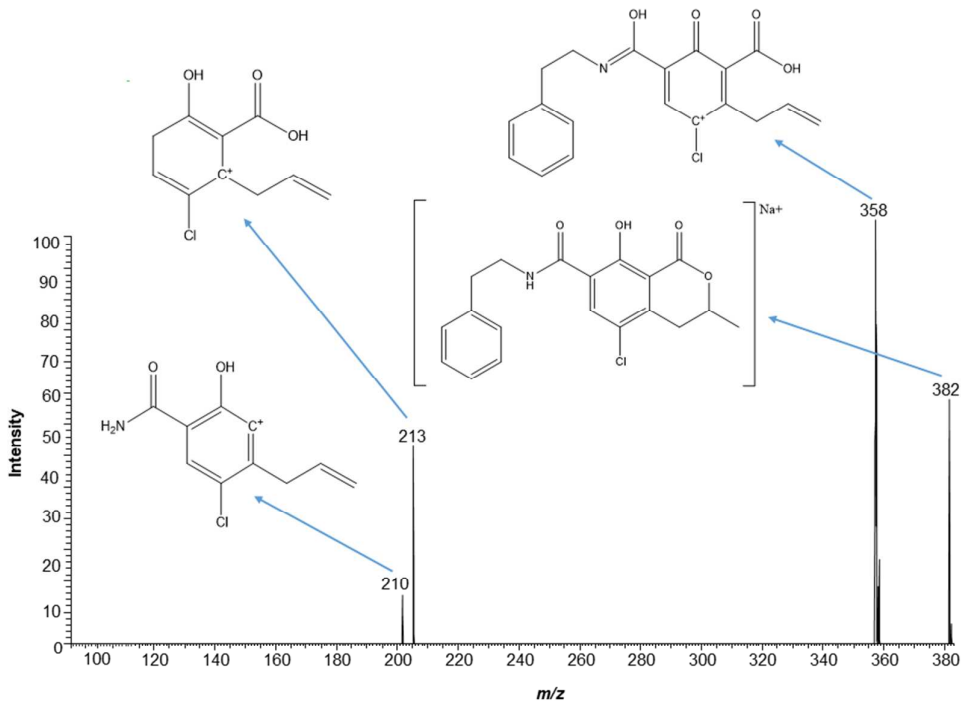
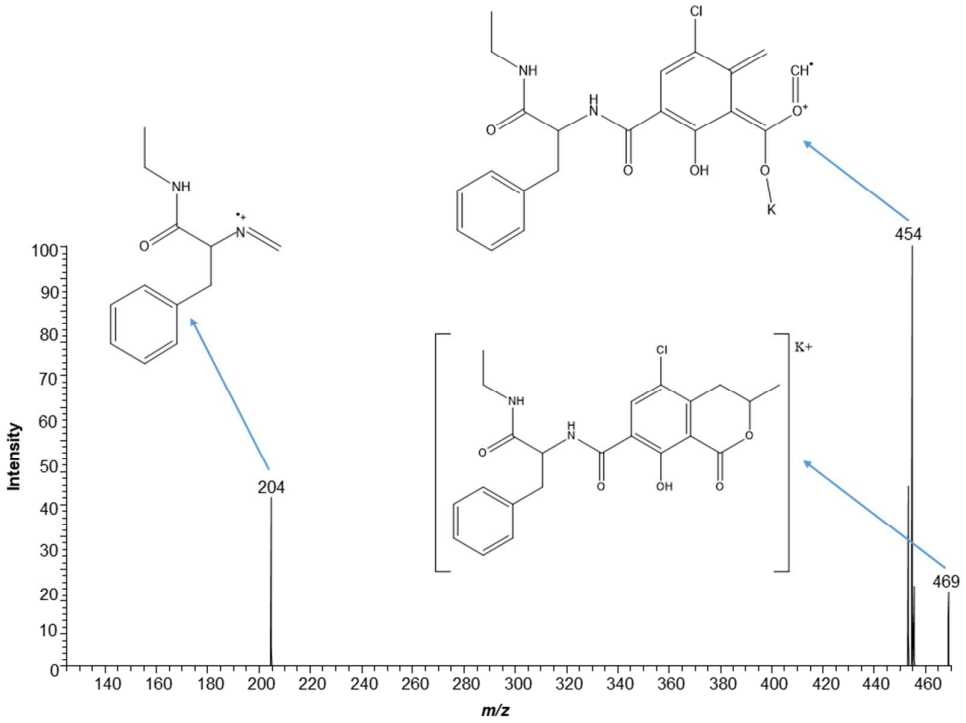
Supporting Information Table of Contents

S1- MS/MS reaction of the 14-decarboxy-ochratoxin A ([M + Na]$^{+}$=382) (Collision-induced dissociation (CID) energy: 120 eV).



S2- MS/MS reaction of the ethylamide-ochratoxin A ($[M + K]^+$=469) (Collision-induced dissociation (CID) energy: 45 eV).



CAPÍTULO 4

The presence of ochratoxin A does not influence *Saccharomyces cerevisiae* growth kinetics but leads to the formation of modified ochratoxins.

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The presence of ochratoxin A does not influence *Saccharomyces cerevisiae* growth kinetics but leads to the formation of modified ochratoxins

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ABSTRACT

Yeasts are able to reduce the levels of ochratoxin A in fermentative processes; and, through their enzymatic complex, these micro-organisms are also capable of forming modified mycotoxins. These mycotoxins are often underreported, and may increase health risks after ingestion of contaminated food. In this sense, this study aims to evaluate whether the presence of ochratoxin A influences yeast growth kinetic parameters and to elucidate the formation of modified ochratoxin by *Saccharomyces cerevisiae* strains during fermentation. Three *S. cerevisiae strains* (12 M, 01 PP, 41 PP) were exposed to OTA at the concentrations of 10, 20 and 30 μg/L. The Baranyi model was fitted to the growth data (Log CFU/mL), and the identification of modified ochratoxins was performed through High Resolution Mass Spectrometry. The presence of ochratoxin A did not influence the growth of *S. cerevisiae* strains. Four pathways were proposed for the metabolization of OTA: dechlorination, hydrolysis, hydroxylation, and conjugation. Among the elected targets, the following were identified: ochratoxin α, ochratoxin β, ochratoxin α methyl ester, ochratoxin B methyl ester, ethylamide ochratoxin A, ochratoxin C, hydroxy-ochratoxin A, hydroxy-ochratoxin A methyl ester, and ochratoxin A cellobiose ester. These derivatives formed from yeast metabolism may contribute to the occurrence of underreporting levels of total mycotoxin in fermented products.

1. Introduction

Ochratoxin A (OTA) is a polyketide-derived secondary metabolite formed by a para-chlorophenolic moiety containing a dihydro-isocoumarin group linked with phenylalanine via a peptide bond (Malir et al., 2016). This mycotoxin is produced by some fungal species belonging to the genera *Penicillium* and *Aspergillus* (Freire et al., 2017; el Khoury and Atoui, 2010), and has been detected in a number raw materials such as coffee, grapes, corn, barley, malt, cocoa, and wheat, as well as products such as beer, wine, chocolate, and breads (Freire et al., 2017; Kawashima et al., 2007; Riba et al., 2008). Due to its stability, OTA is commonly found in processed food products made with contaminated raw materials, and may cause hepatotoxic, neurotoxic, carcinogenic, teratogenic, immunotoxic, and nephrotoxic effects in several animal species after ingestion (el Khoury and Atoui, 2010). Such effects are related to OTA roles in inhibition of protein synthesis, inhibition of cellular energy production, induction of oxidative/nitrosative stress, apoptotic and necrotic cell death, and induction of cell cycle arrest (Malir et al., 2016). Due to these effects, OTA was classified by the International Agency for Research on Cancer (IARC) as a group 2B: possible human carcinogen (IARC, 1993).

Fungal contamination and mycotoxin production in foods may occur at all stages of production and processing, and is dependent on factors such as temperature, relative humidity, presence of insects, application of fungicides and pesticides, microbial competition, water activity, pH, presence of antimicrobial substances, nutrient availability, and substrate structure (Anfossi et al., 2016; Yogendrarajah et al., 2014). Although preventive agricultural practices are adopted, it is not always possible to prevent fungal growth and subsequent production of mycotoxins (Aldred and Magan, 2004). As an alternative to reduce the risk due to the presence of OTA in food, several physical, chemical, and biological strategies have been developed for the degradation or adsorption of this mycotoxin (Massoud et al., 2018). However, the efficiency of these processes is closely correlated with mycotoxin concentration and food composition (Massoud et al., 2018). Additionally, detoxification processes may also lead to the formation of modified mycotoxins, which may also be characterized as potential health hazards (Nathanail et al., 2016).

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Modified mycotoxins may be formed at different stages of food production: in the processing (Berthiller et al., 2013), and through both plant (Freire et al., 2018a) and microorganisms' metabolism (Freire et al., 2018b). Furthermore, recent studies demonstrated that these compounds might also be formed during animal metabolism (Broekaert et al., 2015). Modified mycotoxins present changes in their structure, polarity and solubility, and may not be detected by traditional analytical methods used in the detection of the parent mycotoxin. This impaired assessment, therefore, may result in the underreporting of total mycotoxin levels present in the final product (Berthiller et al., 2013). Thus, this evidences the need for sensitive, selective techniques capable of not only detecting, but also elucidating the structures of various metabolites, even at low concentrations. According to Freire and Sant'Ana (2018), the use of high-performance liquid chromatography coupled to mass spectrometry, as well as the development of new extraction and cleanup techniques, have been fundamental to identify new metabolites and quantify the total mycotoxins in foods. Taking a step further, direct-infusion high-resolution mass spectrometry (DI-HRMS) emerges as an effective strategy to identify and characterize target compounds in complex samples, given its versatility, high-throughput, and simplicity in operation.

Among the biodegradation processes of mycotoxins, yeasts have been widely used in OTA detoxification for fermentative processes due to their defense mechanism and enzymatic complex performance in the hydrolysis and conjugation of mycotoxin (Cecchini et al., 2019; Chen et al., 2018; Petruzzi et al., 2015; Zhang et al., 2016). Nevertheless, the metabolism of these microorganisms may also act in the reconversion of modified mycotoxins present in the raw material, releasing the parent mycotoxin through deconjugation processes, thereby elevating the levels of free mycotoxins in the food (Berthiller et al., 2013). On the other hand, it is not clear whether mycotoxins influence the growth of yeast strains used in fermentative processes, affect their physiological state, resulting in reduced productivity (Jakopović et al., 2018; Kłosowski et al., 2010).

Although some OTA derivatives generated during the biodegradation processes, such as ochratoxin alpha and ochratoxin B, have lower toxicity, other metabolites, such as opened-lactone OTA, presents higher toxicity when compared to the parent mycotoxin (Wu et al., 2011). Moreover, the mechanism of action of OTA and its modified forms in humans and animals still remains unclear (Malir et al., 2016). Therefore, elucidating whether modified ochratoxins are formed during fermentative processes is critical for understanding and managing food contamination and employing preventive strategies.

Although the ability of yeast cells in degrading and adsorbing OTA is known (Cecchini et al., 2019; Chen et al., 2018; Petruzzi et al., 2015; Zhang et al., 2016), the formation of modified ochratoxins throughout fermentation processes has not been investigated. To date, the main derivative identified was ochratoxin α. Assessing the risk of co-occurrence of modified mycotoxins with the parent mycotoxin in food products is a major challenge that must be addressed by regulatory agencies, food producers, control authorities, and the scientific community to protect human and animal health (Stoev and Denev, 2013). In this sense, this study aims to evaluate whether the presence of OTA in fermentation broth influences yeast growth kinetic parameters and to elucidate the formation of modified ochratoxins by *Saccharomyces cerevisiae* strains during fermentation.

2. Material and methods

2.1. Inoculum preparation and fermentation

S. cerevisiae strains were previously isolated from vineyards (Mendes et al., 2017). The strains (12 M, 01 PP, and 41 PP) were reactivated in Yeast, peptone and dextrose agar (YPD agar) [(0.5% yeast extract (Acumedia, Lansing, Michigan, USA), 1% bacteriological peptone (Acumedia, Lansing, Michigan, USA), 2% dextrose (Dinâmica, Diadema, Brazil) and 2% agar (Acumedia, Lansing, Michigan, USA)] for 48 h. Pre-cultures were prepared on a substrate of 5% glucose (Sigma-Aldrich, Darmstadt, Germany) and 0.7% yeast nitrogen base (YNB) (Sigma-Aldrich, Darmstadt, Germany), and shaken on a rotary shaker (Series 25 Shaker/Incubator, New Brunswick Scientific, USA) at 120 rpm for 24 h at 25 °C. The final concentration of cells in the broth was determined in Neubauer's chamber (Sigma-Aldrich, Darmstadt, Germany). An inoculum of 5×10^5 cells/mL was used. Cultures were then inoculated individually into broth containing 6.7% YNB, 20% glucose, 0.1% diammonium phosphate (Synth, Diadema, Brazil), and adjusted to pH 3.6 with tartaric acid (Ecibra, São Paulo, Brazil), according to Angioni et al. (2007).

The fermentation broth was spiked with an appropriate volume of OTA standard solution (100 μg/mL) (Sigma-Aldrich, Darmstadt, Germany) so that final OTA concentrations in the broth were:10, 20 and 30 μg/L. These concentrations were chosen based on the highest levels of OTA already detected in fermented products (CE, 2002). After inoculation, for each strain, the culture media were divided into 500-mL flasks, as two 250-mL replicates. Two controls were prepared for each yeast and OTA concentration studied. The first control comprised of inoculated medium with individual yeast strains, but not spiked with OTA. The second control comprised of OTA-spiked medium, but not inoculated with the individual yeasts. Samples were then incubated in the absence of light at 25 °C and collected at time points of 0, 2, 4, 7, 10, 16, 22, 28, 38, 48 and 58 h for growth evaluation, and at time points 0, 1, 3, 7 and 14 days (end of fermentation), measured by mass loss (Cecchini et al., 2006) for detection of modified ochratoxins.

2.2. Growth assessment and mathematical modeling

The model of Baranyi et al. (1993) (Equations (1)–(3)) was fitted to the growth data (log CFU/mL) using the DMFit add-in for Excel. The growth rate (μ_{max}, 1/h), maximum population (Rg, log CFU/mL) and lag phase (λ, h) of each yeast strain were estimated after the model fit.

$$\ln(N(t)) = \ln(N_0) + \mu_{max} A(t) - \ln\left[1 + \frac{e^{\mu_{max} A(t)} - 1}{e^{(N_{max} - N_0)}}\right] \quad (1)$$

$$A(t) = t + \frac{1}{\mu_{max}} \ln\left(\frac{e^{(-\mu_{max} t)} + q_0}{1 + q_0}\right) \quad (2)$$

$$\lambda = \frac{\ln\left(1 + \frac{1}{q_0}\right)}{\mu_{max}} \quad (3)$$

where: t = time (h), N(t) = number of microorganisms at time (t) (CFU/mL), N_0 = number of microorganisms at time zero (CFU/mL), μ_{max} = maximum growth rate (1/h), and N_{max} = maximum number of microorganisms (CFU/mL), q_0 [-] = parameter expressing the physiological state of the cells when $t = t_0$, λ = lag time (h).

The evaluation of *S. cerevisiae* strains' cell viability was performed by counting in the Neubauer chamber after sample dilution in an indicator solution (Methylene Blue dye). Cells with high physiological activity are not stained, while inactive (dead) cells are stained in blue. Viability was calculated by the equation: viability index (%) = number of colorless cells/(number of colorless cells + number of colored cells) (Lee et al., 1981).

