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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

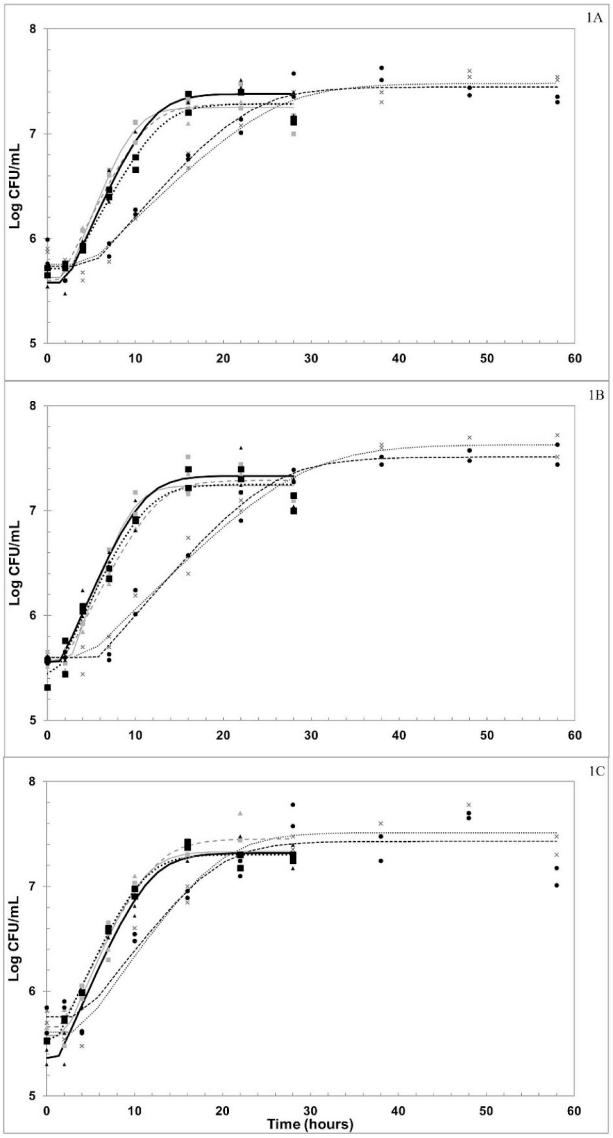

Fig. 1. Growth kinetics of *S. cerevisiae* strains over time in fermentation broths: in the absence of OTA (strains: ×12 M; ····· 12 M after Baranyi model fitting; ■ 01 PP; — 01 PP after Baranyi model fitting; ▲ 41 PP; – – 41 PP after Baranyi model fitting) and presence of OTA (strains: ● 12 M; - - - 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 $p < 0.05$.

