JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

In Vitro Interaction between Ochratoxin A and Different Strains of *Saccharomyces cerevisiae* and *Kloeckera apiculata*

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The interaction of ochratoxin A (OTA) and 20 yeast strains of *Saccharomyces cerevisiae* and *Kloeckera apiculata* during alcoholic fermentation was studied. Levels of OTA were determined in the fermentation liquid and in the yeast cells solid using a high-performance liquid chromatography system with a fluorescence detector. Yeast cells do not adsorb OTA, and for all yeasts, OTA levels did not affect the alcoholic fermentation. Some yeast strains reduced levels of OTA, whereas other strains did not show any effect demonstrating that OTA level reduction is not a genus species characteristic but a strain trait.

KEYWORDS: Ochratoxin A; *Saccharomyces cerevisiae*; *Kloeckera apiculata*; alcoholic fermentation; LC-MS; strain

INTRODUCTION

Ochratoxin A (OTA) is the most important mycotoxin produced by several species of *Aspergillus* and *Penicillium* molds. The occurrence of OTA in wines and grape juices was first reported in a Swiss study in 1996 (*1*). Because OTA showed nephrotoxic, hepatotoxic, and teratogenic properties to humans and animals (*2*), OTA was classified in 1993 by the International Agency for Research on Cancer as an agent of the group 2B, possibly carcinogenic to humans (*3*).

According to the data obtained in several surveys conducted in different countries, both the type of wine and the latitude of the producing region strongly influenced the incidence and concentrations of OTA in wines (*4*−*10*). It has been reported that OTA levels in wines follow the order white < rosé < red < dessert, with the highest levels in wines from the Mediterranean region and southern Europe. The levels of OTA found in wines produced in Europe ranged from not detectable (n.d.) to 10 μg/L. OTA is formed in the vineyard by black *Aspergillus* species, and their incidence changes depending on the degree of maturation of grapes. Only 10% of the molds found on grapes, at veraison, are able to produce OTA, whereas at the ripening stage, the molds that produce OTA are about 47%. Among the OTA-producing molds, 96% belong to the genus *Aspergillus* (95% *Aspergillus carbonarius* and 1% *Aspergillus niger*), with the remaining 4% belong to the genus *Penicillium* (*10*, *11*). Thus, OTA levels can increase dramatically during the phase of withering of the grapes, independently of where this process takes place, either in plants or on a trellis (*12*, *13*).

The tolerable daily intake of OTA is extremely low, 100 ng/kg body weight/week (*14*). The occurrence of OTA in wines has become a problem, especially in the Mediterranean regions, as well as in other wine-producing countries. Europe has recently fixed the maximum residue level of OTA in wines at 2 μg/kg (*15*). In view of previous observations, there is the need to find reliable technological, biological, and industrial methods for removing OTA from wines and food.

The effectiveness of chemical adjuvants on the reduction of OTA levels in food and other food matrices has been investigated. Potassium caseinate and activated carbon were found to be the most effective fining agents for removing OTA from wines (*16*−*18*). Silva et al. (*19*) studied the ability of some lactic bacteria strains to reduce OTA in musts and wines; among them, a strain of *Lactobacillus plantarum* degraded OTA. Piotrowska et al. (*20*, *21*) studied the effect of lactic acid bacteria on OTA and vice versa. The degradation of mycotoxins by microorganisms in ethanol, liquid, and solid YES medium has been reported in the literature (*22*, *23*). Bejaoui et al. (*24*, *25*) reported that *Saccharomyces* strains were able to remove OTA from synthetic and natural grape juice, and Cecchini et al. (*26*) described the reduction of OTA levels by single strains of *Saccharomyces*. Leong et al. (*27*) studied the fate of OTA during vinification, of wine made with grapes inoculated with *A. carbonarius*. Despite these studies, the fate of OTA during fermentation is unclear; it is also unclear whether the mechanism for removal of OTA mediated by yeasts or bacteria is a degradation or adsorption process or both. Moreover, there are few papers dealing with the effects of OTA on the fermentative process. In this study, we carried out in vitro experiments to evaluate whether OTA influenced the fermentation process mediated by different strains of *Saccharomyces cerevisiae* and *Koecklera*

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10.1021/jf062768u CCC: $37.00
Published on Web 02/06/2007

Table 1. Characteristics of the Yeast Strains Used

genus and species	strain		
Kloeckera apiculata	3184	3189	3198
	3187	3191	3199
	3188	3197	3200
Saccharomyces cerevisiae	1090	1164	1237
	1189	1182	1494
	1153	1226	1304
	1161	1236	

apicuculata and if the studied yeasts were able to reduce the levels of OTA in the medium and by which mechanism.

MATERIALS AND METHODS

Reagents. OTA standard was purchased from Sigma Co. (Milan, Italy). Bidistilled water was obtained with a Milli-Q System (Waters, Milford, MA). Acetonitrile, methanol, acetic acid, ethyl acetate, and phosphoric acid (85% purity) were of high-performance liquid chromatography (HPLC) grade, purchased from Carlo Erba (Milan, Italy). The ammonia solution (30% v/v) and ammonium chloride, RPE grade, were from Carlo Erba. An Ochraprep immunoaffinity column was from Orsell (Caroi, Modena, Italy). A PTFE syringe filter of 0.45 μm was from Pall Lifesciences (Ann Arbor, MI); a buffer solution (100 mM) of phosphate monobasic/sodium phosphate bibasic, pH 7.3, was from Normex (Carlo Erba). Standards of ochratoxin α were prepared by cleaving the amidic bond of OTA by carboxypeptidase A (Sigma Co.) according to Stander et al. (*28*). Phenylalanine standard was purchased from Sigma Co.