2.3. Identification of modified ochratoxins by high-resolution mass spectrometry (HRMS)

For the detection of modified ochratoxins formed by the three yeast strains, the fermentation broth was filtered through 0.22 μm PVDF filter membranes units (Jet Biofil, Guangzhou, China) (Tafuri et al., 2008); 10 μL of the filtrate were diluted in 490 μL of methanol and homogenized in the vortex for 30 s. Then, 1 μL formic acid 100% (Sigma-Aldrich, Darmstadt, Germany) was added, and the sample was

submitted to DI-HRMS. An ESI-LTQ-XL Orbitrap Discovery mass spectrometer (Thermo Scientific, Bremen, Germany) with a nominal resolution of 30,000 (FWHM) was used to acquire data in survey scan mode. Parameters were as follows: flow rate of 10 µL/min, capillary temperature 280 °C, spray current of 5 kV, and five arbitrary units of sheath gas. Data were obtained in the positive mode using a mass range of 200–750 *m/z*, in quintuplicates. The following targets were monitored: ochratoxin β (222.0528 g/mol); ochratoxin α (256.0139 g/mol); ochratoxin α methyl ester (270.0295 g/mol); decarboxy ochratoxin A (359.0924 g/mol); ochratoxin B (369.1212 g/mol); ochratoxin A quinone (383.1005 g/mol); ochratoxin B methyl ester (383.1369 g/mol); ochratoxin A hydroquinone (385.1162 g/mol); ochratoxin B ethyl ester (397.1525 g/mol); ochratoxin A (403.0823 g/mol); ochratoxin A methyl ester (417.0979 g/mol); hydroxy-ochratoxin A (419.0772 g/mol); ethylamide ochratoxin A (430.1296 g/mol); ochratoxin C (431.1136 g/mol); hydroxy-ochratoxin A methyl ester (433.0928 g/mol); ochratoxin A glucose ester (565.1351 g/mol); ochratoxin A-methyl-α-glucopyranoside ester (579.1507 g/mol) and ochratoxin A cellobiose ester (727.1879 g/mol).

2.4. Statistical analyses

Analysis of variance (ANOVA), with *a posteriori* Tukey test, was used to evaluate significance in the difference among growth parameters of *S. cerevisiae* strains in fermentation broth as a function of presence and absence of OTA. In order to aid in the identification of modified ochratoxins, the multivariate regression method partial least squares discriminant analysis (PLS-DA) was performed using the online platform MetaboAnalyst 4.0 (Chong et al., 2018; Xia and Wishart, 2011). Interquartile Range was used for data filtering. Data normalization was performed through Quantile normalization.

2.5. Compound characterization

Lipid MAPS (University of California, San Diego, CA) and METLIN (Scripps Center for Metabolomics, La Jolla, CA) online databases, as well as relevant literature references, were consulted for identification of compounds of interest. Structural proposals were provided through mass accuracy, with a maximum considered mass shift of 2 ppm.

3. Results and discussion

3.1. Modeling the growth of S. cerevisiae strains

The growth kinetics obtained for strains 12 M, 01 PP and 41 PP in the absence and presence of OTA showed similar behavior, regardless of the OTA concentration. Nevertheless, variation among strains is observed ($p < 0.05$) (Fig. 1). Table 1 shows the growth kinetic parameters (μ_{max}, λ and Rg) of *S. cerevisiae* strains, estimated after fitting the Baranyi model to the data. An excellent fit of the Baranyi model and a low relative standard deviation is observed, which indicates good repeatability of the experiments (Table 1). Although all yeast strains showed an increase of 2 log cycles (10^7 cells/mL) throughout the fermentation, strain 12 M had a lower growth rate (μ) (0.08–0.12 h^{-1}) and a higher lag phase (λ) (3.8–6.0 h) when compared to strains 01 PP (μ: 0.19–0.23 h^{-1}; λ: 1.5–2.4 h) and 41 PP (μ: 0.15–0.18 h^{-1}; λ: 1.1–2.6 h) in all trials ($p < 0.05$). The maximum population was similar across strains in the presence of OTA, whereas in the control trials strain 12 M had a higher maximum population (7.4–7.6 Log CFU/mL) than strains 01 PP (7.2–7.4 Log CFU/mL) and 41 PP (7.2–7.5 Log CFU/mL) ($p < 0.05$). Additionally, strain 12 M had a longer exponential and stationary phases, reaching 58 h of growth, whereas strains 01 PP and 41 PP already enter the declining phase after 28 h of fermentation.

Through the viability index of yeast cells throughout the fermentation (58 h), it is possible to observe that cell viability is almost constant across all trials for strain 12 M, up to 58 h of the fermentative process (100-85%) (Fig. 2). For strains 01 PP (100–30%) and 41 PP (100–33%), a significant drop is observed after 28 h of fermentation, when these strains enter the decline phase. In some cases, it was not possible to measure the viability index of these strains after 38/48 h, as they were below the count detection limit in the Neubauer chamber. These findings corroborate the values determined for growth parameters after fitting the Baranyi model to data, and indicate the existence of variation between strains, proving the importance of using more than one strain in the experiments in general, so that the results better represent reality (Freire et al., 2018b). Even if subjected to similar conditions, the variation between strains is an inherent phenomenon of the microorganisms (Whiting and Golden, 2002). Cell history, physiological state, genetic and phenotypic variability, and diversity between strains belonging to the same species will influence fitness and robustness of strains (den Besten et al., 2017). Therefore, overestimation or underestimation of the predicted data may occur if the model fitting is obtained from data a single strain that is not

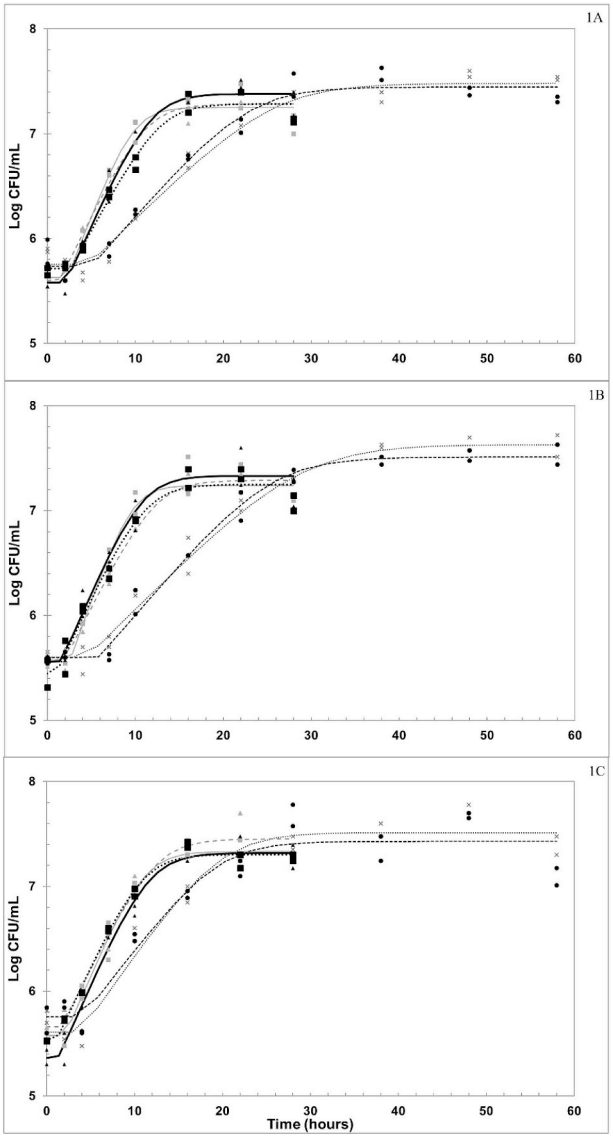

Fig. 1. Growth kinetics of *S. cerevisiae* strains over time in fermentation broths: in the absence of OTA (strains: ×12 M; ····· 12 M after Baranyi model fitting; ■ 01 PP; — 01 PP after Baranyi model fitting; ▲ 41 PP; – – 41 PP after Baranyi model fitting) and presence of OTA (strains: ● 12 M; - - - 12 M after Baranyi model fitting; ▲ 01 PP; ▬ 01 PP after Baranyi model fitting; ■ 41 PP; •••• 41 PP after Baranyi model fitting) at concentrations 10 (1A), 20 (1B) and 30 (1C) µg/L.

Table 1
Growth parameters of *S. cerevisiae* strains obtained after fitting to the Baranyi model.

Growth parameters	Strains	Control		OTA (10 μg/L)		Control		OTA (20 μg/L)		Control		OTA (30 μg/L)	
Growth rate (μ) (1/h)	12 M	0.08^{Aa}	± 0.01	0.09^{Aa}	± 0.00	0.08^{Aa}	± 0.00	0.10^{Aa}	± 0.01	0.12^{Aa}	± 0.01	0.11^{Aa}	± 0.00
	01 PP	0.22^{Ba}	± 0.04	0.19^{Ba}	± 0.03	0.23^{Ba}	± 0.03	0.19^{Ba}	± 0.02	0.20^{Ba}	± 0.00	0.19^{Ba}	± 0.03
	41 PP	0.17^{Ba}	± 0.01	0.15^{ABa}	± 0.00	0.17^{Ba}	± 0.03	0.18^{Ba}	± 0.03	0.17^{Ba}	± 0.02	0.18^{Ba}	± 0.00
Lag phase (λ) (h)	12 M	4.6^{Ba}	± 0.3	5.0^{Ba}	± 0.9	4.5^{Ba}	± 0.0	6.0^{Bb}	± 0.1	3.8^{Ba}	± 0.0	4.0^{Ba}	± 0.3
	01 PP	2.4^{Aa}	± 0.7	2.1^{Aa}	± 0.2	2.3^{Ab}	± 0.6	1.5^{Aa}	± 0.1	2.1^{Ab}	± 0.4	1.3^{Aa}	± 0.3
	41 PP	1.3^{Aa}	± 0.4	2.6^{Aa}	± 0.4	1.9^{Ab}	± 0.2	1.0^{Aa}	± 0.0	1.7^{Aa}	± 0.0	1.1^{Aa}	± 0.0
Maximum population (Rg) (CFU/mL)	12 M	7.5^{Ba}	± 0.0	7.4^{Aa}	± 0.0	7.6^{Ba}	± 0.1	7.5^{Aa}	± 0.1	7.5^{Ba}	± 0.0	7.4^{Aa}	± 0.0
	01 PP	7.2^{Aa}	± 0.0	7.4^{Aa}	± 0.1	7.2^{Aa}	± 0.0	7.3^{Aa}	± 0.2	7.3^{Aa}	± 0.0	7.3^{Aa}	± 0.0
	41 PP	7.3^{Aa}	± 0.1	7.3^{Aa}	± 0.0	7.5^{Aa}	± 0.1	7.2^{Aa}	± 0.0	7.5^{ABb}	± 0.1	7.3^{Aa}	± 0.1
R^2	12 M	0.98		0.97		0.98		0.98		0.96		0.92	
	01 PP	0.97		0.98		0.96		0.96		0.98		0.98	
	41 PP	0.97		0.98		0.97		0.96		0.97		0.99	

*Means followed by uppercase letters compare the values of each growth parameter between the strains in the column, and averages followed by the lowercase letters compares the value of the growth parameter between the control and the OTA contaminated assay for each concentration. Different letters show a statistical difference at $p < 0.05$.

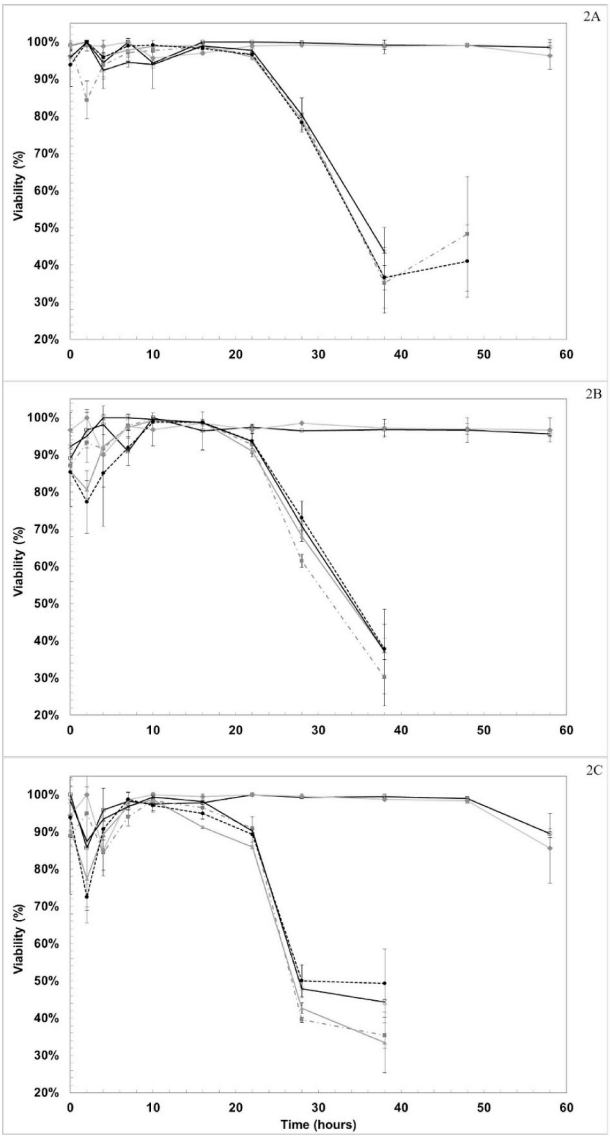

Fig. 2. Viability of *S. cerevisiae* strains' cells over time in fermentation broths: in the absence of OTA (strains: 12 M; 01 PP; 41 PP) and presence of OTA (strains: 12 M; 01 PP; 41 PP) at concentrations 10 (2A), 20 (2B) and 30 (2C) μg/L.

representative of most strains of the same species (Romero et al., 2010). Also, *S. cerevisiae* strains are commonly used in various fermentative processes (Jakopović et al., 2018), and these variations among strains may reflect the standardization of these processes.

The presence of 10 μg/L of OTA did not influence the lag phase for any of the strains. In the presence of 20 μg/L of OTA, strain 12 M presented a higher lag phase (6.0 h) when compared to the control, while the opposite was observed for strain 01 PP (1.5 h) and 41 PP (1.0 h), in which a higher lag phase is observed in control. In the presence of 30 μg/L of OTA, only the second strain presented a lower lag phase (1.3 h) when compared to the control. The growth rate (μ) of the three yeast strains assessed were neither affected by the presence of OTA in the fermentation broth, nor by the different concentrations of this mycotoxin. The same occurred for the maximum population, except for strain 41 PP in the presence of 30 μg/L of OTA, in which the control group presented a higher maximum population (7.31 Log CFU/mL) ($p < 0.05$).

In corn fermentation, the presence of 177.5 μg/L of OTA affects the performance of *S. cerevisiae* (Kłosowski et al., 2010). However, the assessed concentration is much higher than that usually found in fermented foods naturally contaminated with OTA (Freire et al., 2017; Kawashima et al., 2007; Riba et al., 2008). In contrast, growth during fermentation by *S. cerevisiae* and *Kloeckera apiculata* strains were not affected in the presence of OTA (6.0 μg/L) (Angioni et al., 2007). Similar results were found by Cecchini et al. (2006) and Jakopović et al. (2018). Although some authors (Donèche, 1993; Dziuba et al., 2007) demonstrate a negative influence of metabolites produced by fungi on the growth of yeasts in fermentative processes, OTA does not seem to be one of these metabolites for the strains evaluated in our study.

According to Boeira et al. (2000), the inhibitory effect on the growth of *S. cerevisiae* strains is dependent on the type and concentration of the mycotoxin, the evaluated strain, and incubation time and temperature. Moreover, it is possible that cell integrity, composition of yeast cell walls and the ability to bind to mycotoxin are determinant factors in strain sensitivity or insensitivity (Jakopović et al., 2018; Piotrowska and Masek, 2015). Chemical stressors may induce structural changes in proteins, resulting in dysfunctional cell compartments, which impact growth ability (Jakopović et al., 2018; Holubářová et al., 2000). Probably, the tested yeast strains can adapt to the presence of OTA; some yeast genes respond by coding for greater resistance, using stress response pathways, mycotoxin degradation mechanisms and DNA repair (Jakopović et al., 2018; Ianiri et al., 2013). The difference found between some evaluated parameters is possibly related to experimental variability. If there is any influence by mycotoxin presence, it varies from strain to strain, which may either favor or delay the adaptation phase of the strain.

