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Ochratoxin A removal in synthetic and natural grape juices by selected oenological *Saccharomyces* strains

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ABSTRACT

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Aims: To assess, for the first time the efficiency in removing ochratoxin A (OTA) from laboratory medium [yeast peptone glucose (YPG)], synthetic grape juice medium (SGM) and natural grape juice by viable and dead (heat and acid-treated) oenological *Saccharomyces* strains (five *S. cerevisiae* and one *S. bayanus*) compared with a commercial yeast walls additive.

Methods and Results: Levels of OTA during its interaction with six oenological *Saccharomyces* strains (five *S. cerevisiae* and one *S. bayanus*) or with a commercial yeast walls additive in YPG medium, in SGM or in natural grape juices was assessed by HPLC after appropriate extraction methods. A significant decrease of OTA levels in YPG medium and SGM was observed for many of the growing strains reaching a maximum of 45%, but no degradation products were detected. With both heat and acid pretreated yeasts, OTA removal was enhanced, indicating that adsorption, not catabolism, is the mechanism to reduce OTA concentrations. Adsorption was also improved when the yeast concentration was increased and when the pH of the medium was lower. Approximately 90% of OTA was bound rapidly within 5 min and up to 72 h of incubation with heat-treated cells of either *S. cerevisiae* or *S. bayanus*. A comparative study between heat-treated cells (HC) and commercial yeast walls (YW) (used as oenological additive), introduced at two different concentrations (0·2 and 6·7 g l^{-1}) in an OTA-contaminated grape juice, showed the highest efficiency by HC to adsorb rapidly within 5 min the total amount of the mycotoxin.

Conclusions: Oenological *S. cerevisiae* and *S. bayanus* were able to remove ochatoxin A from synthetic and natural grape juices. This removal was rapid and improved by dead yeasts having more efficiency than commercial yeast walls.

Significance and Impact of the Study: The efficiency of heat-treated yeasts to remove OTA gives a new hope for grape juice and must decontamination avoiding negative impacts on human health.

Keywords: adsorption, biodegradation, grape juice, ochratoxin A, *Saccharomyces*.

INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin produced by two genera of fungi *Aspergillus* and *Penicillium*. A big concern is about its occurrence in many commodities (feeds, foods and beverages), because it is suspected to be nephrotoxic, teratogenic, hepatotoxic and carcinogenic (Marquardt and Frohlich 1992). This toxin has been detected in human blood in many countries after the consumption of contami nated foods (Petkova Bocharova *et al.* 1988).

Since 1996, the presence of this toxin has been reported in grapes and grape products such as grape juice

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(<3 311 ng l ^{1}) and wine (<3 388 ng l ^{1}) (Zimmerli and Dick 1996). In fact, wine is considered as the second major source of OTA intake after cereals (Anon. 1998). Available results show that wines from the Mediterranean area contain higher levels of OTA than wines from northern areas in Europe (Zimmerli and Dick 1996). Grape juices can also show OTA contamination. It seems they contain more OTA than some wines and so contribute to OTA intake by children (Zimmerli and Dick 1996).

In order to protect consumer health from the risk of exposure to this mycotoxin, reliable methods to reduce OTA levels are highly desirable. Decontamination procedures based on physical or chemical removal were proposed on a laboratory scale (McKenzie *et al.* 1997; Laciakova *et al.* 1999). Different assays for biological removal of OTA on laboratory media or on different commodities have been developed (El Nezami *et al.* 1998; Piotrowska and Zakowska 2000a,b; Skrinjar *et al.* 2002) and seemed to be the most promising ones (Skrinjar *et al.* 2002), but no study for OTA removal in grape products was undertaken.

Regarding mechanisms of mycotoxins removal, degrada tion by micro organisms has been reported in literature. In laboratory media: *Acinetobacter calcoaceticus* (Hwang and Draughon 1994) and *Aspergillus* species (Varga *et al.* 2000; Abrunhosa *et al.* 2002) are able to degrade OTA in ethanol minimal salts medium, and in liquid and solid YES medium, respectively. OTA degradation has also been observed in foods and beverages such as yoghurt by *Lactobacillus acidophilus* (Skrinjar *et al.* 2002) and in milk by *Lactobacillus*, *Streptococcus* and *Bifidobacterium* (Skrinjar *et al.* 1996).

Different *Saccharomyces* species completely degraded zearalenone present as a sole carbon source into zearalenone α and zearalenone β (Boswald *et al.* 1995). *Saccharomyces cerevisiae* has been reported to be able to degrade patulin in apple juice during cider fermentation (Moss and Long 2002).

An adsorption mechanism has also been suggested for OTA removal in MRS medium by lactic acid bacteria (Piotrowska and Zakowska 2000a), for aflatoxin B1 also adsorbed by lactic acid bacteria (El Nezami *et al.* 1998) and by probiotic bacteria (Peltonen *et al.* 2000), and finally for zearalenone and α zearalenol by lactic acid bacteria (El Nezami *et al.* 2002).