Yeasts and Culture Media. The yeasts used were 11 *S. cerevisiae* and 9 *K. apiculata* from the collection of the Department of Scienze Ambientali Agrarie and Biotecnologie Agroalimentari (Di.S.A.AB.A.), University of Sassari, Italy (**Table 1**). The yeast strains were used alone and in combination (3197 + 1090 yeasts). The culture media broth contained 6.7% yeast nitrogen base (YNB, Biolife, Italy), 20% glucose, and 0.1% diammonium phosphate, adjusted to pH 3.6 with tartaric acid.

Yeast cultures were prepared by growing the strains inoculated with YPD (1% bactoyeast extract, 2% bactopeptone, 2% bactoagar, and deionized water) for 48 h. Precultures were prepared with a substrate of 5% glucose and 0.7% YNB and agitated on a rotary shaker at 120 rpm for 24 h at 25 °C. The inoculated suspension contained 5×10^6 cells/mL, while for the test in combination were 1×10^7 cells/mL for *K. apiculata* and 1×10^5 cells/mL for *S. cerevisiae*, respectively. The culture media were fortified at 0.2, 0.6, 3.0, and 6.0 μg/L of OTA. After inoculation, for each strain and concentration, the culture media were divided in 500 mL flasks into three 150 mL replications.

For each concentration of OTA and yeast strain, two controls were prepared. One was prepared with inoculated media not containing OTA and the other was prepared with media containing OTA but without yeast to evaluate the stability of OTA during the experiment time. The samples were incubated in the dark at 25 °C. Samples were collected at 0, 1, 3, and 7 days and at end of fermentation: 7 days for *K. apiculata* and 20 days for *S. cerevisiae*, respectively.

Three parameters were assessed on the culture media: the percent of alcohol produced, the total count of yeast, and colony-forming units (CFUs). The percent of alcohol produced was determined at the end of fermentation by ponderal method. The total count of yeasts was carried out after 1, 3, 7, and 20 days (only for *S. cerevisiae*) using a Thoma counting chamber. The CFUs were determined as follows: A sample was spread on a surface of an YPD plate and was left to incubate at 30 °C for 48 h, and the number of colonies formed were counted. The investigations were carried out in triplicate for each strain.

HPLC−Fluorescence Analysis. A LaChrom-Merck-Hitachi liquid chromatograph (Hitachi Ltd., Tokyo, Japan) consisting of a D-7000 System Manager, a L-7100 pump, a L-7200 autosampler, and a L-7485 Fluorescence Detector was used. A Spherisorb ODS2 column (4.6 mm × 250 mm; 5 μm; Waters, Milan, Italy) was employed. The injection volume was 50 μL, and the flow rate was 1 mL/min. Isocratic elution was with 0.2 M phosphoric acid and acetonitrile (55:45, v/v). In this chromatographic condition, the retention time for OTA was 24.85 min. The fluorescence detector was set at 333 and 440 nm for the excitation and the emission wavelengths, respectively. Quantitative determinations were performed following the external standard method measuring peak area vs concentrations. The calibration graphs were constructed by injecting standard solutions prepared in bidistilled water at five concentration levels. The limit of detection (LOD) calculated as S/N = 3:1 was 0.1 μg/L, and the limit of quantification (LOQ) as S/N = 10:1 was 0.2 μg/L.

HPLC-MS Analysis. An HPLC system (Shimadzu, Milan, Italy) equipped with an SPD11 Avp DAD detector, an SIL 11 AD vp autoinjector, and a LC 10 AD binary pump coupled on line with an MS2010 mass spectrometer (Shimadzu) was used. UV and MS data were acquired and processed using Shimadzu "LCMS solution" software. Isocratic elution was with acetonitrile−aqueous 0.1% trifluoroacetic acid (99% purity) (50: 50, v/v) for 20 min. The used column was a 150 × 2.1 i.d., 3.5 μm particle size Waters Symmetry C18. The injection volume was 20 μL, and the flow rate was 0.2 mL/min. According to the maximum of the UV spectra of OTA, UV-DAD detection was carried out at 215 nm for the detection of phenylalanine. In this chromatographic condition, the retention time for OTA was 9.58 min. The ESI-MS interface was operated in the positive mode: ESI CDL, 200 °C; block at 200 °C; flow gas (N_2) at 4.5 mL/min; probe voltage, 3 kV; scan mode, 100−600 amu; selected ion monitoring, 404; 445 amu for OTA; and 257 amu for α-OT (the analysis for the determination of α-OT residues was done in the flow injection mode), respectively.

Sample Preparation. An aliquot of 5 mL of the culture media inoculated with different strains was transferred into a glass tube and centrifuged at 4000 rpm for 5 min. The aqueous phase was separated from the yeasts. One milliliter of the aqueous layer was diluted with 20 mL of phosphate buffer (pH 7.3). Using a syringe, the solution was applied to the Ocraprep immunoaffinity column at the flow rate of 3 mL/min. The column was washed with 20 mL of the phosphate buffer at the flow rate of 5 mL/min. OTA was eluted from the column using 1.5 mL of a mixture of methanol/acetic acid (98:2; v/v) followed by 1.5 mL of bidistillate water. The eluted solution was submitted to HPLC for analysis.