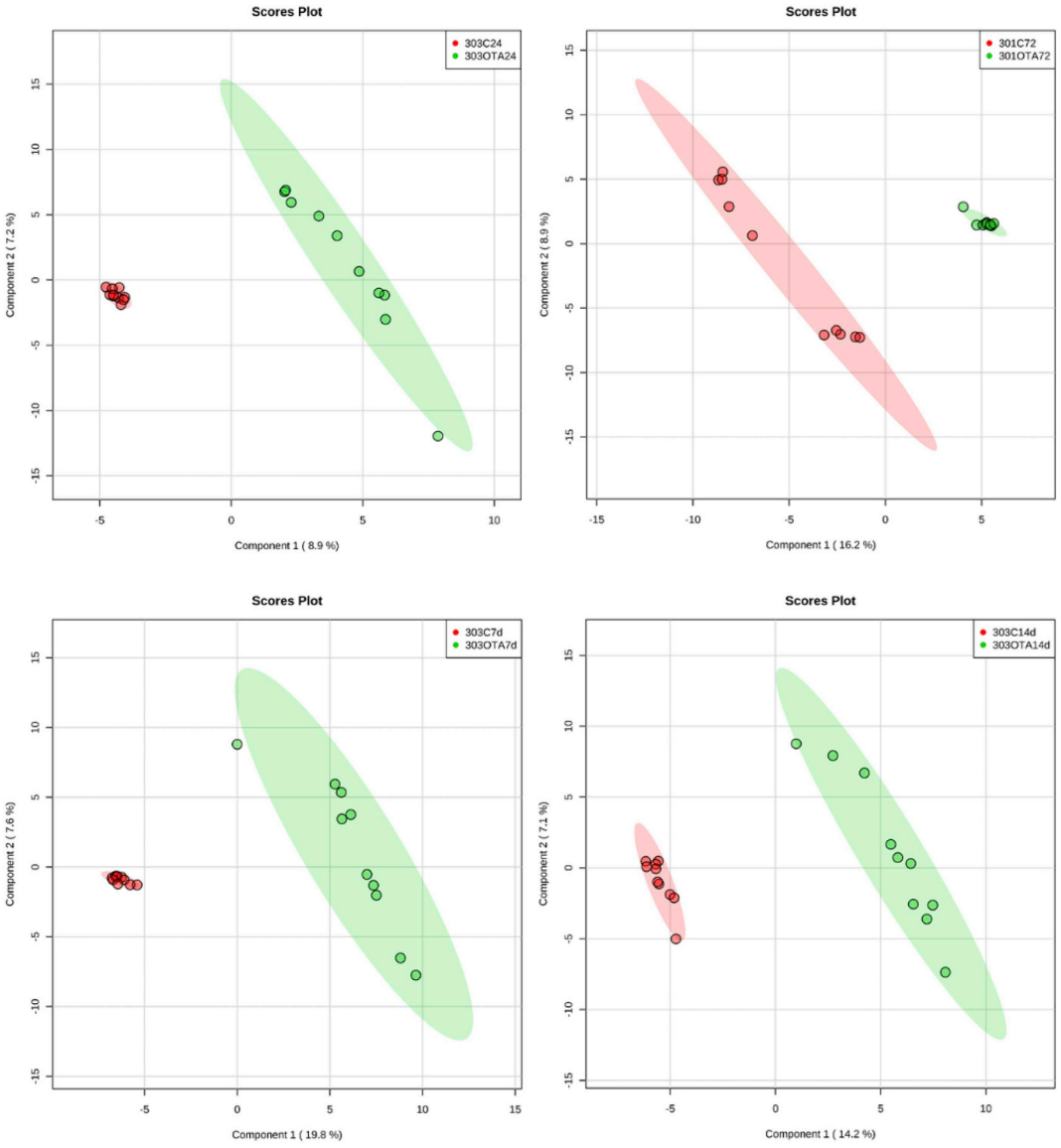

Fig. 3. Scores plot generated from PLS-DA analysis for strain 41 PP in the absence (red) and presence (green) of OTA (30 μg/L). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.2. Formation of modified ochratoxins by strains of S. cerevisiae

To detect the formation of modified ochratoxins from the metabolism of *S. cerevisiae* strains, high-resolution mass spectrometry and PLS-DA were combined into a metabolomics-based targeted approach. Although the presence of OTA did not influence growth parameters of yeast strains ($p < 0.05$), a discrimination of produced metabolites was observed, grouping samples with similar ionic content, with the formation of 2 clusters: strains in the presence of OTA (green cluster) and in the absence of OTA (red cluster) (Fig. 3). Such behavior is observed for all strains, at all concentrations tested. Additionally, variability is also observed within each cluster (i.e., the more distant the samples, the greater the variability), which seems to be more representative when the strain is in the presence of a mycotoxin.

Among the targets sought, several candidates derived from ochratoxin were identified (Tables 2 and 3). The formation of these metabolites occurred at all concentrations, for all strains, in most evaluated time points. However, the transformation of mycotoxin into derivatives on the fourteenth day was not detected in any of the strains tested in the presence of 10 μg/L of OTA. The highest number of metabolites was detected in the presence of 20 μg/L, followed by 30 μg/L OTA, suggesting that there is a possible correlation between the levels of OTA and the metabolism of mycotoxin by the strains (i.e., the higher the OTA levels, the more compounds are formed). However, there is likely to be a maximum OTA concentration threshold in which yeast strains metabolize mycotoxin. Among the detected compounds, the formation of different metabolites occurs through the same strain at different times. Furthermore, metabolites detected at one time point are not detected at subsequent analysis timepoints, and it is not possible to describe a pattern of occurrence of the formation of modified ochratoxins. It is possible that some formed compounds are intermediate metabolites that are converted into other derivatives, which were not identified in this study, over time.

Strain 12 M was able to form: ochratoxin B methyl ester, ochratoxin α and hydroxy-ochratoxin A; strain 01 PP: ochratoxin C, ochratoxin α, ochratoxin β, ochratoxin α methyl ester, hydroxy-ochratoxin A methyl ester and ethylamide ochratoxin A; and strain 41 PP: ochratoxin C, ochratoxin α, hydroxy-ochratoxin A and ochratoxin A cellobiose ester.

Ochratoxin α was the most commonly found compounds throughout the fermentation. The ochratoxin B methyl ester was detected only at the start of the fermentation. Strain 12 M was able to form ochratoxin B methyl ester and ochratoxin α at all concentrations evaluated.

Table 2
Ochratoxin A derivatives identified over 14 days of fermentation in the presence of ochratoxin A.

OTA concentration (μg/L)	Strains	Metabolites			
		24 h	72 h	7 days	14 days
10	12 M	ochratoxin B methyl ester	–	ochratoxin α	–
	01 PP	–	–	ochratoxin C	–
	41 PP	ochratoxin α	hydroxy-ochratoxin A	–	–
20	12 M	ochratoxin B methyl ester	ochratoxin α	hydroxy-ochratoxin A	ochratoxin α
	01 PP	–	ochratoxin α and ethylamide ochratoxin A	ochratoxin C; ochratoxin α and ochratoxin β	ochratoxin β
	41 PP	ochratoxin A cellobiose ester	ochratoxin α and hydroxy-ochratoxin A	ochratoxin α	–
30	12 M	ochratoxin B methyl ester	–	–	ochratoxin α
	01 PP	ochratoxin α methyl ester	–	–	hydroxy-ochratoxin A methyl ester and ochratoxin β
	41 PP	–	ochratoxin α	ochratoxin C and ochratoxin α	–

Ochratoxin α was the only common compounds formed by strain 41 PP at all concentrations, whereas the metabolites formed by strain 01 PP varied.

Modified mycotoxins may be formed by the action of the enzymatic complex of the yeast strains, possibly through degradation, conjugation, oxidation, and hydrolysis reactions, among others (Berthiller et al., 2013; Nathanail et al., 2016). Evidences in the literature are found for the ability of strains from the genera *Trichosporon*, *Rhodotorula*, and *Cryptococcus* to degrade OTA, generating ochratoxin α and phenylalanine (Schatzmayr et al., 2003). Conversely, while *S. cerevisiae* strains are considered efficient in reducing OTA levels, derived forms were not identified by Angioni et al. (2007), Cecchini et al. (2006) and Piotrowska and Zakowska (2000) in previous studies. According to these authors, the OTA reduction was linked to an adsorption mechanism by the yeast cells. In contrast, throughout alcoholic fermentation, some strains of *S. cerevisiae* and *Kloeckera apiculata* were not able to reduce OTA, and OTA residues were not detected in the biomass, excluding an adsorbing effect from the yeast cell walls of the strains studied (Angioni et al., 2007). Furthermore, ochratoxin α and phenylalanine were not detected in trials in which OTA was reduced (Angioni et al., 2007). According to the authors, these compounds probably reacted with molecules from the media and were converted into new compounds. All these events may further hinder the detection of OTA and its modified forms by conventional methods used for the quantification, thus causing underreporting and low recovery of the mycotoxin. Therefore, robust targeted methods for the detection of modified mycotoxin such as high-resolution mass spectrometry are much-desired tools to solve this issue. The experimental simplicity of DI-HRMS has already been successfully explored in previous contributions, and showcase the potential of this approach in tackling molecular identification issues in complex matrices (Freire and Sant'Ana, 2018).

The main transformation reactions of OTA into detoxification processes are hydrolysis, hydroxylation, lactone opening and conjugation (Wu et al., 2011). However, most studies indicate OTA degradation through hydrolysis reactions (Loi et al., 2017), generating metabolites with lower toxicity when compared to parent mycotoxin. Studies indicate that the toxicity of OTA is probably related to the isocoumarin moiety and the lactone carbonyl group (Heussner and Bingle, 2015; Xiao et al., 1996). Although not directly responsible for the toxicity of OTA, the presence of chlorine and phenylalanine may have a significant influence on biological reactivity of mycotoxin (Heussner and Bingle, 2015). Therefore, changes in these structures may have a significant impact on the toxicity of compounds generated from the parent mycotoxin. In our study, given the profile of the modified mycotoxins we found, as well as the biocatalytic/enzymatic nature of a yeast culture medium such as the one used in our experiments, we employed a retrosynthetic rationale to analyze the structure of each molecule and hypothesize that OTA underwent metabolism through four different pathways: dechlorination, hydrolysis, hydroxylation, and conjugation (Fig. 4).

Ochratoxin B is formed from dechlorination (chlorine loss) of OTA (Heussner and Bingle, 2015), followed by enzymatic hydrolysis with the release of phenylalanine moiety, resulting in the formation of ochratoxin β. Ochratoxin α is also a compound generated from hydrolysis reaction and loss of a phenylalanine moiety (Stander et al., 2001), and is a less toxic derivative (Wu et al., 2011).

Studies on the toxic effects of ochratoxin β were not found. However, as its structure is similar to ochratoxin α, it is possible that any toxic effect is similar or even milder (Heussner and Bingle, 2015; Xiao et al., 1996). The primary concern regarding these compounds is related to the possibility of reconversion of these hydrolysis products into the parent mycotoxin (OTA). Literature reports bring light into the ability of some microorganisms to perform such reconversion: *A. ochraceus* strains were able to produce OTA from the metabolites ochratoxin β and ochratoxin α in shaken solid substrate (shredded wheat breakfast cereal) fermentation (Harris and Mantle, 2001).

Hydroxy-ochratoxin A is formed through the hydroxylation of OTA (OH group addition); this reaction, however, does not seem to reduce

Table 3
Species elucidated by HRMS.

Compound	Molecular formula	Experimental Mass	Theoretical Mass	Mass Error	Adduct
ochratoxin α	$C_{11}H_9ClO_5$	257.0222	257.0217	−1.94	$[M+H]^+$
ochratoxin β	$C_{11}H_{10}O_5$	261.0170	261.0165	1.79	$[M+K]^+$
ochratoxin α methyl ester	$C_{12}H_{11}ClO_5$	293.0191	293.0193	0.58	$[M+Na]^+$
ochratoxin B methyl ester	$C_{21}H_{21}NO_6$	384.1440	384.1447	1.86	$[M+H]^+$
ochratoxin A	$C_{20}H_{18}ClNO_6$	404.0893	404.0901	1.96	$[M+H]^+$
ethylamide ochratoxin A	$C_{22}H_{23}ClN_2O_5$	431.1366	431.1374	1.80	$[M+H]^+$
ochratoxin C	$C_{22}H_{22}ClNO_6$	432.1206	432.1214	1.83	$[M+H]^+$
hydroxy-ochratoxin A	$C_{20}H_{18}ClNO_7$	442.0668	442.0670	0.34	$[M+Na]^+$
hydroxy-ochratoxin A methyl ester	$C_{21}H_{20}ClNO_7$	472.0557	472.0565	1.78	$[M+K]^+$
ochratoxin A cellobiose ester	$C_{32}H_{38}ClNO_{16}$	728.1943	728.1957	1.98	$[M+H]^+$

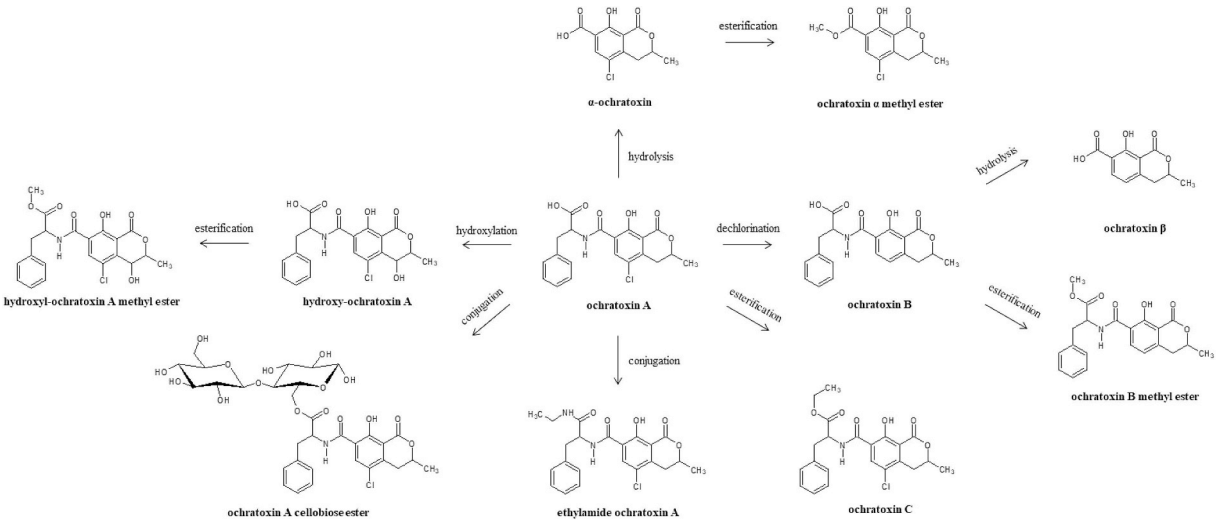

Fig. 4. Proposed metabolic pathways of OTA by *S. cerevisiae*.

the toxicity of the molecule (Heussner and Bingle, 2015). Methyl esters formed from these compounds, on the other hand, have much lower toxicity when compared to the parent mycotoxin (Heussner and Bingle, 2015; Xiao et al., 1996). The formation of OTA methyl esters, ochratoxin B, hydroxy-ochratoxin A, and ochratoxin α by esterification (methyl group addition) in the presence of a strong acid and high methanol concentration has been described in the literature (Li et al., 2000). In our study, we hypothesize that either the tartaric acid present in the fermentation medium or the methanol used for sample dilution before analysis might have acted as methyl sources for the formation of ester compounds.

Ochratoxin C is an OTA ethyl ester that appears to have toxic effects similar to OTA, although its mechanism is yet to be revealed (Heussner and Bingle, 2015). Despite that, the transformation of ochratoxin C into OTA by the metabolism of Wistar rats was demonstrated by Fuchs et al. (1984).