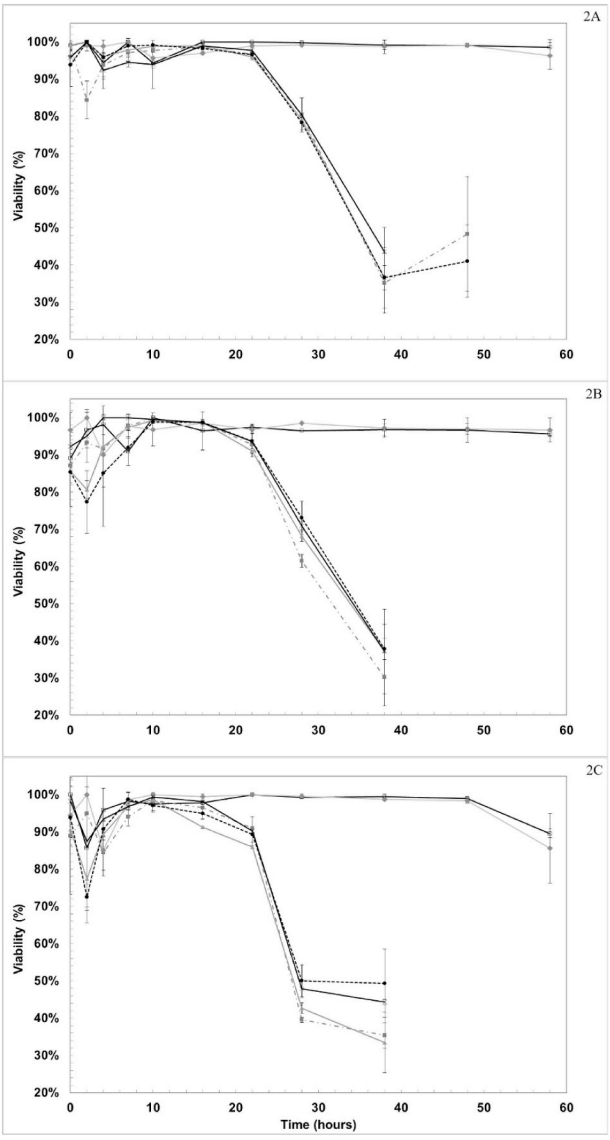

Fig. 2. Viability of *S. cerevisiae* strains' cells over time in fermentation broths: in the absence of OTA (strains: 12 M; 01 PP; 41 PP) and presence of OTA (strains: 12 M; 01 PP; 41 PP) at concentrations 10 (2A), 20 (2B) and 30 (2C) μg/L.

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.