The pellet was extracted twice with 5 mL of ethyl acetate in a Vortex mixer for 30 s. The extracts were combined, and 2 mL of the resulting solution was gently evaporated to dryness with nitrogen. The residue was dissolved in 1 mL of bidistilled water, filtered through a PTFE filter, and injected for HPLC analysis. The samples with OTA levels under 0.1 μg/L were concentrated ten times before HPLC analysis.

Recovery Assays. YNB solution, YNB fermented sample, and the pellet were fortified with known amounts of OTA at 0.2, 1.0, 5.0, and 20.0 μg/L and, after 1 h, were processed according to the above procedure. Recovery experiments were done in four replicates. Recoveries ranged from 78 to 91%, with a maximum coefficient of variation (CV) of 7.6%, and from 79 to 104% with a maximum CV of 11.4% for the liquid layer and the pellet, respectively.

Statistical Analysis. Analysis of variance was performed by MSTAT-C (1991); analysis was followed by the Tukey posthoc test, when appropriate ($p < 0.05$).

RESULTS AND DISCUSSION

Yeasts and Fermentation Process. Tables 2 and **3** show the effect of the different concentrations of OTA on the fermentation process. The analysis of the total count, the CFU, and the alcohol production did not change in the fermentation media for both *S. cerevisiae* and *K. apiculata*, even at high OTA levels (6.0 μg/L).

OTA Residues. Calibration graphs were calculated by injecting standard solutions prepared in the eluent mixture 0.2 M phosphoric acid−acetonitrile (55:45 v/v); a good correlation (0.9990) was obtained in the range of concentration of OTA from 0.2 to 20 μg/L. The two phases, fermentation media and pellet, were analyzed separately at the end of the fermentation process.

Table 2. Effect of the Different Levels of OTA (μg/L) on the Fermentation Activity of Different Strains of *K. apiculata*

	yeast counts at different fermentation time								
		total count cell/mL $\times 10^7$					CFU/mL $\times 10^7$		
	OTA level	day			alcohol	OTA level	day		
strain	(μg/L)	1	3	7	(%)	(μg/L)	1	3	7
3184	control	3.50	8.50	6.00	4.7	control	0.90	2.50	1.20
	0.2	3.00	5.50	5.00	4.7	0.2	1.10	2.80	1.50
	0.6	2,50	8.00	6.50	4.5	0.6	1.20	3.10	1.10
	3.0	2.00	9.50	5.00	4.5	3.0	0.80	2.60	1.90
	6.0	2.00	8.00	5.50	4.5	6.0	1.20	2.70	1.60
3187	control	8.00	14.00	10.00	5.2	control	1.20	3.70	1.60
	0.2	7.00	17.00	6.50	5.0	0.2	1.10	3.50	1.80
	0.6	10.00	12.50	7.50	5.5	0.6	1.30	3.40	1.60
	3.0	7.00	19.80	5.40	5.5	3.0	1.40	3.10	1.40
	6.0	9.50	18.00	8.50	5.2	6.0	1.50	4.10	1.70
3188	control	7.50	10.00	4.50	5.7	control	0.50	3.60	1.40
	0.2	7.50	10.00	3.75	4.7	0.2	0.60	3.80	1.70
	0.6	7.00	9.50	5.00	5.0	0.6	0.70	4.20	1.50
	3.0	6.50	9.00	3.75	5.0	3.0	1.20	3.70	1.60
	6.0	6.00	8.00	4.30	5.7	6.0	0.80	2.50	1.10
3189	control	3.00	7.50	5.00	6.0	control	0.90	2.50	1.20
	0.2	3.00	8.00	5.50	5.5	0.2	0.80	2.70	1.70
	0.6	3.00	6.00	4.00	6.0	0.6	0.80	2.60	1.50
	3.0	4.00	6.00	3.00	6.0	3.0	1.10	3.10	1.90
	6.0	3,50	5.50	3.50	6.5	6.0	1.00	2.90	1.80
3191	control	4.00	7.10	3.21	3.5	control	0.70	2.56	1.20
	0.2	4.42	5.70	2.46	3.0	0.2	0.78	2.11	1.40
	0.6	4.25	6.50	2.08	2.5	0.6	1.08	4.86	1.23
	3.0	3.80	5.90	3.23	2.2	3.0	0.69	4.26	1.37
	6.0	4.30	6.60	3.94	3.7	6.0	1.01	3.89	1.53
3197	control	4.50	7.50	3.00	5.0	control	0.70	2.60	1.30
	0.2	4.00	6.00	5.50	5.0	0.2	1.10	2.90	1.60
	0.6	4.00	6.00	4.60	6.0	0.6	0.80	3.10	1.90
	3.0	2.00	5.00	4.20	6.0	3.0	0.90	3.20	1.40
	6.0	2.50	4.00	3.50	5.5	6.0	0.60	2.50	1.50
3198	control	3.75	6.00	5.00	5.0	control	1.20	3.10	1.60
	0.2	3.00	6.00	5.50	5.7	0.2	0.90	3.80	1.80
	0.6	3.50	5.50	5.00	5.2	0.6	0.80	4.10	2.00
	3.0	3.50	12.00	5.00	5.5	3.0	0.60	3.10	1.80
	6.0	3.00	5.00	4.30	5.5	6.0	0.90	2.90	2.10
3199	control	6.00	7.50	7.00	5.5	control	1.10	4.20	1.50
	0.2	5.50	7.50	6.00	6.0	0.2	0.60	3.50	1.80
	0.5	4.00	5.50	5.00	5.5	0.5	1.20	4.10	1.50
	2.0	4.50	6.50	4.50	5.7	2.0	0.90	4.50	1.70
	5.0	5.00	6.50	4.00	5.7	5.0	0.80	3.90	1.60
3200	control	4.50	6.00	3.00	6.2	control	0.90	2.90	1.50
	0.2	5.50	6.50	4.30	5.7	0.2	1.10	3.10	1.60
	0.6	4.50	7.50	4.00	6.0	0.6	0.70	2.90	1.90
	3.0	5.00	6.00	5.00	5.7	3.0	1.00	2.60	2.10
	6.0	6.00	8.00	5.00	6.0	6.0	0.80	2.70	2.00