Conjugation reactions are also considered detoxification stages in the metabolism of microorganisms, although bioactivation may occur (Heussner and Bingle, 2015). OTA has high protein affinity (Duarte et al., 2012) and sugar-binding properties (Bittner et al., 2013), which indicates the possibility of conjugation and formation of derived species such as ethylamide ochratoxin A (amine addition) and ochratoxin A cellobiose ester (cellobiose addition).

OTA may also be metabolized into other compounds that were either not investigated in our study, or were not detected by the method used. Additionally, the reduction in OTA levels during fermentation processes may also be related to the strong adsorption of mycotoxin in yeast cell walls (Cecchini et al., 2019; Chen et al., 2018; Petruzzi et al., 2014; Abrunhosa et al., 2010) or the matrix (fermentation broth), making it impossible to extract them. It is possible that up to 50% of the mycotoxins bind to the wall of yeast cells or components of the fermentation medium, and are therefore not detected (Kakeya et al., 2002). Such adsorption possibly occurs due to the presence of glucogalactan exopolysaccharide, β- (1,3 and 1,6) -D-glucans, mannoproteins, and mannans in the cell wall, as well as cell surface properties that allow ionic and electrostatic interactions to occur (Cecchini et al., 2019; Chen et al., 2018). Petruzzi et al. (2017) have demonstrated that, not only OTA removal by *S. cerevisiae* strains is strain-dependent, OTA removed from the medium may also be released later, even after adsorption.

Even if OTA did not influence the growth of the strains evaluated, the presence of these modified ochratoxins formed from yeast metabolism may contribute to the underreporting of total mycotoxin levels in fermented products, or derivatives made from contaminated raw materials (Berthiller et al., 2013). Although most compounds are generated from a defense mechanism of these microorganisms, as an attempt to reduce the toxicity caused by OTA, some formed compounds may still present some level of toxicity, even if milder (Freire and Sant'Ana, 2018). Furthermore, some of these compounds may be more toxic if they are more bioaccessible and bioavailable when compared to the parent mycotoxin (Berthiller et al., 2013; Freire and Sant'Ana, 2018). Additionally, modified mycotoxins may be reconverted into the parent mycotoxin not only by the microorganism itself, but also by the industrial process, or in the digestive system, after the ingestion of the food containing these compounds, recovering the toxicity responsible for health effects in humans and animals (Berthiller et al., 2013; Plasencia and Mirocha, 1991). In this sense, biosynthetic routes and the biotransformation of OTA derivatives still need to be elucidated, as well as their toxic effects. An alternative is the use of radiolabeled OTA standards to trace and understand the routes that OTA and modified ochratoxins undergo during the fermentative processes (Abrunhosa et al., 2010).

Although the use of strains of yeast, bacteria and filamentous fungi are widely studied as a biological strategy in reducing OTA levels in food, and the tolerance of *S. cerevisiae* strains to OTA is of great benefit to industry, the application of microorganisms in food processing must be carried out with caution, with particular attention to their biosafety. Our findings demonstrate it is not possible to ensure food safety by assessing only the presence of the parent mycotoxin. The formation of modified mycotoxins during processing may generate a final product with high total levels of mycotoxins (free and modified), even if the raw material used is recognized as safe. Studies on the stability and fate of these modified mycotoxins along processing are necessary to estimate the total levels and propose legislation covering all potential mycotoxins and derivatives present in fermented foods. Finally, the prevention of the toxigenic fungi growth in food remains the best strategy for reducing human and animal health risks, and preventing economic losses.

Conflicts of interest

The authors have declared no conflict of interest.

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CAPÍTULO 5

From grape to wine: Fate of ochratoxin A during red, rose, and white winemaking process and the presence of ochratoxin derivatives in the final products.

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From grape to wine: Fate of ochratoxin A during red, *rose*, and white winemaking process and the presence of ochratoxin derivatives in the final products

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ABSTRACT

The presence of ochratoxin A (OTA) in wine is mainly due to the contamination of grapes by *Aspergillus carbonarius* and *A. niger*, still in the vineyard or at stages prior to winemaking. Throughout winemaking process, although there is a reduction in OTA levels, modified mycotoxins may also be formed. In fact, modified mycotoxins are compounds that normally remain undetectable during the conventional analysis used for the parent toxin. In this context, the current study aimed to evaluate the effect of grape variety and winemaking steps on OTA fate as well as the formation of modified ochratoxins. White, *rose* and red wines were prepared from Muscat Italia, Syrah and Touriga Nacional varieties, respectively. OTA was determined during different steps of winemaking by UHPLC-ESI-MS/MS. Identification of ochratoxin derivatives was performed using tandem MS experiments. A reduction of 90.72, 92.44 and 88.15% in OTA levels was observed for white, *rose* and red wines, respectively. Among the sought targets, the following ochratoxin-derived candidates were identified: ochratoxin β, ochratoxin α methyl ester, ochratoxin B methyl ester, ochratoxin A methyl ester, ethylamide ochratoxin A, ochratoxin C and ochratoxin A glucose ester. These results indicate that the formation of ochratoxin derivatives leads to an underestimation of total mycotoxin levels in wine and, therefore, the inclusion of techniques for multi-mycotoxins detection should be considered.

1. Introduction

Moderate consumption of wine has shown beneficial health effects due to the presence of compounds with antioxidant activity. However, components that cause negative effects on human health such as sulfites, toxic metals, pesticide residues and mycotoxins may also be present (Čepo et al., 2018).

Ochratoxin A (OTA) is a mycotoxin produced by some fungal species belonging to genera *Aspergillus* and *Penicillium*. Because OTA has several toxic effects such as carcinogenic, genotoxic, immunotoxic and hepatotoxic (Chen et al., 2018; Luo, Liu, & Li, 2018), it has been classified by the International Cancer Research Agency [IARC] (1993) as a possible carcinogen for humans (group 2B). Moreover, the European Food Safety Authority [EFSA] (2006) established that tolerable weekly intake of OTA is 120 ng/kg body weight.

Among different foods possibly contaminated with fungi and subsequently with OTA, wine is known as the second most important source of OTA considering mean European total dietary intake, only behind cereals (European Commission [EC], 2002). To this end, the European Union has set a maximum tolerable OTA limit of 2 μg/kg in wines (EC, 2006). This same limit was also established by Brazilian legislation (BRASIL, 2011). In addition to the possible toxic effects caused by the ingestion of wines contaminated with OTA, high concentrations of this mycotoxin, still present in grapes, can also impair the fermentative capacity of yeast(s) and result in changes in wine composition, altering taste and color (Bizaj, Curtin, Cadez, & Raspor, 2014).

The presence of this mycotoxin in wine is mainly due to the contamination of grapes still in the vineyard or at stages prior to winemaking by *Aspergillus carbonarius* and *Aspergillus niger*. Factors such as grape variety, damages in grape berries, vineyard location,

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temperature, relative humidity, precipitation, microclimate, harvesting period, pesticide application and good agricultural practices in the vineyard, oenological steps and good manufacturing practices during winemaking will be decisive for OTA levels present in wine (Freire et al., 2017; Quintela, Villarán, López de Armentia, & Elejalde, 2013).

In general, red wines have higher OTA levels in comparison to *rose* and white wines due to differences in applied oenological practices: especially the maceration step (Dachery, Veras, Dal Magro, Manfroi, & Welke, 2017; Lasram et al., 2008). During winemaking, crushing of grape berries and the maceration steps favor OTA release to must, suggesting grape skin is the main source of OTA (Lasram et al., 2008), while its content tends to decrease during fermentation and stabilization steps (Cecchini, Morassut, Garcia-Moruno & Di Stefano, 2006).

The reduction in OTA levels during winemaking has been justified by two main approaches: 1) the partition of OTA between liquid and solid phases, due to the adsorption of OTA in the solid particles of the must and subsequent natural sedimentation (Mariño-Repizo, Gargantini, Manzano, Raba & Ceruttia, 2017) and 2) adsorption or degradation by yeasts and/or lactic acid bacteria (LAB) (Cecchini et al., 2006); with varying degrees of success. However, this reduction is limited and, due to its partial stability, OTA is not completely eliminated throughout the oenological steps and residues of this mycotoxin are still detected in the final product (Cecchini et al., 2006; Cecchini, Morassut, Saiz, & Garcia-Moruno, 2019; Csutorás et al., 2013). A third justification for the reduction may be related to OTA degradation or transformation into modified mycotoxins during fermentation processes (Freire et al., 2019).

Modified mycotoxins cannot be detected when conventional analytical methods are used to quantify the parent mycotoxin due to changes in their structure, polarity and solubility (Berthiler et al., 2013). These forms were first reported by Gareis (1994), who observed that the severity of mycotoxicosis in animals did not correlate with mycotoxin levels detected in the diet. Possibly, the toxin conjugated to another molecule (glucose, amino acids or sulfate) was not detected in the animal feed by the analytical method used and was subsequently hydrolyzed and released into the gastrointestinal tract, thereby increasing animal exposure (Gareis, 1994).

Modified mycotoxins can be formed: 1) in the plant, as part of the detoxification mechanism; 2) during processing, through physical, chemical and biological processes and; 3) animal metabolism (Berthilher et al., 2013; Freire & Sant'Ana, 2018). The most relevant OTA derivatives already identified include ochratoxin B (dechloro analog of OTA), ochratoxin C (ethyl ester of OTA), ochratoxin α (isocoumaric derivative of OTA) and ochratoxin β (dechloro analog of ochratoxin α) (El Khoury & Atoui, 2010). It is possible that these and other derivatives not yet elucidated co-occur with OTA in wines and result in synergistic and/or additive effects to human and animal health. Furthermore, microbial, animal and human metabolism may act on the conversion of modified mycotoxins to parent mycotoxin, increasing OTA bioavailability (Berthiller et al., 2013; Freire & Sant'Ana, 2018).

Since the first discovery of OTA in wines (Zimmerli & Dick, 1996), a number of studies have focused on the reduction of OTA levels during winemaking by degradation and/or adsorption mechanisms (Cecchini et al., 2006, 2019; Csutorás et al., 2013). Nevertheless, the effects of grape variety and different winemaking steps on reduction of OTA levels have not been systematically evaluated. In addition, little information is available regarding the presence of OTA derivatives in wines. Such an investigation is highly relevant to avoid underestimation of the total ochratoxin intake and its possible adverse effects. In this regard, the present study aimed to evaluate the effect of grape variety and winemaking steps on OTA fate as well as the formation of modified ochratoxins. The latter is considered a challenging objective as commercial standards and reference methods are not yet available.

2. Material and methods

2.1. *A. carbonarius* and *A. niger* strains

A. carbonarius 10614 and *A. niger* 10443, isolated from wine grapes and obtained from the Culture Collection of the Department of Food Science/CCDCA-UFLA, were used in the assays. Both strains were previously selected in grape-based medium (Freire, Guerreiro, Pia et al., 2018). A conidia suspension from each strain was prepared individually and their concentration determined in a Neubauer chamber (Sigma-Aldrich, Darmstadt, Germany). The concentration of each suspension was standardized at 10^6 conidia/mL. Following, a mixture containing 50% of suspension from *A. carbonarius* 10614 and 50% of suspension from *A. niger* 10443 was prepared.

2.2. Wine grapes

Grapes were obtained in the wine region of the Vale Submédio São Francisco (Pernambuco, Bahia, Brazil). For each grape variety, a total of 15 kg was collected, to know: Syrah, Touriga Nacional and Muscat Italia. Only healthy grapes with no signs of mechanical damage or fungal growth were used. Grape berries were inoculated by spraying the conidia suspension mixture (*A. carbonarius* and *A. niger*). The final inoculum concentration was 10^4 conidia/g of grapes. As control, grapes were inoculated with sterile distilled water not added of the conidia suspension. Grapes were incubated for 7 days at 25 °C (Freire, Guerreiro, Carames et al., 2018).

2.3. *Saccharomyces cerevisiae* strain

S. cerevisiae 41 PP, used as inoculum in micro-winemaking, has been previously isolated from the viniferous environment (Mendes, Ramírez-Castrillón, Feldberg, Bertoldi, & Valente, 2017). The strain was reactivated in formulated yeast, peptone and dextrose agar (YPD agar) containing 0.5% of yeast extract (Acumedia, Lansing, Michigan, United States of America), 1% of bacteriological peptone (Acumedia, Lansing, MI, USA), 2% of dextrose (Diadema, Brazil) and 2% of agar (Acumedia, Lansing, MI, USA) and incubated for 48 h at 25 °C (Freire et al., 2019). The pre-culture was prepared in grape broth (obtained by macerating the grapes) and agitated on a rotary shaker at 120 rpm for 24 h at 25 °C (Series 25 Shaker/Incubator, New Brunswick Scientific, USA). After, it was inoculated into a new grape broth and incubated on a rotary shaker at 120 rpm for 24 h at 25 °C. This procedure was repeated once more. The final cell concentration in the broth was determined in a Neubauer chamber (Sigma-Aldrich, Darmstadt, Germany). An inoculum containing 10^8 cells/mL was used.

2.4. Micro-winemaking

Micro-winemaking was performed according to Guerra and Baranbé (2005), with adaptations. Red, *rose* and white wines were produced, respectively, from Touriga Nacional (red grape), Syrah (red grape) and Muscat Italia (white grape) grape varieties. After incubation period of grapes with the fungal suspension, the destemming (berry separation from rachis) and the crushing of grapes were performed to obtain the mash. Then, a 0.01% potassium metabisulphite solution (Dinâmica, Diadema, Brazil) was added and after 2 h the mash obtained from each grape variety was inoculated with *S. cerevisiae* strain (10^6 cells/g of must). After inoculation, the mash was divided into 5L-flasks in two repetitions. A control, containing mash prepared from non-contaminated grapes, was also prepared. The primary fermentation was carried out at 25 °C with daily remounting (mixture of liquid and solid parts). For red wine, crush/pressing (pomace separation: skins and seeds from must – must extraction) was performed at the end of primary fermentation. For *rose* and white wines, crush/pressing was carried out 48 h after primary fermentation. The end of primary fermentation was

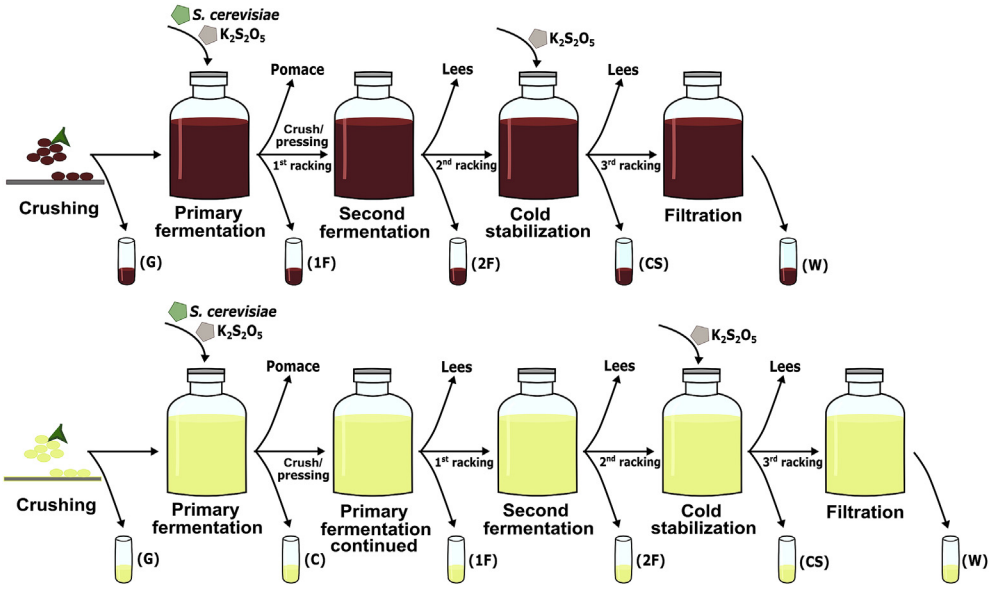


Fig. 1. Different steps for red, *rose*, and white micro-winemaking process evaluated in the present study. Samples were collected in the following steps: G: grape, after crush; C: must, after crush/pressing; 1F: must, at the end of primary fermentation; 2F: must, at the end of second fermentation; CS: must, at the end of cold stabilization; W: wine, after filtration. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

detected by measurement of density (0.992–1.050 g/L) in all wines. Thereafter, a first racking (translocation from one container to another for the separation of solid phases-lees and liquid-must) was performed. The secondary fermentation was carried out at 20 °C for 20 days. Subsequently, a second racking was performed and a 0.005% potassium metabisulphite solution was added, followed by cold stabilization at 4 °C for 30 days. After this period, a third racking and a filtration step for clarification were performed (Fig. 1).