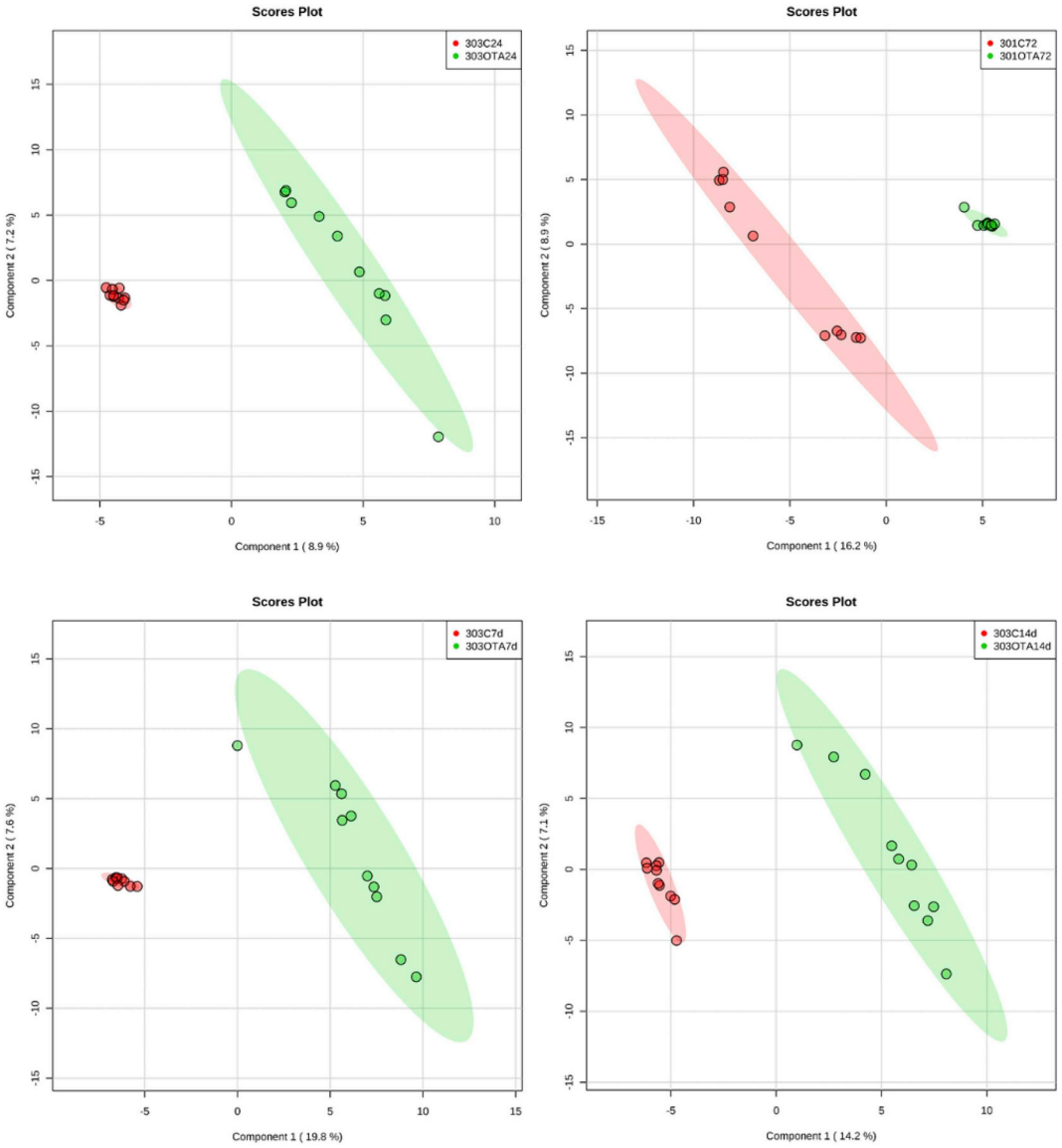

Fig. 3. Scores plot generated from PLS-DA analysis for strain 41 PP in the absence (red) and presence (green) of OTA (30 μg/L). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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	$[M+H]^+$

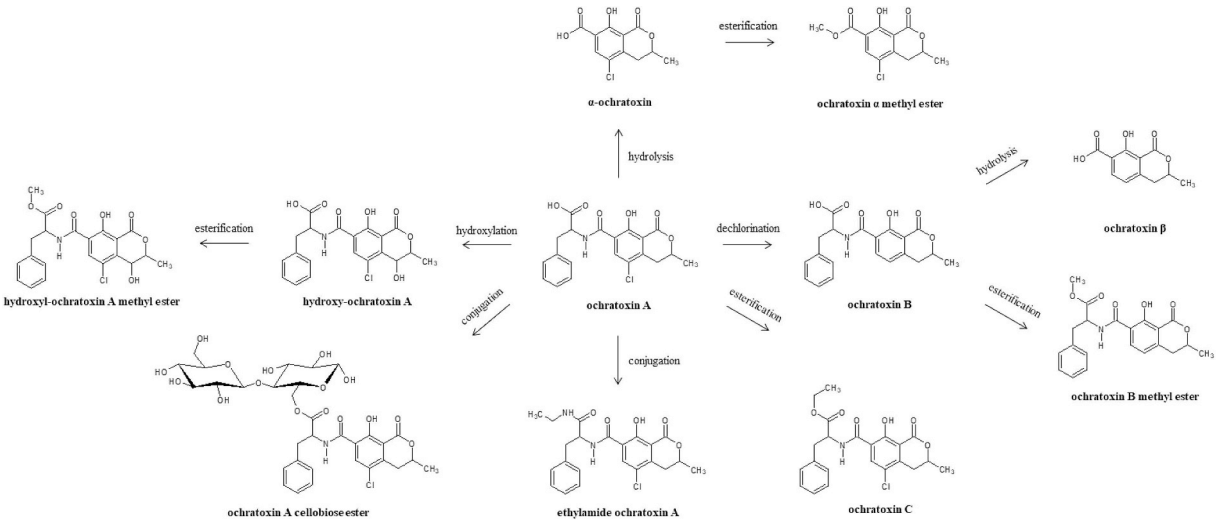

Fig. 4. Proposed metabolic pathways of OTA by *S. cerevisiae*.

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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