Mycotoxin detoxification mediated by microorganisms has been studied by many authors (*29*, *30*). Varga et al. (*23*) and Abrhunosa et al. (*22*) studied the degrading activity in ethanol medium. Piotrowska et al. (*20*) and Silva et al. (*19*) studied OTA decrease in the presence of lactic acid bacteria comparing the initial value with that of sample taken during the fermentation process. It was found that a higher decrease was achieved after 16−20 h of incubation. In another study, the same authors evaluated (*21*) the sensitivity of lactic acid bacteria to OTA and their removing ability from the medium. These authors found that one strain was sensitive to OTA and that all strains were capable of reducing OTA but at different levels. They also studied the adsorbing capacity of the biomass produced; the data obtained allowed the authors to conclude that the ability of lactic acid bacteria in reducing OTA levels was strain-dependent and that a small amount of OTA was adsorbed by the biomass, but the main amount of OTA was removed following a different pathway of degradation, still not known. The OTA degradation ability of lactic acid bacteria has also been studied by Skrinjar et al. (*31*) in yogurt. Considering only the retention time of the compounds obtained after reaction with carboxypeptidase A or by chloridric acid hydrolysis, Abrunhosa et al. (*19*) concluded that the OTA main demolition products, fungi-mediated, were α-OT from the scission of the amidic bond and phenylalanine (**Figure 1**).

HPLC-MS was the critical method for compounds identification, matching t_R values and ESI fragmentation patterns with the authentic standard. The analysis performed in the positive ESI mode gave the following fragments and percent abundances adducts: *m*/*z* 404 (100) $[M + H]^+$, *m*/*z* 445 (*14*) $[M + H + CH_3CN]^+$. Confirmation of OTA was assessed in selected ion monitoring (SIM), recording the ions 404 and 445 (**Figure 2**).

Phenylalanine was not found in the fermentative media or in the pellet extract nor α-OT. The absence of α-OT and phenylalanine in the media and in the pellet did not ensure that the degradation pathway suggested by Abrunhosa did not occur, because phenylalanine and α-OT could also react with other compounds in the media. In order to understand the fate of OTA during the fermentation process, it should be carried out in an experiment using a labeled standard of OTA, which is unfortunately not available in the market. The data reported in this paper do not agree with Bejaoui et al. (*24*). These authors found that the 90% of OTA rapidly bound to both strains of *S. cerevisiae* and *Saccharomyces bayanus*. They studied the decrease of OTA levels mediated by yeasts, in YPG e synthetic grape juice, finding high differences in the amount of OTA between the inoculation step and the end of fermentation. The degradation products searched, not specified by the authors, were not found, allowing them to conclude that OTA was adsorbed by the yeast. In another paper, the same authors (*25*) studied the ability to remove OTA residues of conidia from *Aspergillus* species. This experiment carried out on both SGM and natural red wine led to a decrease of OTA content and in some cases to the occurrence of α-OT, but the removing effects were ascribed to adsorption on the pellet. On the other hand, no analysis was carried out on the yeast cell walls, and the degradation products may potentially be chemicals that are not detectable, neither with the UV nor with the fluorescence detector. Nevertheless, the decrease of OTA levels found by Bejaoui et al. is comparable with those reported in this paper. Cecchini et al. (*26*) reported OTA residues in the lees after extraction with methanol. They also reported a higher removal capacity in red wines if compared with white wines, but they did not find significant differences in the biomass produced, ascribing the OTA decrease to an adsorbing effect of yeast. Even in this case, no degradation products were found in the medium, and little information was given in the paper on the nature of the degradation products searched. The difference in the balance of OTA removed from the medium with that extracted from the biomass was explained, assessing that it was irreversibly bound to the yeast walls. Caridi (*32*) reported on the ability of mannoproteins to remove OTA from the medium, acting like a sponge. Ringot et al. (*33*) reported on in vitro experiments of the spontaneous nature of OTA to adsorb on vinaccia with 16% of yeast, dry yeast cell walls, and β-glucan. In the wine, at pH 3.5, OTA is partially dissociate, showing a positive charge on the amino group (NH_4^+); for these reasons, the OTA phenolic groups and carboxyl groups could be involved in different mechanisms of absorption by phenolic substances present in the lees. Leon et al. (*27*) reported the fate of OTA during vinification, after inoculation with two different strains of *A. carbonarius*. Vinification was carried out with two different