2.5. Determination of OTA concentration

To determine OTA levels throughout red winemaking process, grape samples were collected after destemming and crushing (G) and from the must at the end of primary fermentation (after first racking) (1F); at the end of secondary fermentation (after second racking) (2F); after cold stabilization (after third racking) (CS) and from wine after clarification (filtration) (W). Throughout white and *rose* winemaking process, besides the aforementioned samples (G; 1F; 2F; CS and W), must samples were also collected after crush/pressing (C) performed 48 h after the beginning of primary fermentation.

2.5.1. Sample preparation and extraction

Sample preparation was performed based on the original QuEChERS method and dispersive solid-phase extraction (d-SPE) cleanup, described by Anastassiades, Lehotay, Stajnbaher, and Schenck (2003), with minor modifications. In the first step, 10 g of grapes or 10 mL of wine/must was put into a 50-mL polypropylene tube. Subsequently, 10 mL of acetonitrile (J. T. Baker, Phillipsburg. New Jersey, USA) + 1% formic acid 98% (Sigma-Aldrich, St. Louis, Missouri, USA) was added and tubes were vortexed for 10 min. Then, 4 g of $MgSO_4$ (Sigma-Aldrich, St. Louis, MO, USA) and 1 g of NaCl (Sigma-Aldrich, St. Louis, MO, USA) were added, and the tube was shaken for 5 min before centrifugation carried out at 8000 x *g* for 5 min at 20 °C (model 5810R, Eppendorf, Hamburg, Germany). For cleanup step, an aliquot of 1.5 mL of the supernatant was transferred to a microtube containing $MgSO_4$ and PSA (primary secondary amine) (Sigma-Aldrich, St. Louis, MO, USA), (150:50 mg/mg) and the mixture was vortexed for 5 min, being centrifuged at 5000 x *g* for 3 min at 20 °C (Mini model centrifuge, Gyrozen, Seoul, Republic of Korea). The supernatant was filtered through polytetrafluoroethylene (PTFE) membranes (0.22 μm) (Nova Analítica, São Paulo, Brazil) directly into the vial.

A standard stock solution was previously prepared by dissolving OTA (commercial standard from Sigma-Aldrich, St. Louis, MO, USA) in acetonitrile (100 mg/L). Thereafter, standard OTA solutions were prepared, by dilution in solvent (acetonitrile + 1% formic acid 98%), at concentrations: 1.0, 2.0, 5.0, 10.0, 20.0, 40.0 and 80.0 μg/L. Accordingly, matrix-matched standard OTA solutions of 1.0, 2.0, 5.0, 10.0, 20.0, 40.0 and 80.0 μg/L were obtained by adding an appropriate volume of a sample extract (wine/grape) to each serially diluted standard solution.

2.5.2. OTA quantification by UHPLC-ESI-MS/MS

Chromatographic separation was performed with a Poroshell 120 EC-C18 column (2.7 μm, 2.1 mm × 50 mm) (Agilent Technology, Santa Clara, California, USA, p/n 699775-902) using a mobile phase containing 0.2% formic acid solution (A) and acetonitrile + 0.2% formic acid (B). A linear gradient program was applied, starting with 70% A and 30% B, decreasing linearly the proportion until 5% A and 95% B in 1 min, kept constant until 2.50 min and then returning to the initial proportion. A post run interval of 1.5 min was necessary to re-equilibrate the column for the initial condition. Elution conditions were optimized at a constant flow rate of 0.5 mL/min, injection volume used was 2 μL and column oven temperature was 40 °C. The analyses were performed in a UHPLC system coupled with a 6460 triple quadrupole tandem mass spectrometer with electrospray ionization (ESI) source in the positive mode (Agilent Technology, Santa Clara, CA, USA). The ionization source operation conditions were as follows: gas temperature, 350 °C; gas flow, 11 L/min; nebulizer, 30 psi; sheath gas flow, 12 L/min; sheath gas temperature, 380 °C; capillary voltage, 3.5 kV and nozzle voltage, 0.0 kV. Sample analyses were performed in MRM (multiple reaction monitoring) scan mode, using a dwell time of 200 ms per channel) and MassHunter software workstation, version B.08.00. Two transitions were monitored for OTA to obtain at least three identification points, as recommended by the European Commission Decision 2002/657/EC (EC, 2002). The following optimized mass spectrometric parameters were established: fragmentor, 118 V and collision energy of 9 V for transitions m/z 404.1 → 357.9 (qualifier transition) and 21 V for transitions m/z 404.1 → 238.9 (quantifier transition), which was the high-intensity fragment and, for this reason, chosen for quantitation.

2.5.3. Validation of the analytical method

The analytical method developed for the analysis of OTA in grapes and wine was validated following the recommendations of the

European Commission Decision 2002/657/EC (EC, 2002) and European Commission Regulation 401/2006 (European Commission, 2006), which lay down rules on the use of sampling and analysis methods for the official control of mycotoxins levels in foodstuffs. The parameters evaluated included linearity, sensitivity, selectivity, matrix effect, precision (repeatability and within-laboratory reproducibility), trueness (recovery), limit of quantitation (LoQ) and robustness. The best fit of the analytical curve was achieved using weighted linear regression (1/x), which showed lower precision and accuracy deviations. The matrix effect was determined according to Sapozhnikova and Lehotay (2013), taking into account the slope obtained in analytical curves obtained for OTA prepared in the extract and in the solvent. For OTA identification, two product ions were monitored and the ratio of their abundance was observed. The ion ratios for MRM remained consistent both in matrix and solvent, being within the expected deviation of ± 30%, adding another layer of selectivity to the method.

2.6. Identification of ochratoxin derivatives

The search and identification of the possible elected ochratoxin derivatives present in white, red and *rose* wines (W) was performed using ESI-LTQ-XL Discover (Thermo Scientific, Bremen, Germany) mass spectrometer. Samples were filtered in polyvinylidene fluoride (PVDF) filter membranes (0.22 μm) (Jet Biofil, Guangzhou, China) (Tafuri, Meca, & Ritieni, 2008). An aliquot of 10 μL of the sample was diluted in 490 μL of methanol (J. T. Baker, Phillipsburg, NJ, USA) and homogenized under vortex for 30 s and then added of 1 μL of formic acid (Sigma-Aldrich, Darmstadt, Germany). Next, direct injection of the sample was performed. The following parameters were used: flow rate at 10 μL min^{-1}, spray current at 5 kV, capillary temperature at 280 °C, and sheath gas at 5 arbitrary units. Data were acquired in the positive mode using mass range of 200–750 *m/z* in the survey scan mode, in five replicates.

The identification of elected targets was performed using tandem MS experiments and helium as the collision gas, with energies for collision-induced dissociation (CID) ranging from 18 to 28 eV. The fragmentation analysis profile spectra of MS/MS were analyzed using XCalibur software (v. 2.4, Thermo Scientific, San Jose, CA, USA). The structures of the elected ochratoxin derivatives were proposed using our MS/MS data and by comparison with both literature data and theoretical mass fragmentation obtained with Mass Frontier software (v. 6.0, Thermo Scientific, San Jose, CA).

2.7. Statistical analysis

The analysis of variance (ANOVA), followed by the Tukey test was used to evaluate differences in OTA levels determined throughout winemaking. The significance level was set at 5% ($p < 0.05$) for all analyses performed. The analyses were performed using Sisvar software (version 5.6, 2015, Brazil) (Ferreira, 2011).

3. Results and discussion

3.1. Validation of the analytical method

The optimized method was used to quantify OTA levels in grapes and wines. The parameters used in the method validation are shown in Table 1. Selectivity assays demonstrated the viability of blank samples used in the validation procedure, as no interference signals were observed. The matrix effect observed ranged from 2.7 to 9.1%. Linear correlation coefficients (*r*) of analytical curves were higher than 0.99. Precision (repeatability and within-laboratory reproducibility) (% CV) observed was less than 19.5% and accuracy (expressed as recovery) was determined as 79.3–105.2% for all matrices at all fortification levels tested. LoD and LoQ values observed for both red and white grapes and white wine were 1 μg/kg and 2 μg/kg, respectively; whereas for red wine, the levels determined for the same parameters were, respectively, 0.5 μg/kg and 1 μg/kg. Thus, the analytical methods employed to determine OTA levels in grapes and wines were shown to be in agreement with the validation guides used (EC, 2002; EC, 2006) and, therefore, suitable for the intended purpose.

3.2. Fate of OTA

The highest OTA level produced by strains of *A. carbonarius* and *A. niger* mixture was detected in grapes from Muscat Italia (240.13 μg/kg) variety, followed by Touriga Nacional (12.33 μg/kg) and Syrah (7.97 μg/kg) varieties ($p < 0.05$) (Table 2). These results indicate a direct influence of grape variety on OTA levels produced by the strains tested.

In a previous study, lower OTA levels were detected in Syrah variety, artificially contaminated with *A. niger* (148.04 μg/g) and *A. carbonarius* (93.93 μg/g), in comparison with the values assessed for Touriga Nacional (235.52 μg/g by *A. niger* and 159.99 μg/g by *A. carbonarius*) and Muscat Italia (246.74 μg/g by *A. niger* and 115.21 μg/g by *A. carbonarius*) varieties (Freire, Guerreiro, Carames et al., 2018).

The physicochemical composition and natural metabolites of grapes can affect the metabolites produced by the fungus and the overall regulation of the synthesis of mycotoxins (Kumar, Barad, Sionov, Keller, & Prusky, 2017). A positive correlation between OTA levels produced by *A. niger* and *A. carbonarius* and parameters such as pH, total soluble solids, total glycosides in glucose and total anthocyanin; and a negative correlation with titratable acidity, pectic acid, total phenolic compounds and antioxidant capacity (DPPH and ORAC values) in grapes has been previously established (Freire, Guerreiro, Carames et al., 2018).

At the end of the winemaking, white wine, made from Muscat Italia variety, presented higher OTA levels (22.28 μg/kg), followed by red wine, made from Touriga Nacional variety (1.46 μg/kg), and *rose* wine, made from Syrah variety (< LoQ) ($p < 0.05$). These figures correlate positively with OTA levels detected in grapes.

In addition to the influence of the initial levels of grape contamination, the steps of the winemaking process will also influence the final OTA concentration determined in wine (Fig. 2). However, it is important to highlight that the reduction of free mycotoxin (OTA) levels during winemaking is due in part to physical removal, degradation, transformation into new forms or association with food components and, therefore, it does not necessarily indicate a decrease in total levels of the mycotoxin (free mycotoxin plus modified mycotoxin) in the final product (Humpf & Voss, 2004).

A 90.7% reduction in OTA levels was observed, when grapes (G) and final product (wine – W) were compared for white wine. Throughout winemaking process, the highest reduction of OTA levels occurred in crush/pressing (C) step (76.6%), followed by secondary fermentation (2F) (45.0%). Primary fermentation (1F) also contributed to lower OTA levels (26.9%), while cold stabilization (1.3%) and filtration (0.2%) had little influence ($p < 0.05$).

Throughout *rose* wine-making process, a 92.4% reduction in OTA levels was observed, when grapes (G) and final product (wine – W) were compared. Most of the reduction in OTA levels had already occurred in crush/pressing (C) step (83.4%), followed by primary fermentation (1F) (18.7%). Since initial OTA concentration was lower in grapes and a significant reduction had already occurred in the early steps (C and 1F), OTA levels were below LoQ (1.0 μg/kg), but above LoD (0.5 μg/kg) in secondary fermentation (2F), cold stabilization (CS) and filtration (W) steps. Thus, these steps (2F, CS and W) had little or no influence on reduction of the mycotoxin levels ($p < 0.05$).

During red winemaking process, an 88.2% reduction in OTA levels was observed comparing grapes (G) and final product (wine - W). Since there is no early crush/pressing (C) step (48h) in red wine manufacturing (this step is performed only at the end of primary fermentation [1F] together with a 1st racking), the highest reduction in OTA

Table 1
Parameters used in method validation for quantitation of ochratoxin A in grapes and wine.

Parameters	Criterion[a]	Red wine		White wine		Red grape		White grape	
Range of work (μg/kg)	–	1–80		2–80		2–80		2–80	
Equation of the analytical curve[b]	–	y = 366.8 x + 12.0		y = 258.9 x + 97.5		y = 248.2 x + 26.9		y = 212.3 x + 103.7	
Linearity	–	r = 0.9997		r = 0.9996		r = 0.9987		r = 0.9965	
Specificity	Interferences in blank ≤30% of RL[c]	No interferences		No interferences		No interferences		No interferences	
Matrix effect	–	7.5%		2.7%		4.6%		9.1%	
LOQ	–	1 μg/kg		2 μg/kg		2 μg/kg		2 μg/kg	
Retention time	± 0.1 min	≤0.1 min		≤0.1 min		≤0.1 min		≤0.1 min	
Ion ratio	± 30%	Complies		complies		Complies		Complies	
Robustness	Recovery between 70 and 120 CV ≤ 20%	Complies		Complies		Complies		Complies	
		Analyte concentration	**%CV**	**Analyte concentration**	**%CV**	**Analyte concentration**	**%CV**	**Analyte concentration**	**%CV**
Precision (Repeatability) (n = 5)	%CV ≤ 20%	1 μg/kg	2.2	2 μg/kg	5.4	2 μg/kg	3.9	2 μg/kg	8.3
		10 μg/kg	1.8	10 μg/kg	3.3	10 μg/kg	3.4	10 μg/kg	12.4
		80 μg/kg	3.5	80 μg/kg	1.5	80 μg/kg	1.9	80 μg/kg	16
Precision (within-laboratory reproducibility) (n = 15)	%CV ≤ 30%	1 μg/kg	19.5	2 μg/kg	10.3	2 μg/kg	12.1	2 μg/kg	8.9
		10 μg/kg	4.3	10 μg/kg	2.9	10 μg/kg	6	10 μg/kg	7.7
		80 μg/kg	6.2	80 μg/kg	1.2	80 μg/kg	5.7	80 μg/kg	9.6
Accuracy (n = 15)	% Recovery 70 - 110 (≤20%)	**Analyte concentration**	**% Recovery (%CV)**	**Analyte concentration**	**% Recovery (%CV)**	**Analyte concentration**	**% Recovery (%CV)**	**Analyte concentration**	**% Recovery (%CV)**
		1 μg/kg	102.4 (10.7)	2 μg/kg	105.2 (8.2)	2 μg/kg	101.2 (6.8)	2 μg/kg	99.3 (7.0)
		10 μg/kg	87.0 (4.6)	10 μg/kg	94.2 (3.9)	10 μg/kg	88.4 (8.8)	10 μg/kg	83.4 (8.2)
		80 μg/kg	90.3 (6.8)	80 μg/kg	93.1 (3.4)	80 μg/kg	83.5 (4.0)	80 μg/kg	79.3 (8.7)

[a] Criterion from the European Commission Decision 2002/657/EC and European Commission Regulation EC/401/2006.
[b] All analytical curves are presented using a weighed (1/x) in the linear regression.
[c] RL: reporting limit.

Table 2
Ochratoxin A (OTA) levels (expressed as μg/kg and percentages [%] of reduction) determined throughout red, *rose*, and white winemaking process.