Table 3. Effect of the Different Levels of OTA (μg/L) on the Fermentation Activity of the Different Strains of *S. cerevisiae*

		yeast counts at different fermentation time									
		total count cell/mL $\times 10^7$						CFU/mL $\times 10^7$			
	OTA level	day				alcohol	OTA level	day			
strain	(μg/L)	1	3	7	end of F.	(%)	(μg/L)	1	3	7	end of F.
1090	control	4.37	9.60	19.30	12.40	14.7	control	0.42	6.00	1.42	0.46
	0.2	4.14	1.90	14.20	11.20	14.5	0.2	0.34	6.33	2.27	0.60
	0.6	5.58	3.00	18.80	10.60	14.5	0.6	0.07	5.63	2.46	0.65
	3.0	4.64	11.20	17.70	12.30	15.2	3.0	0.11	4.80	4.00	0.80
	6.0	5.73	12.50	18.40	12.90	14.2	6.0	0.04	6.06	5.00	0.71
1189	control	5.98	13.70	15.70	12.70	14.5	control	0.50	2.70	0.90	0.14
	0.2	6.98	13.60	15.60	13.90	13.7	0.2	0.64	2.02	1.06	0.51
	0.6	5.83	13.50	19.90	14.20	13.2	0.6	0.03	1.04	0.90	0.01
	3.0	4.71	12.60	18.60	12.10	13.2	3.0	0.04	0.66	0.74	0.05
	6.0	6.89	15.60	17.60	12.50	14.2	6.0	0.07	0.55	0.61	0.01
1153	control	4.60	9.20	25.70	16.60	12.7	control	0.01	1.74	1.12	0.66
	0.2	4.25	10.40	18.50	15.70	12.5	0.2	0.01	2.75	2.20	1.76
	0.6	4.62	12.40	21.20	15.00	12.0	0.6	0.04	2.85	2.44	1.70
	3.0	5.50	16.40	19.40	14.20	12.5	3.0	0.09	2.58	2.05	2.52
	6.0	6.20	15.40	21.20	14.20	12.5	6.0	0.04	3.48	2.87	2.80
1161	control	6.90	13.50	16.70	15.80	13.0	control	0.06	2.50	3.10	1.50
	0.2	2.50	10.80	16.00	14.00	13.7	0.2	0.08	2.80	4.10	1.80
	0.6	5.20	14.80	19,.60	16.30	13.7	0.6	0.10	2.90	3.20	1.40
	3.0	3.30	10.80	15.90	12.70	13.5	3.0	0.09	3.10	3.50	1.70
	6.0	5.00	12.12	17.40	15.60	13.5	6.0	0.08	2.90	3.70	1.60
1164	control	4.40	27.00	19.00	14.40	9.8	control	0.90	2.10	4.00	2.10
	0.2	3.80	16.00	27.00	21.90	10.0	0.2	1.00	2.00	3.60	1.90
	0.6	4.00	28.00	18.00	16.30	8.5	0.6	0.80	2.20	4.10	2.20
	3.0	5.90	28.00	21.00	8.00	9.5	3.0	0.70	1.90	4.20	1.70
	6.0	4.50	25.00	15.00	15.00	8.8	6.0	0.60	1.90	3.90	1.90
1182	control	5.80	13.70	21.20	13.80	12.2	control	0.90	2.10	4.00	2.10
	0.2	5.40	11.40	18.50	16.30	13.0	0.2	1.00	2.00	3.60	1.90
	0.6	7.90	10.40	17.90	15.00	11.7	0.6	0.80	2.20	4.10	2.20
	3.0	7.70	11.00	16.40	11.00	11.7	3.0	0.70	1.90	4.20	1.70
	6.0	6.90	11.00	17.70	10.80	13.0	6.0	0.60	1.90	3.90	1.90
1226	control	7.00	7.30	15.70	13.80	13.7	control	0.08	2.00	3.50	1.50
	0.2	6.50	8.80	14.80	14.00	14.0	0.2	0.08	2.10	3.40	1.40
	0.6	6.00	7.30	16.20	14.30	13.7	0.6	0.11	1.80	4.10	1.60
	3.0	6.20	7.90	14.40	12.70	14.7	3.0	0.10	1.70	3.50	1.70
	6.0	6.00	7.00	16.60	14.60	13.7	6.0	0.09	1.90	3.70	1.30
1236	control	6.20	9.50	12.30	11.20	13.5	control	0.90	2.00	4.10	1.50
	0.2	6.40	7.30	12.50	12.30	14.0	0.2	1.20	2.10	4.00	1.60
	0.6	3.70	6.90	14.40	11.20	13.5	0.6	0.70	1.90	3.00	1.60
	3.0	4.30	8.60	14.60	11.80	12.7	3.0	0.80	1.80	3.90	1.40
	6.0	4.00	7.90	11.60	11.40	13.7	6.0	1.10	2.20	4.20	1.80
1237	control	8.10	13.30	17.50	14.80	12.7	control	0.08	2.00	4.10	1.30
	0.2	7.70	12.30	17.00	15.60	11.2	0.2	0.12	1.50	3.50	1.20
	0.6	6.00	8.00	13.50	12.00	14.0	0.6	0.09	1.70	3.90	1.50
	3.0	6.60	10.20	13.50	14.60	14.7	3.0	0.10	1.40	4.30	1.10
	6.0	6.00	9.60	17.30	16.00	14.5	6.0	0.10	1.40	4.20	1.60
1494	control	8.30	11.00	17.00	15.30	13.2	control	0.90	1.60	3.60	2.10
	0.2	6.30	11.50	15.50	12.20	15.2	0.2	1.00	1.50	3.90	2.10
	0.6	4.60	12.50	19.40	9.60	14.0	0.6	0.89	1.40	3.70	2.20
	3.0	4.60	12.90	15.40	10.00	14.2	3.0	1.20	1.30	4.10	1.90
	6.0	5.20	10.80	14.60	11.60	14.7	6.0	1.10	1.70	3.80	1.80
1304	control	3.70	11.60	22.90	13.10	14.2	control	0.08	1.70	3.50	1.80
	0.2	8.30	10.20	18.70	14.80	14.7	0.2	0.08	1.60	4.20	1.50
	0.6	8.30	9.30	19.30	14.40	14.5	0.6	0.11	1.50	3.80	1.80
	3.0	4.60	7.90	21.40	17.00	14.0	3.0	0.10	1.80	3.90	1.50
	6.0	5.80	8.70	17.70	15.10	13.5	6.0	0.09	2.10	4.10	1.60

strains, and no OTA decrease was reported during this step by the authors. During the solid−liquid separation stage, they observed a higher repartition of OTA in the solid phase; however, they concluded that further research was needed on the nature of OTA binding precipitated by grape constituents and yeasts.

Our experiment was carried out on a synthetic wine, and no adsorbing effect from other constituent present in a real must could affect OTA residues. This experiment allowed us to understand if in real fermentation conditions *Saccharomyces* and *Kloeckera* strains were able to degrade or adsorb OTA. Because the pellet separated by centrifugation from the reaction media consisted of dead yeast cells, the lack of OTA residues indicated the absence, in our experiment, of an adsorption phenomenon of yeast cell walls.

Tables 4 and **5** showed the effect of the fermentation process on the levels of OTA at the end of fermentation in the samples and in the control. The reduction percentages were not correlated to OTA concentration and strain type. *K. apiculata* strains 3184 and 3191 did not cause any decrease of the OTA content at all concentrations studied, while strain 3199 did not show any effect at high concentrations of OTA (6.0, 3.0, and 0.6 μg/L) and a

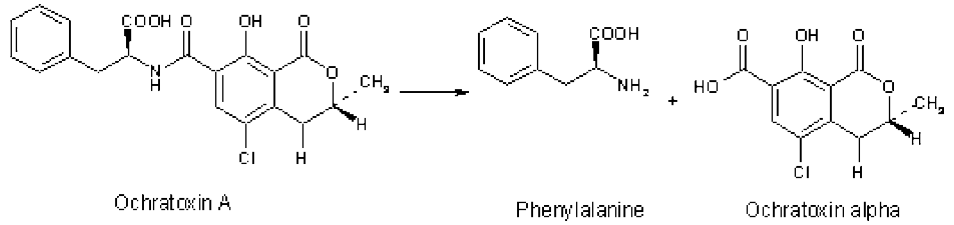

Figure 1. OT degradation reaction, mediated by fungi-producing α-OT and phenylalanina.

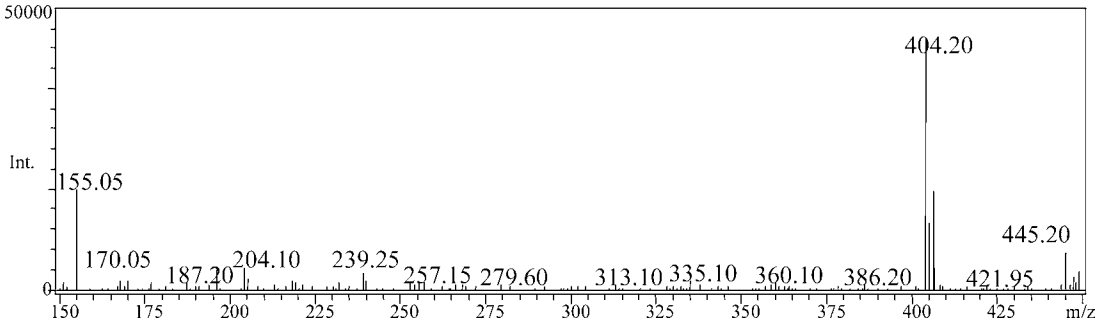

Figure 2. ESI(+)-MS mass spectra of OTA; *m*/*z* 404 $[M + H]^+$ molecular ion, *m*/*z* 445 $[M + H + CH_3CN]^+$ adduct.

Table 4. OTA Concentration (μg/L) and Its Reduction (%) in the Culture Medium with *K. apiculata* Strains at the End of the Fermentation (20 Days)

		6.0 mg/L[b]		3.0 mg/L		0.6 mg/L		0.2 mg/L	
	sample	mg/L	R%[c]	mg/L	R%	mg/L	R%	mg/L	R%
1	control	6.14 a[a]		2.84 ab		0.68 a		0.22 a	
2	3184	6.64 a		3.07 a		0.71 a		0.26 a	
3	3187	3.68 c	40	1.49 b	47	0.37 b	45	0.12 b	45
4	3188	4.12 bc	33	1.52 b	46	0.69 a		0.08 b	64
5	3189	4.38 bc	29	1.80 ab		0.54 ab		0.21 a	
6	3191	5.44 ab		2.20 ab		0.51 ab		0.16 ab	
7	3197	4.57 bc	25	2.12 ab		0.64 a		0.25 a	
8	3198	4.61 bc	25	2.39 ab		0.41 b	40	0.10 b	54
9	3199	6.15 a		2.63 ab		0.49 ab		0.14 b	36
10	3200	4.49 bc	27	1.63 b	43	0.53 ab		0.21 a	

[a] Different letters denote significant differences within a column group at $P \leq 0.05$ by Tukey's test. [b] Treatment concentration. [c] Percent of reduction.