Step	White wine			Red wine			*Rose* wine		
	OTA (μg/kg)[b]		% reduction[a]	OTA (μg/kg)[b]		% reduction[a]	OTA (μg/kg)[b]		% reduction[a]
Grape (G)	240.13^{cD}	± 0.71	–	12.32^{bC}	± 0.64	–	7.97^{aB}	± 0.19	–
Crush/pressing (C)	56.26^{bC}	± 0.28	76.57^{aD}	Na	Na	Na	1.32^{aA}	± 0.03	83.44^{bC}
End of primary fermentation (1F)	41.14^{cB}	± 1.80	26.87^{bB}	4.72^{bB}	± 0.04	61.70^{cC}	1.07^{aA}	± 0.09	18.76^{aB}
End of secondary fermentation (2F)	22.62^{cA}	± 0.74	45.02^{bC}	2.00^{bA}	± 0.09	57.57^{cC}	< LoQ		$> 6.70^{aAc}$
End of cold stabilization (CS)	22.32^{bA}	± 0.26	1.31^{aA}	1.43^{aA}	± 0.04	28.50^{bB}	< LoQ		
After filtration (W)	22.28^{bA}	± 0.55	0.17^{aA}	1.46^{aA}	± 0.00	-1.87^{aA}	< LoQ		
% Total reduction			90.72^{b}			88.15^{a}			92.44^{c}

Na: not available (step not performed).

[a] Reduction in OTA levels, expressed as percentage (%) and calculated in comparison to the previous step. Average values followed by lowercase letters compare OTA reduction (%) determined in a same micro-winemaking step for different wine varieties (white; *rose*; red); uppercase letters compare OTA reduction (%) observed at different micro-winemaking steps (G; C; 1F; 2F; CS; W) for a same wine variety. Different letters show statistically significant difference ($p < 0.05$).

[b] Average values followed by lowercase letters compare OTA levels (μg/kg) determined in a same micro-winemaking step for different wine varieties (white; *rose*; red); uppercase letters compare OTA levels (μg/kg) observed at different micro-winemaking steps (G; C; 1F; 2F; CS; W) for a same wine variety. Different letters show a statistically significant difference ($p < 0.05$).

[c] Below LoQ (Limit of quantification) and above LoD (Limit of detection); LoQ was considered for the calculation of the reduction in OTA levels.

levels occurred in primary (61.7%) and secondary (57.6%) fermentation steps (1F and 2F, respectively). Cold stabilization (CS) also contributed to lower the mycotoxin levels (28.5%), but not filtration step (-1.9%) ($p < 0.05$).

Although all wines showed a remarkable reduction in OTA levels as observed at the end of winemaking, *rose* and white wines had higher reduction rates (92.4% and 90.7%, respectively) in comparison with red wine (88.2%). The absence of the maceration step in *rose* and white wines, due to early crush/pressing (C) step, seems to be the most relevant factor for the reduction of the mycotoxin levels observed throughout the process.

In contrast, several studies (Cecchini et al., 2019; Csutorás et al., 2013; Dachery et al., 2017) have observed a more pronounced reduction of OTA levels in red wines when compared to *rose* and white wines throughout fermentation process. For instance, a reduction of 73, 85, and 90% in white, *rose*, and red wine musts, respectively, was observed by Csutorás et al. (2013) during 90 days of fermentation. It is possible that the interaction between polyphenols (such as anthocyanins) and OTA interferes in the percentage reduction during fermentation. Cecchini et al. (2019) observed a higher reduction in OTA levels in red wines (39–51.6%) in comparison to white wines (around 29%), although the decrease in OTA content observed was not proportional to anthocyanin concentration in wine.

In these studies, the most significant factor in the reduction of OTA levels seems to be a higher presence of particles from maceration step that will act as OTA adsorbents. These particles act as a sponge coated with negative charges interacting with the acidic feature of toxins (Huwig, Freimund, Käppeli, & Dutler, 2001; Ponsone, Chiotta, Combina, Dalcero, & Chulze, 2009). An ionic bonding between OTA and anthocyanins may occur, as well as an esterification reaction between the carboxyl group of the OTA molecule and the hydroxyl group of anthocyanins (Cecchini et al., 2019). However, it is likely that these complexes formed will not be completely removed during winemaking (in racking steps) and will remain in the final product, resulting in masked OTA (Freire & Sant'Ana, 2018). This alteration on OTA structure (binding to must components) alters the characteristics of the molecule, such as structure, polarity and solubility, and makes impossible its quantification by the use of conventional analytical

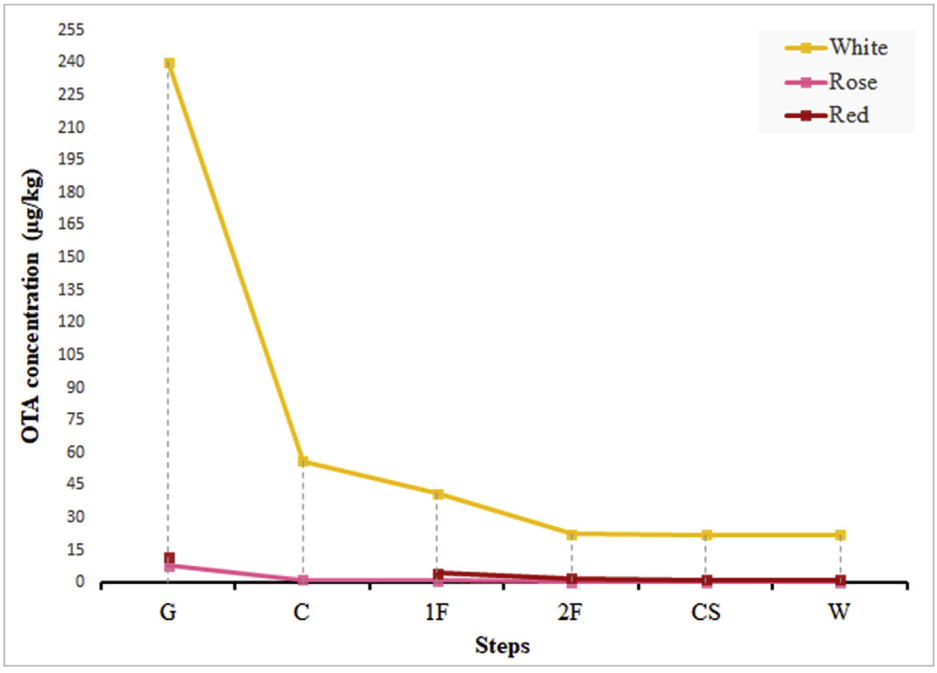


Fig. 2. OTA levels (μg/kg) obtained during red, *rose*, and white micro-winemaking process. G: grape, after crush; C: must, after crush/pressing; 1F: must, at the end of primary fermentation; 2F: must, at the end of second fermentation; CS: must, at the end of cold stabilization; W: wine, after filtration. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

methods, leading to a false reduction of OTA levels and, therefore, underreporting of total mycotoxin levels in wine (Berthiller et al., 2013).

According to our data, by comparison of OTA levels initially present in grapes (G) and those obtained up to primary fermentation step (1F), a reduction of 82.9% was observed in white wine and 86.6% in *rose* wine, whereas this figure corresponded to only 61.7% in red wine. The separation of the pomace in white and *rose* wines occurred after 48h (crush/pressing [C]), while in red wine it only took place at the end of the first fermentation (1F) (7 days), which resulted in a longer contact time between the pomace and the must. In this regard, a higher extraction rate of OTA from seeds (pomace) to must occurred increasing initial levels of OTA in red wines. According to Battilani, Pietri, and Logrieco (2004) an increase of approximately 20% in OTA levels can be observed after the maceration step. The increase of the alcohol concentration in the partially fermented must may favor the extraction of OTA from the solid phase to the liquid phase. However, at later steps, the increase of yeast biomass helps to reduce toxin levels (Lasram et al., 2008). Therefore, when winemaking process was assessed as a whole, the percentages of reduction observed for OTA levels were 90.7, 92.4 and 88.2% for white, *rose* and red wines, respectively.

The results obtained in the present study corroborate the findings of Lasram et al. (2008). According to these authors, after 1 day of the alcoholic fermentation, a significant increase (59.5% of the initial OTA content) in OTA levels was initially observed in red must. However a significant decrease (50.9% of the initial OTA content) in OTA levels was determined in *rose* must. Despite that, at the end of the fermentation process a similar decrease in the initial OTA content was observed in both red (41.0%) and *rose* (44.0%) wines.

In addition to solid-liquid separation (crush/pressing and racking) steps, other factors that occur throughout winemaking process, such as OTA transformation into other metabolites, mycotoxin adsorption by yeasts and components from musts, pomace and lees, will also be responsible for the reduction of OTA levels in wine.

The reductions in OTA levels determined in primary and secondary fermentation steps (1F and 2F, respectively) are closely related to the performance of the present microorganisms. Filamentous fungi species such as *A. niger* and *A. cabonarius* (Bejaoui, Mathieu, Taillandier, & Lebrihi, 2005), lactic bacteria (Abrunhosa et al., 2014), as well as yeasts, including *S. cerevisiae* (Petruzzi et al., 2015), were shown to reduce OTA levels in grape juices and musts (30–80%), growth medium (50–90%) and must (20–76%), respectively. This reduction has been related to the phenomenon of adsorption by cell wall components.

Glucogalactans, glucans, mannoproteins and mannans have been named as responsible for the binding of OTA to yeast cell wall (Chen et al., 2018). Conversely, exopolysaccharides and peptidoglycans appear to be responsible for such binding in LAB (Dalié, Deschamps, & Richard-Forget, 2010). At last, the adsorption phenomenon has been associated with hydrophobic interactions in filamentous fungi (Bejaoui et al., 2005).

However, such ability seems to be variable among strains. Angioni et al. (2007) did not detect OTA residues adsorbed on the cell wall of yeasts tested. The authors suggested that the reduction in OTA levels observed after fermentation was due to OTA degradation to other undetected metabolites. Furthermore, the *S. cerevisiae*-OTA binding may be reversible, with subsequent release of up to 85% of the initially adsorbed OTA (Petruzzi et al., 2015). A slight increase in OTA level was observed in culture medium after being adsorbed by *A. japonicus* and *A. carbonarius* conidia (Bejaoui et al., 2005).

After fermentation, cold stabilization (CS) is performed to remove suspended solids that make the wine cloudy (Gil-Serna, Vázquez, González-Jaén, & Patiño, 2018). This step influenced the reduction of OTA levels only in red wine (28.50%). The present study demonstrated that the percentage reduction in OTA levels throughout the process is cumulative until reaching a maximum reduction (approximately 90%). For white and *rose* wines this maximum reduction was already reached by the end of fermentation. For red wine, the reduction still continued during cold stabilization (CS) due to adsorption of residual mycotoxin by lees and subsequent separation of the liquid and solid phases through racking.

According to other studies (Gentile et al., 2016; Quintela, Villarán, Lopez de Armentia, & Elejalde, 2011) lower OTA levels were observed in commercial *rose* and white wines in comparison with red wines, because the pomace has been separated from the must earlier (anticipated crush/pressing [C]). In contrast, Čepo et al. (2018) determined the highest OTA concentrations (0.24 μg/L) in white wine. The authors justified the higher concentration due to the lack of good winemaking practices. In the present study, three different grape varieties were contaminated with the same amount of inoculum. However, different OTA concentrations were detected in Muscat Italia (240.13 μg/kg) (used to make white wine), Touriga Nacional (used to make red wine) (12.33 μg/kg) and Syrah (used to make *rose* wine) (7.97 μg/kg) grape varieties ($p < 0.05$). Consequently, white wine presented higher OTA levels (22.28 μg/kg), followed by red (1.46 μg/kg) and *rose* (< LoQ) wines ($p < 0.05$). Therefore, the influence of grape variety on final OTA levels in wines may be more relevant than the different steps used in red, *rose*, and white winemaking process.

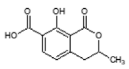
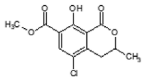
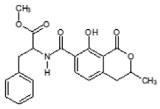
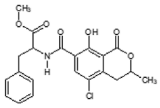
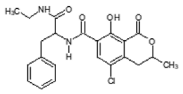
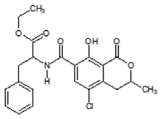
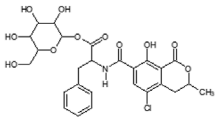
An efficient reduction of OTA levels is due to a set of factors, such as process steps and initial contamination levels. However, if the initial OTA concentration is high, the process steps will not be capable to result in safe levels of OTA. Considering the smallest reduction in OTA level observed in this study (88%), it is proposed that the maximum OTA level in grapes should be less than 16 μg/kg to ensure that the levels of this mycotoxin are below the maximum limit allowed by legislation from several countries (2 μg/kg) (Brazil, 2011; EC, 2006). However, although a recognized safe raw material is used, OTA initially present in grapes can be transformed into other metabolites still on the vine and throughout winemaking, which makes it impossible to determine the real fate of total mycotoxins (free plus modified) during winemaking and final product. Therefore, control of OTA levels in the raw material alone is not sufficient to guarantee the toxicological safety of the end product (Freire & Sant'Ana, 2018).

The best strategy to reduce health risks associated with the ingestion of mycotoxin-contaminated wine is to prevent contamination of the grapes by toxigenic fungi still in the field and throughout the process. Practices such as the use of biological control methods in the vineyard, avoiding the storage of grapes after harvest and commencement of fermentation right after removal of grapes from bunches drastically reduce the probability of growth of toxigenic fungi and, consequently, ochratoxins production. If contamination has already occurred, the following strategies can be used to reduce OTA levels, although its complete elimination is considered impossible: use of selected strains of yeasts and LAB during fermentation, which are able to adsorb and metabolize OTA to less toxic compounds; use of non-contaminated pomace and lees obtained from other processes to act as an adsorbent and other allowed chemical adsorbents, and dilution of contaminated grapes/wine with non-contaminated grapes/wine.

3.3. Formation of ochratoxin derivatives throughout winemaking

The reduction of OTA levels in wines has been justified by several studies (Angioni et al., 2007; Cecchini et al., 2006; Chen et al., 2018; Petruzzi et al., 2015) as a phenomenon of adsorption of mycotoxin onto the yeast cell wall and must components, since ochratoxin derivatives were not detected in musts evaluated by those authors. However, most of these studies only evaluated derivatives ochratoxin α and phenylalanine using HPLC with fluorescence detection (HPLC-FL). In fact, the combined use of HPLC-FL and immunoaffinity column clean-up (IAC) or solid phase extraction (SPE) is considered the most used method for detection of ochratoxin A in several foods (Alcaide & Aguilar, 2008; Cecchini et al., 2019; Petruzzi et al., 2015). Nonetheless, it is known that modified mycotoxins may not be detected by traditional analytical

Table 3
Tandem Mass Spectrometry acquisition parameters for mycotoxin.

Mycotoxin	Structure	Molecular formula	Theoretical Mass	Adduction ion	Precursor ion (m/z)	MS/MS fragmentation
Ochratoxin β		$C_{11}H_{10}O_5$	222.05	$[M + H]^+$	223	163, 177, 205
Ochratoxin α methyl ester		$C_{12}H_{11}ClO_5$	270.03	$[M + Na]^+$	293	187, 233, 247
Ochratoxin B methyl ester		$C_{21}H_{21}NO_6$	383.14	$[M + H]^+$	384	252, 352, 366
Ochratoxin A methyl ester		$C_{21}H_{20}ClNO_6$	417.10	$[M + Na]^+$	440	348, 404, 422
Ethylamide ochratoxin A		$C_{22}H_{23}ClN_2O_5$	430.13	$[M + Na]^+$	453	361, 417, 435
Ochratoxin C		$C_{22}H_{22}ClNO_6$	431.11	$[M + K]^+$	470	342, 434, 452
Ochratoxin A glucose ester[a]		$C_{26}H_{28}ClNO_{11}$	565.14	$[M + H]^+$	566	298, 534, 548

[a] Molecule detected only in red and *rose* wines.

methods used to quantify the parent mycotoxin due to structural and physical modifications of the molecule (Berthiller et al., 2013). Such transformations imply changes in chromatographic parameters and even in extraction efficiency (Freire & Sant'Ana, 2018). Therefore, it is extremely important to search for sensitive and selective techniques capable to elucidate and detect molecules derived from the parent mycotoxin. In this respect, mass spectrometry is an effective strategy for the detection and elucidation of modified mycotoxins in foods due to its versatility and sensitivity (Freire et al., 2019).