Table 5. OTA Concentration (μg/L) and Its Reduction (%) in the Culture Medium with *S. cerevisiae* Strains at the End of the Fermentation (20 Days)

		6.0 mg/L[b]		3.0 mg/L		0.6 mg/L		0.2mg/L	
	sample	mg/L	R%[c]	mg/L	R%	mg/L	R%	mg/L	R%
1	control	6.06 ab[a]		3.35 ad		0.64 a		0.18 ae	
2	1090	5.31 ab		2.60 abc		0.43 b	32	0.10 c	44
3	1153	5.83 a		2.86 abc		0.70 a		0.23 e	
4	1161	4.58 abc		2.22 bc	34	0.52 ab		0.17 abe	
5	1164	5.26 ab		3.26 ab		0.62 a		0.11 c	39
6	1182	3.40 c	44	2.01 c	40	0.25 c	61	0.03 d	83
7	1189	4.78 abc		3.36 ad		0.65 a		0.27 e	
8	1226	4.93 abc		2.29 abc		0.41 b	35	0.11 c	39
9	1236	4.02 bc	34	2.08 c	38	0.43 b	32	0.13 bc	28
10	1237	4.65 abc		2.28 abc		0.52 ab		0.14 ac	
11	1304	5.15 ab		2.52 abc		0.45 b	30	0.00 d	100
12	1494	5.99 a		2.84 abc		0.57 ab		0.13 ac	

[a] Different letters denote significant differences within a column group at $P \leq 0.05$ by Tukey's test. [b] Treatment concentration. [c] Percent of redaction.

lessening effect at the lower concentration (36% at 0.2 μg/L). All of the others strains showed different behaviors. The strain with the higher decreasing effect was 3187 (~45%). At the end of fermentation (20 days), the lessening effects of *S. cerevisiae* strains generally increase when the concentration of OTA in the fermentation media decreases. Strain 1182 had the highest decreasing effect, and strain 1236 showed a good decreasing effect but lower than the former. Strains 1090, 1226, and 1304 showed activity only at low concentrations of OTA. The other strains did not show any reducing activity on OTA residues.

The data obtained in this paper allowed us to conclude that OTA residues did not affect the fermentative process and *S. cerevisae* and *K. apiculata* activity. The lessening activity of *S. cerevisae* and *K. apiculata* on OTA is not a genus but a strain peculiarity; yeasts decreased the amount of OTA present in the synthetic must from 0.25- to 0.5-fold, especially when low amounts of OTA were present. The absence of OTA residues in the biomass excluded an adsorbing effect from the yeast cell walls of the strains studied, and the absence of α-OT and phenylalanine suggested other degradation pathways of OTA. The uses of OTA isotopically labeled could help us to understand the real degradation pathway of this mycotoxin. The selection of yeasts may have a fundamental impact not only for the flavor characteristics of the wine but also for its toxicological safety.

LITERATURE CITED

(1) Zimmerli, B.; Dick, R. Ochratoxin A in table wine and grape-juice: Occurrence and risk assessment. *Food Addit. Contam.* **1996**, *13*, 655−668.

(2) Marquardt, R. R.; Frohlich, A. A. Review of recent advances in understanding ochratoxicosis. *J. Anim. Sci.* **1992**, *70*, 3968−3988.

(3) IARC (International Agency for Research on Cancer). Ochratoxin A. Monographs on the evaluation of carcinogenic risks to humans: some naturally occurring substances, food items and constituents, heterocyclic aromatic amines and mycotoxines. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, **1993**, *56*, 489−521.

(4) Burdaspal, P. A.; Legarda, T. M. Ochratoxina en vinos, mostos y zumos de uva elaborados en España y en otros paises europeos. *Alimentaria* **1999**, *36*, 107−113.

(5) Festas, I.; Herbert, P.; Santos, L.; Cabral, M.; Barros, P.; Alves, A. Ochratoxin A in some Portuguese wines: Method validation and screening in Porto wine and vinho verde. *Am. J. Enol. Vitic.* **2000**, *51*, 150−154.

(6) Ospital, M.; Cazabeil, J. M.; Betbeder, A. M.; Tricard, C.; Creppy, E.; Medina, B. Ochratoxin A in wines. *Rev. Fr. Oenol.* **1998**, *169*, 16−18.