Among the sought targets, a number of ochratoxin-derived candidates were identified: ochratoxin β, ochratoxin α methyl ester, ochratoxin B methyl ester, ochratoxin A methyl ester, ethylamide ochratoxin A, ochratoxin C and ochratoxin A glucose ester (Table 3). The putative identification of these compounds occurred in all wines, with the exception of ochratoxin A glucose ester, which was detected only in red and *rose* wines.

These derivatives identified may have been formed by the fungus itself present in the grapes (Freire, Guerreiro, Pia et al., 2018), by the action of the yeast used in the fermentation (Freire et al., 2019) or through reactions with components from both grapes and must (Freire, Guerreiro, Carames et al., 2018).

A. carbonarius and *A. niger* strains isolated from grapes were shown to degrade OTA to ochratoxin α in synthetic grape juice (Bejaoui, Mathieu, Taillandier, & Lebrihi, 2006). These microbial species are also able to produce other derivatives, including ochratoxin β, ochratoxin B, ochratoxin C and methyl esters through their enzymatic complex (Remiro, Irigoyen, González-Peñas, Lizarraga, & López de Cerain, 2013).

The yeast *S. cerevisiae* produces extracellular enzymes such as glucosidase, pectinase and xylanase (Strauss, Jolly, Lambrechts, & van Rensburg, 2001) that can act on the hydrolysis of OTA or its conjugation with components present in the must during fermentation, leading to the formation of several derivatives (Freire et al., 2019).

The formation of ochratoxin derivatives may also be related to the matrix association phenomenon. In this case, acid conditions of the must favor the ionization of the amino group of OTA molecule and an interaction with medium components (Cecchini et al., 2006). Polysaccharides, pectic substances, lignin and proteins present in grapes and must can also bind to OTA through ionic interactions (Cecchini et al., 2019; Valenta, 1998).

Ochratoxin C, ochratoxin B methyl ester and ochratoxin A methyl ester derivatives may have been formed by dechlorination and esterification reactions carried out by the fungus metabolism itself or by the yeast (Freire et al., 2019). Moreover, pH of the must, pectin hydrolysis and the presence of acids may also favor the esterification reaction (methyl and ethyl group addition). These same derivatives have been previously detected in wines (Remiro, González-Peñas, Lizarraga, & López de Cerain, 2012; Remiro et al., 2013). The formation of OTA methyl esters, ochratoxin B, and ochratoxin α in the presence of a strong acid and high methanol concentration has also been observed (Li, Marquardt, & Frohlich, 2000).

The conjugation reactions of OTA and proteins and sugars present in the must may have been responsible for the formation of ethylamide ochratoxin A and ochratoxin A glucose ester due to the high affinity between OTA and proteins (Duarte, Lino, & Pena, 2012) and its binding properties with sugar (Bittner, Cramer, & Humpf, 2013). Ethylamide ochratoxin A has been previously identified in grape-based medium after *A. niger* inoculation (Freire, Guerreiro, Pia et al., 2018) and also in grapes from Syrah variety (Freire, Guerreiro, Carames et al., 2018). This same molecule has been also detected in fermentation broth in the presence of *S. cerevisiae* (Freire et al., 2019).

The putative identification of ochratoxin A glucose ester only in red and *rose* wines, made with red grapes from Touriga Nacional and Syrah varieties, respectively, suggests a possible influence of the grape variety on the formation of modified mycotoxins throughout processing. However, further studies are needed to quantify these compounds and

to evaluate their correlation with physicochemical characteristics of wine grapes.

Although the ochratoxin α derivative was not detected, it was most likely formed during the process and then transformed into ochratoxin α methyl ester. In fact, ochratoxin α is formed due to the hydrolysis of the amide bond by the action of hydrolytic enzymes such as carboxypeptidases, proteases, lipases and ochratoxinase (Abrunhosa, Santos & Venancio, 2006; Dobritzsch, Wang, Schneider, & Yu, 2014; Stander, Bornscheuer, Henke, & Steyn, 2000). Bejaoui et al. (2006) also reported the conversion of ochratoxin α to unidentified derivatives. Yeasts and the fungus itself, still present in grapes, may have been responsible for this conversion due to their extensive enzymatic machinery (Freire et al., 2019).

Although most derivatives, which toxicological properties were previously investigated, present a minor deleterious effect on health (Freire et al., 2019), a major issue is the possible conversion of the modified mycotoxin onto the parent mycotoxin during processing or by human and animal metabolism (Freire & Sant'Ana, 2018). Ochratoxin C has been shown to be converted to OTA throughout storage, increasing OTA levels in wine (Remiro et al., 2012). Furthermore, the co-occurrence of these compounds may act additively or synergistically with OTA, increasing the levels of total mycotoxins in the food and, consequently, the health risk due to the consumption of contaminated wine.

Despite the effort for mycotoxin detoxification, several ochratoxin-derived candidates were detected in wines. In this sense, it is necessary to consider that wine may be contaminated with a mixture of mycotoxins. These results indicate an underestimation of total mycotoxin levels in wine and the need to include techniques for detection and quantification of multi-mycotoxins.

This study contributed significantly to the elucidation of the presence of modified mycotoxins in foods. However, further investigations including the use of naturally contaminated samples and isotopic patterns are needed to establish the fate and the stability of these mycotoxins throughout winemaking process, as well as to estimate possible health risks.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2020.107167.

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DISCUSSÃO GERAL

As cepas de *A. carbonarius* e *A. niger* avaliadas neste estudo tiveram um excelente ajuste do modelo de Baranyi em meio de cultivo a base de uva. Todas as cepas de *A. carbonarius* e *A. niger* foram produtoras de OTA em todos os dias avaliados (3, 6, 15 e 21). A maior produção desta micotoxina foi detectada no dia 15, tempo em que todas as cepas já haviam atingido o diâmetro máximo. A partir de então, observou-se uma redução dos níveis da micotoxina (dia 21). Os maiores níveis de OTA foram produzidos pelas cepas de *A. carbonarius* e não se correlacionaram com a taxa de crescimento em meio de cultivo a base de uva.

Existe uma variabilidade (experimental, biológica e entre cepas) de multiplicação e produção de OTA por *A. carbonarius* e *A. niger* em meios de cultivo a base de uva. A variabilidade entre cepas foi maior do que a biológica e experimental para o parâmetro taxa de crescimento (S>B=E) para a espécie *A. niger.* Para *A. carbonarius* as variabilidades entre cepas e biológica foram semelhantes e maiores que a variabilidade experimental (S=B>E). No que concerne à produção de OTA, observou-se uma maior variabilidade entre as cepas ao longo dos dias avaliados (3, 6, 15 e 21), para ambas as espécies.

Modelos desenvolvidos para bactérias, como o modelo de Baranyi, e ajustados a partir de experimentos realizados em meio de cultivo podem ser utilizados para avaliar a cinética do crescimento fúngico e para extrapolar o comportamento e fisiologia destes micro-organismos em um alimento, a fim de melhorar a qualidade e segurança. No entanto, as especificidades destes micro-organismos devem ser levadas em consideração (DANTIGNY; GUILMART; BENSOUSSAN, 2005; GOUGOULI; KOUTSOUMANIS, 2010). Nossos achados demonstram a existência de variabilidade inter e intra-espécie, indicando que a extrapolação de modelos obtidos a partir de dados de apenas uma cepa individual podem não ser representativo da maioria das cepas de uma determinada espécie, levando a superestimativa ou subestimativa dos dados previstos (ROMERO et al., 2010). Portanto é de extrema importância que ocorra uma seleção de cepas representativas, que possam abranger uma maior gama de condições, considerando seus comportamentos cinéticos distintos e que reflitam cada vez mais a realidade.

Dentre os fungos filamentosos detectados em uvas, as espécies *A. carbonarius* e *A. niger* são as mais comumente encontradas (ROUSSEAUX et al., 2014), sendo que, cepas de *A. carbonarius* possuem maior relevância, devido aos altos níveis de OTA produzidos, apesar de cepas de *A. niger* serem detectadas com maior incidência nas uvas (BELLÍ et al., 2004; LEONG et al., 2006; OLIVERI; TORTA; CATARA, 2008). Além disto, é possível que as cepas da

espécie *A. carbonarius* tenham se adaptado ao meio de cultivo a base uva de forma diferente do que as cepas de *A. niger*, e por isto, maiores níveis de OTA foram detectados. Os requisitos para produção de OTA variam entre as espécies e são mais restritos quando comparado aos requisitos para o seu crescimento (MITCHELL et al., 2004).

Uma redução nos níveis de OTA, produzidos pelas cepas de *A. carbonarius* e *A. niger,* foi observada entre os dias 15 e 21. Tal redução pode estar relacionada com a formação de micotoxinas modificadas através da atuação do complexo enzimático do próprio fungo (ASTORECA et al., 2009). Além disto, parte da OTA pode tornar-se fortemente adsorvida pela matriz do meio de cultivo com o passar dos dias de incubação (15 para 21) e não ter sido extraída pelo método de extração utilizado (BRODEHL et al., 2014).

Para avaliar a influência do estádio de maturação e da variedade das uvas na formação de OTA e micotoxinas modificadas, uma cepa de *A. carbonarius* (CCDCA10614) e uma de *A. niger* (CCDCA10443) foram utilizadas.

Em contraste com os resultados encontrados no meio de cultivo a base de uva, a cepa de *A. niger* produziu maiores níveis de OTA nas uvas em todas as variedades e estádios de maturação, exceto no estádio de pintor (período em que inicia a maturação do fruto), em que *A. carbonarius* produziu maiores níveis da micotoxina.

Embora na maior parte dos estudos *A. carbonarius* seja considerada a principal espécie produtora de OTA em níveis mais elevados, algumas cepas de *A. niger* podem ser maiores produtoras (PERRONE et al., 2006). Além da espécie *A. niger* ser uma boa competidora, esta adapta-se extremamente bem no ambiente vinífero e nas uvas (EINLOFT et al., 2017). Neste sentido, a maior produção de OTA por esta espécie nas variedades de uvas aqui testadas pode estar relacionada há uma melhor adaptação ao substrato. No entanto, as melhores condições de crescimento nem sempre estão relacionadas à maior produção de metabólitos secundários. Portanto, apesar de experimentos realizados em meio de cultivo serem utilizados para extrapolar o comportamento dos fungos em um alimento (DANTIGNY; GUILMART; BENSOUSSAN, 2005), tal comportamento pode variar, demonstrando a importância de experimentos realizados diretamente no alimento.

Uma correlação positiva entre os níveis de OTA produzidos por *A. niger* e *A. carbonarius* e os parâmetros: pH, sólidos solúveis totais, total de glicosídeos em glicose e antocianinas totais; e uma correlação negativa com acidez titulável, ácidos pécticos, compostos fenólicos totais e capacidade antioxidante em uvas foi estabelecida. Portanto, o favorecimento ou inibição do crescimento fungico e da produção de metabolitos primários e secundários está intimamente relacionada aos níveis que estes compostos estão presentes no substrato.

Embora maiores níveis de OTA têm sido detectados nos estágios finais de maturação, esta micotoxina esteve presente desde os estágios iniciais. Portanto, apesar dos estágios de maturação mais avançados favorecerem o crescimento do fungo, se ele estiver presente nos estádios iniciais a micotoxina será produzida (LASRAM et al., 2012).

Nossos resultados demonstram a influência da variedade e do estádio de maturação das uvas na produção de OTA. Além disto, a espécie fungica também possui influência direta nos níveis de micotoxinas produzidos. Cada espécie interage de forma diferente frente aos compostos presentes na planta. Ao encontrar os componentes da uva o fungo pode metaboliza-lo, responder com sinais (produção de outras substâncias) ou ambos (SHALABY; HORWITZ, 2015). No entanto, a relação entre a composição físico-química das uvas e sua contaminação por fungos toxigênicos é algo bastante complexo.

Além da produção de OTA, alguns micro-organismos também são capazes de produzir micotoxinas modificadas, através de seu complexo enzimático agindo na hidrólise/conjugação da micotoxina mãe. Adicionalmente, plantas através de seus mecanismos de defesa: metabolismo de fase I e II também podem transformar a micotoxina mãe (BERTHILLER, et al., 2013; FREIRE; SANT'ANA, 2018). Para identificarmos estes metabólitos utilizamos espectrometria de massas de alta resolução (HRMS).

Dentre os *targets* buscados, identificamos apenas o candidato etilamida ocratoxina A como um biomarcador produzido pela cepa CCDACA10443 (*A. niger*) no dia 21 em meio de cultivo a base de uva. Nos ensaios com as uvas, foram identificados os candidatos decarboxi ocratoxina A na variedade Moscato Itália nos estádios de maturação: pintor e 15 dias após o início do pintor; e etilamida ocratoxina A na variedade Syrah no ponto ótimo de colheita (em que a uva atinge seu ponto ótimo de maturação).

Estes achados indicam a influência dos estádios de maturação e da variedade das uvas também na presença de micotoxinas modificadas. No entanto, não se pode afirmar como estes metabólitos foram formados: se a partir do metabolismo do próprio fungo ou a partir do mecanismo de defesa da planta. É possível que outras micotoxinas modificadas também estejam presentes, embora não tenha sido possível identificá-las com o método utilizado.

Devido a possibilidade de formação de micotoxinas modificadas por micro-organismos, também avaliamos a formação destes compostos por cepas de *Saccharomyces cerevisiae* ao longo da fermentação e a influência da presença da OTA no crescimento das leveduras através de modelagem matemática.

Os níveis de OTA não influenciaram no crescimento das cepas de leveduras avaliadas. No entanto, uma variação nos parâmetros de crescimento entre as cepas foi

observada. A variação entre as cepas é um fenômeno inerente aos micro-organismos, mesmo que submetida a condições semelhantes (WHITING; GOLDEN, 2002). O estado fisiológico, variabilidade genética e fenotípica influenciarão a aptidão e a robustez das cepas (DEN BESTEN et al., 2017).

O efeito inibitório de micotoxinas no crescimento de cepas de *S. cerevisiae* depende do tipo e concentração da micotoxina, da cepa avaliada, do tempo e temperatura de incubação (BOEIRA et al., 2000). Além disso, é possível que a integridade celular, a composição da parede celular da levedura e a capacidade de se ligar à micotoxina sejam fatores determinantes na sensibilidade ou insensibilidade da cepa (PIOTROWSKA; MASEK, 2015; JAKOPOVIĆ et al., 2018). Provavelmente, as cepas de leveduras testadas neste estudo se adaptaram à presença de OTA. Algumas cepas de leveduras possuem genes que respondem codificando para maior resistência, usando vias de resposta ao estresse, reparo do DNA e mecanismos de degradação de micotoxinas (IANIRI et al., 2013; JAKOPOVIĆ et al., 2018).

Embora a presença da OTA não tenha influenciado os parâmetros de crescimento das cepas de leveduras, diversos candidatos derivados de ocratoxina foram detectados: éster metílico de ocratoxina B, ocratoxina α, hidroxi ocratoxina A, ocratoxina C, ocratoxina β, éster metílico de ocratoxina α, éster metílico de hidroxi ocratoxina A, etilamida ocratoxina A e éster de celobiose ocratoxina A.

De acordo com o perfil dos candidatos identificados, bem como a natureza biocatalítica/enzimática do caldo de fermentação, empregamos uma lógica retrosintética para analisar a estrutura de cada molécula e hipotetizar que a OTA sofreu metabolismo através de quatro vias diferentes: decloração, hidrólise, hidroxilação e conjugação. Embora a maioria dos compostos seja gerada a partir de um mecanismo de defesa desses micro-organismos, como uma tentativa de reduzir a toxicidade causada pela OTA, alguns compostos formados ainda podem apresentar algum nível de toxicidade, mesmo que mais suave (FREIRE; SANT'ANA, 2018).

O maior nível de OTA produzido pelo mix das cepas *A. carbonarius* (CCDCA10614) e *A. niger* (CCDCA10443) foi detectado no vinho elaborado a partir da variedade Moscato Itália (vinho branco) seguido por Touriga Nacional (vinho tinto) e Syrah (vinho *rose*). As porcentagens de redução da OTA foram de 90,72, 92,44 e 88,15 % no vinho branco (Moscato Itália), *rose* (Syrah) e tinto (Touriga Nacional), respectivamente.