(7) Otteneder, H.; Majerus, P. Occurrence of ochratoxin A in wines: Influence of the type of wines and its geographical origin. *Food Addit. Contam.* **2000**, *17*, 793−798.
(8) Pietri, A.; Bertuzzi, T.; Pallaroni, L.; Piva, G. Occurrence of ochratoxin A in Italian wines. *Food Addit. Contam.* **2001**, *18*, 647−654.
(9) Visconti, A.; Pascale, M.; Centonze, G. Determination of ochratoxin A in wine by means of immunoaffinity column clean-up and HPLC. *J. Chromatogr. A* **1999**, *864*, 89−101.
(10) Battilani, P.; Pietri, A.; Bertuzzi, T.; Languasco, L.; Giorni, P.; Kozakiewicz, Z. Occurrence of ochratoxin A-producing fungi in grape grown in Italy. *J. Food Prot.* **2003**, *66*, 633−636.
(11) Rousseau, J.; Blateyron, L. Ochratoxine A dans le vins: Pas de solutions curative sur vin, prioritè à la maitrise sanitarire au vignoble. *Revue. Des. Cenolougues.* **2002**, *104*, 14−15.
(12) Da Rocha, C. A. R.; Palacios, V.; Combina, M.; Fraga, M. E.; De Oliveira, A. R.; Magnoli, C. E.; Dal Cero, A. M. Potential ochratoxin A producers from wine grapes in Argentina and Brasil. *Food Addit. Contam.* **2002**, *19*, 408−414.
(13) Rousseau, J. Ochratoxin A in wine: Overview. *Wine Internet Tech. J.* 2004. Available at http://www.infowine.com/.
(14) FAO. *Food and Nutrition*; 56th meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA); FAO: Rome, Italy, 2001; Report 74.
(15) GUUE, L25, January 28, 2005; Commission Regulation (EC) No. 123/2005 of 26 January 2005.
(16) Castellari, M.; Versari, A.; Fagiani, G.; Parpinello, P.; Galassi, S. Removal of ochratoxin A in red wines by means of adsorption treatments with commercial fining agents. *J. Agric. Food Chem.* **2001**, *49*, 3917−3921.
(17) Laciakova, A.; Cabadaj, R.; Pastorova, B.; Conkova, E.; Kovac, G.; Laciak, V. The effect of feed additives on ochratoxin A. *Folia Vet.* **1999**, *42*, 71−73.
(18) Savino, M.; Morassut, M.; Cecchini, F.; Garcia Moruno, E.; Beccaccino, B. *Ocratossina A: Decontaminazione Fisica dei Vini Mediante uso di Carbone Enologico*; Atti Congresso OMS: Roma, 2004.
(19) Silva, A.; Galli, R.; Grazioli, B. Metodi di riduzione di residui di ocratossina nei vini. *Ind. Bevande* **2003**, *32*, 467−472.
(20) Piotrowska, M.; Zakowska, Z. The biodegradation of ochratoxin A in food products by lactic acid bacteria and baker's yeast. *Food Biotechnol.* **2000**, 307−310.
(21) Piotrowska, M.; Zakowska, Z. The elimination of ochratoxin A by lactic acid bacteria. *Pol. J. Microbiol.* **2005**, *4*, 279−286.
(22) Abrunhosa, L.; Serra, R.; Venancio, A. Biodegradation of ochratoxin A by fungi isolated from grapes. *J. Agric. Food Chem.* **2002**, *50*, 7493−7496.
(23) Varga, J.; Rigò, K.; Téren, J. Degradation of ochratoxin A by *Aspergillus* species. *Int. J. Food Microbiol.* **2000**, *59*, 1−7.
(24) Bejaoui, H.; Mathieu, F.; Taillandier, P.; Lebrihi, A. Ochratoxin A removal in synthetic and natural grape juice by selected oenological *Saccharomyces* strains. *J. Appl. Microbiol.* **2004**, *97*, 1038−1044.
(25) Bejaoui, H.; Mathieu, F.; Taillandier, P.; Lebrihi, A. Conidia of black aspergilli as new biological adsorbents for ochratoxin A in grape juice and musts. *J. Agric. Food Chem.* **2005**, *53*, 8224−8229.
(26) Cecchini, F.; Morassut, M.; Garcia Moruno, E.; Di Stefano, R. Influence of yeast strain on ochratoxin A content during fermentation of white and red must. *Food Microbiol.* **2006**, *23* (5), 411−417.
(27) Leong, S. L.; Hocking, A. D.; Varelis, P.; Giannikopoulos, G.; Scott, E. S. Fate of ochratoxin A during vinification of Semillon and shiraz grapes. *J. Agric. Food Chem.* **2006**, *54*, 6460−6464.
(28) Stander, M. A.; Pieter, S. S.; Francois, H. v. d. W.; Barry, E. P. A kinetic study into the hydrolysis of the ochratoxins and analogues by carboxypeptidase *A. Chem. Res. Toxicol.* **2001**, *14*, 302−304.
(29) Hwang, C. A.; Draughon, F. A. Degradation of ochratoxin A by Acinetobacter calcoaceticus. *J. Food Prot.* **1994**, *57*, 410−414.
(30) Boswald, C.; Engelhardt, G.; Vogel, H.; Wallnofer, P. R. Metabolism of Fusarium mycotoxins zearalenone and deoxynivalenol by yeast strains of technological relevance. *Nat. Toxins* **1995**, *3*, 138−144.
(31) Skrinjar, M.; Rasic, J. L.; Stojicic, V. Detoxification of ochratoxin A in Yoghurt by Lactobacillus acidophilus. *Prehrambena Ind.* **2002**, *13*, 28−30.
(32) Caridi, A. Enological functions of parietal yeast mannoproteins. *Antonie van Leeuwenhoek* **2006**, *89*, 417−422.
(33) Ringot, D.; Lerzy, B.; Bonhoure, J. P.; Auclair, E.; Oriol, E.; Larondelle, Y. Effect of temperature on in vitro ochratoxin A biosorption onto yeast cell wall derivatives. *Process Biochem.* **2005**, *40*, 3008−3016.

Received for review October 3, 2006. Revised manuscript received November 24, 2006. Accepted November 28, 2006.

JF062768U