A redução nos níveis de OTA ao longo da vinificação deve-se:1) a separação sólido-líquido (descuba e trasfegas); 2) adsorção da micotoxina pelas leveduras e componentes dos

mosto, bagaço e borra e; 3) a transformação da OTA em outros compostos (CECCHINI et al., 2006; LASRAM et al., 2008).

Além da influência do processo na redução dos níveis de OTA, os níveis iniciais de contaminação são determinantes na concentração final da micotoxina no vinho. Uma correlação positiva entre os níveis de OTA detectados na uva e os níveis de OTA detectados no respectivo vinho foi observada. Portanto, se a concentração inicial de OTA for alta, as etapas do processo não serão capazes de reduzir a micotoxina para níveis seguros. Neste sentido, uma eficiente redução da OTA no vinho deve-se há um conjunto de fatores: etapas do processo e níveis de contaminação inicial.

Entre os *targets* buscados, foram identificados vários candidatos derivados de ocratoxina: ocratoxina β, éster metílico de ocratoxina α, éster metílico de ocratoxina B, éster metílico de ocratoxina A, etilamida ocratoxina A, ocratoxina C e éster de glicose ocratoxina A. Estes derivados podem ter sido formados pelo próprio fungo presente nas uvas (REMIRO et al., 2013), pela ação da levedura utilizada na fermentação (STRAUSS et al., 2001), ou, através de reações com componentes da uva e do mosto (CECCHINI et al., 2006).

Diversos derivados de ocratoxina foram detectados neste estudo. Embora a maior parte dos derivados possuam um menor efeito deletério a saúde quando comparado a micotoxina mãe, tais compostos podem atuar de forma sinérgica com a OTA elevando os níveis totais de micotoxinas no alimento e o risco para saúde. Além disto, é possível que ocorra uma reconversão da micotoxina modificada para a micotoxina mãe ao longo do processo ou pelo metabolismo de micro-organismos, humanos e animais (BERTHILLER et al., 2013; FREIRE; SANT'ANA, 2018).Ademais, alguns desses compostos podem ser mais tóxicos se forem mais bioacessíveis e biodisponíveis quando comparados à micotoxina mãe (BERTHILLER et al., 2013; FREIRE; SANT'ANA, 2018).

CONCLUSÃO

Neste estudo a produção de OTA não se correlacionou com a taxa de crescimento e observou-se a existência de uma variabilidade (experimental, biológica e entre cepas) de multiplicação e produção de OTA por *A. carbonarius* e *A. niger* em meio de cultivo a base de uva. O candidato derivado etilamida ocratoxina A foi identificado em meio de cultivo a base de uva na presença de *A. niger,* sugerindo a formação de micotoxinas modificadas.

A variedade e o estádio de maturação das uvas influenciaram os níveis de OTA produzidos pelas espécies *A. carbonarius* e *A. niger*. O candidato decarboxi ocratoxina A foi identificado nas uvas da variedade Moscato Itália nos estádios de maturação: pintor e 15 dias após o início do pintor e; o candidato etilamida ocratoxina A na variedade Syrah no ponto ótimo de colheita, ambos na presença de *A. niger*.

A presença de OTA não influenciou os parâmetros de crescimento das cepas de *S. cerevisiae* e diversos candidatos derivados de ocratoxina foram identificados no caldo de fermentação: éster metílico de ocratoxina B, ocratoxina α, hidroxi ocratoxina A, ocratoxina C, ocratoxina β, éster metílico de ocratoxina α, éster metílico de hidroxi ocratoxina A, etilamida ocratoxina A e éster de celobiose ocratoxina A.

As etapas de separação sólido-líquido (descuba e trasfegas); a adsorção da micotoxina pelas leveduras e componentes do mosto, bagaço e borra e; a transformação da OTA em outros compostos são fatores responsáveis pela redução de OTA nos vinhos. Os candidatos derivados de ocratoxina: ocratoxina β, éster metílico de ocratoxina α, éster metílico de ocratoxina B, éster metílico de ocratoxina A, etilamida ocratoxina A, ocratoxina C e éster de glicose ocratoxina A foram detectados nos vinhos.

Os derivados identificados neste estudo podem ter sido formados pelo próprio fungo, pela ação da levedura ou através de reações com componentes do meio, uva, mosto e vinho. Estes resultados indicam uma possível subestimação dos níveis totais de micotoxinas nos alimentos e a necessidade da inclusão de técnicas para detecção de multi-micotoxinas. Nesse sentido, as rotas biossintéticas e a biotransformação destes derivados de ocratoxina ainda precisam ser elucidadas, bem como seus efeitos tóxicos.

Este estudo contribui significativamente na elucidação da presença de micotoxinas modificadas em alimentos. No entanto, investigações adicionais com amostras naturalmente contaminadas e padrões marcados são necessárias para estabelecer o destino destas micotoxinas ao longo do processo produtivo de alimentos e de possíveis riscos à saúde.

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ANEXO 1

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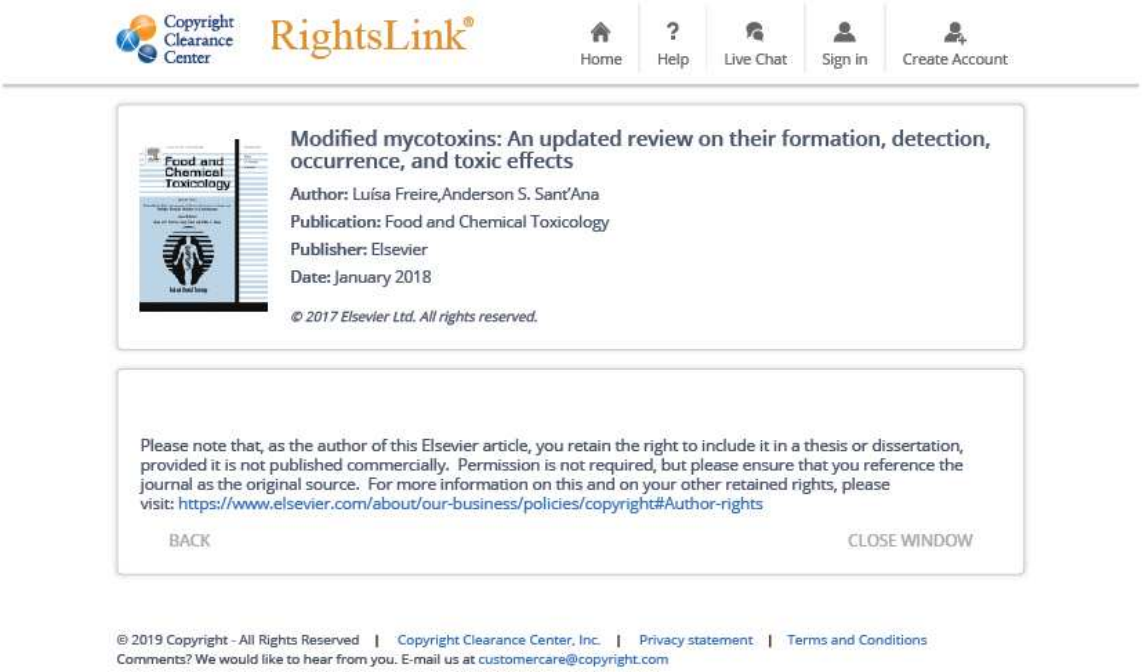

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Modified mycotoxins: An updated review on their formation, detection, occurrence, and toxic effects

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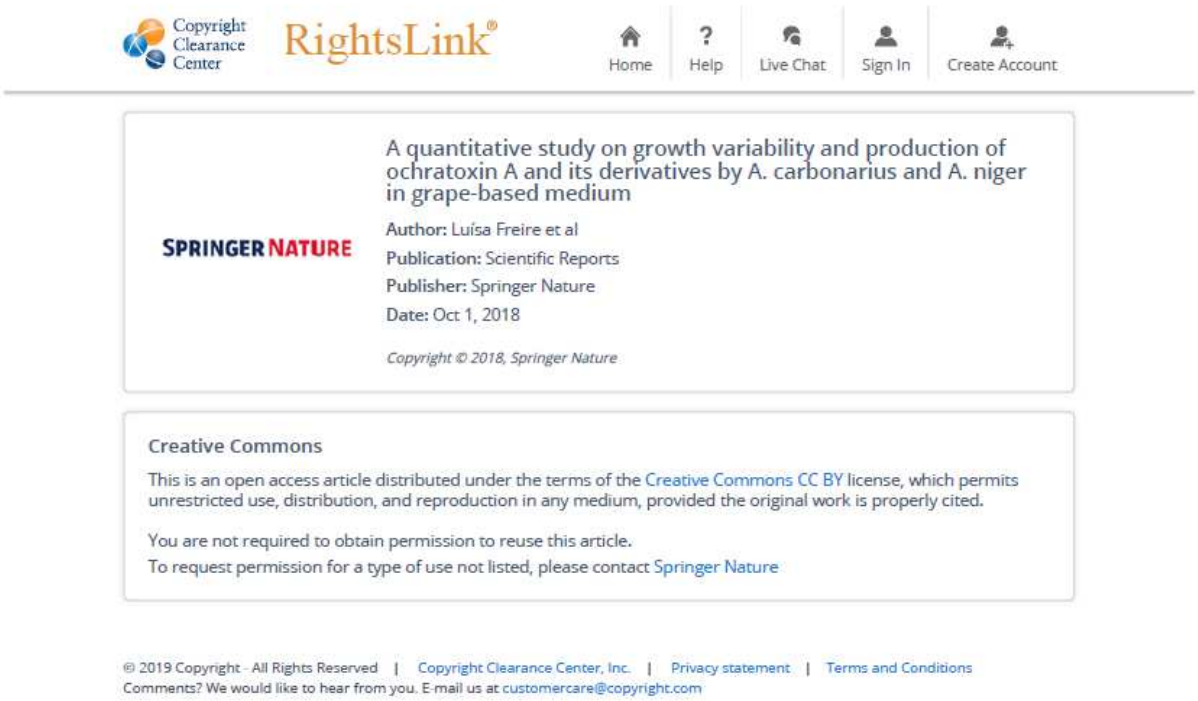

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A quantitative study on growth variability and production of ochratoxin A and its derivatives by A. carbonarius and A. niger in grape-based medium

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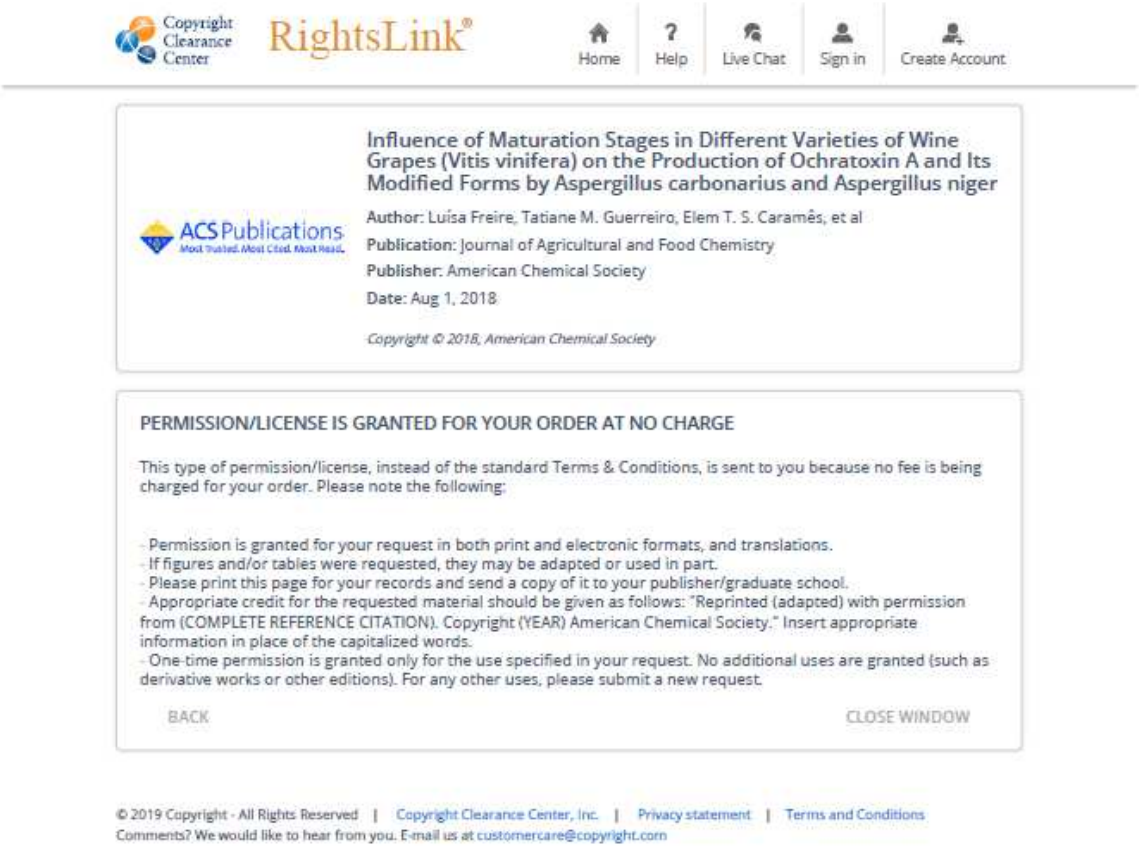
ANEXO 3

DECLARAÇÃO DE AUTORIZAÇÃO DE USO DE CONTEÚDO



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Influence of Maturation Stages in Different Varieties of Wine Grapes (Vitis vinifera) on the Production of Ochratoxin A and Its Modified Forms by Aspergillus carbonarius and Aspergillus niger

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Author: Luísa Freire, Tatiane M. Guerreiro, Elem T. S. Caramês, et al
Publication: Journal of Agricultural and Food Chemistry
Publisher: American Chemical Society
Date: Aug 1, 2018

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ANEXO 4

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The presence of ochratoxin A does not influence Saccharomyces cerevisiae growth kinetics but leads to the formation of modified ochratoxins

Author:
Luísa Freire,Marianna M. Furtado,Tatiane M. Guerreiro,Juliana S. da Graça,Beatriz S. da Silva,Diogo N. Oliveira,Rodrigo R. Catharino,Anderson S. Sant'Ana

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ANEXO 5

DECLARAÇÃO DE AUTORIZAÇÃO DE USO DE CONTEÚDO

ANEXO 6

DECLARAÇÃO CADASTRO SISGEN

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Certidão

Cadastro nº AB17223

Declaramos, nos termos do art. 41 do Decreto nº 8.772/2016, que o cadastro de acesso ao patrimônio genético ou conhecimento tradicional associado, abaixo identificado e resumido, no Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado foi submetido ao procedimento administrativo de verificação e não foi objeto de requerimentos admitidos de verificação de indícios de irregularidades ou, caso tenha sido, o requerimento de verificação não foi acatado pelo CGen.

Número do cadastro:	**AB17223**
Usuário:	**UNICAMP**
CPF/CNPJ:	**46.068.425/0001-33**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa**

Espécie

Aspergillus niger

Aspergillus carbonarius

Título da Atividade:	**Fungos e micotoxinas modificadas em uvas e vinhos: modelagem da variabilidade de formação, estabilidade ao longo do processamento e efeitos à saúde**

Equipe

Luisa Freire	**UNICAMP**
Anderson de Sousa Sant'Ana	**Unicamp**
Rodrigo Ramos Catharino	**Unicamp**
Giuliano Elias Pereira	**Embrapa Semiárido**

Resultados Obtidos

Divulgação de resultados em meios científicos ou de comunicação

Identificação do meio onde foi divulgado:	**Periódicos: Journal of Agricultural and Food Cl**

Data do Cadastro:	**30/10/2018 18:35:55**
Situação do Cadastro:	**Concluído**

Conselho de Gestão do Patrimônio Genético
Situação cadastral conforme consulta ao SisGen em **14:15** de **12/01/2020**.

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO - SISGEN