Other studies have mentioned that *S. cerevisiae* removed OTA when it was mixed with two other mycotoxins: fumonisin B1 and B2 (Scott *et al.* 1995), or present alone in sour dough (Piotrowska and Zakowska 2000a), but no details were mentioned about the removal mechanism.

Our objective in this study is to understand OTA removal by oenological *Saccharomyces* strains from different syn thetic media [yeast peptone glucose (YPG) and synthetic grape juice (SGM)] initially contaminated with OTA and from a red grape juice compared with a commercial yeast walls additive commonly used as an alcoholic fermentation activator.

MATERIALS AND METHODS

Yeast strains

Five strains of *S. cerevisiae* (LALVIN BM45; LALVIN Rhône 2226; UVAFERM 43; LALVIN Rhône 2323 and LALVIN Rhône 2056) and one strain of *S. bayanus* (LALVIN QA23) were used in this study. These strains were supplied by LALLEMAND S.A. company (Montreal, Canada) as dry active yeasts. The *S. cerevisiae* strains have been previously selected for red winemaking in Mediterra nean areas.

Yeast walls additive

A oenological yeast walls additive supplied by Springer Oenologie (Bioferm E.C.L., Maisons Alfort, France) was used in this study.

Culture media

Yeast peptone glucose was prepared by dissolving 5 g bacto peptone (Difco), 5 g yeast extract (Difco) and 40 g glucose D(+) (Fisher Labosi, Elancourt Cedex, France), in 1 l distilled water and the pH was adjusted at 5 5·2. SGM was prepared by dissolving: 70 g glucose D(+) (Fisher Labosi), 30 g fructose D(−) (Sigma), 7 g tartaric acid L(−) (Rectapur Prolabo, Paris, France), 10 g malic acid L(−) (Fisher Labosi), 0·67 g $(NH_4)_2HPO_4$ (Prolabo Rhône Poulenc, Paris, France), 0·67 g KH_2PO_4 (Acros), 1·5 g $MgSO_4 \cdot 7H_2O$ (Acros), 0·15 g NaCl (Fisher Labosi), 0·15 g $CaCl_2$ (Acros, Geel, Belgium), 0·0015 g $CuCl_2$ (Prolabo, Paris, France), 0·021 g $FeSO_4 \cdot 7H_2O$ (Riedel de Haën, Seelze, Germany), 0·0075 g $ZnSO_4 \cdot 7H_2O$ (Fisher Labosi) and 0·05 g hydrated catechin (Sigma); in 1 l distilled water and the pH was adjusted at 3·8 4·0 with KOH 2 N. Phosphate buffered saline (PBS) (lot 83230 003; 0·2 g l ^{1} potassium dihydrogen phosphate, 2·9 g l ^{1} disodium hydrogen phosphate 12 H_2O, 0·2 g l ^{1} potassium chloride, 8 g l ^{1} sodium chloride, 0·1 g l ^{1} thimerosal, pH 7·4) (Rhône diagnostics Techno logies, Glasgow, UK). These laboratory media were supplemented with OTA at a concentration of 2 μg ml ^{1}. A red grape juice was also used in this experiment, and was contaminated with OTA at a concentration of 10 μg l ^{1}.

Growing yeasts assay

The degradation capacity of yeasts was tested in screw cap tubes in 2 ml appropriate medium (YPG and SGM media), under agitation (240 rev min ^{1}), at 30°C and for 6 days.

Each medium was inoculated at 1% from a preculture containing *ca* 10^7 CFU ml $^{-1}$. A negative control (control 1), an OTA contaminated medium without yeast, was used to calculate OTA removal percentage. A positive control (control 2), a growing yeast culture in a medium without OTA, was also used. All assays were performed in triplicate.

Yeast preparation

An overnight culture of yeast in YPG medium at 30°C was centrifuged at 16 000 *g*, for 5 min and washed twice with PBS buffer, the resulting yeasts constitute the viable cells (VC). A part of those cells was boiled in PBS buffer for 1 h to obtain heat treated cells (HC), or incubated in 2 M HCl (v/v) for 1 h to obtain acid treated cells (AC). After these treatments yeast suspensions were washed twice with PBS buffer and were ready for use.

Adsorption assay

Treated or not treated *S. cerevisiae* LALVIN Rhône 2056 cells were inoculated, at a concentration of 6·7 g l $^{-1}$ ± 2% in PBS, YPG and SGM containing 2 μg ml $^{-1}$ OTA. After 2 h of incubation at 30°C, cells were removed and OTA was analysed in the supernatant.

In SGM, another concentration of viable and heat treated *S. cerevisiae* LALVIN Rhône 2056 (27·4 g l $^{-1}$) was also tested. A kinetic of OTA adsorption with heat treated *S. cerevisiae* LALVIN Rhône 2056 and with *S. bayanus* (LALVIN QA23) at a concentration of 27·4 g l $^{-1}$ was performed in SGM over 72 h.

An OTA contaminated red grape juice (10 μg l $^{-1}$) was inoculated with heat treated *S. cerevisiae* LALVIN Rhône 2056 or with yeast walls additive at two concentrations of 6·7 and 0·2 g l $^{-1}$ and OTA removal kinetics were established over 2 h.

Only control 1 was performed and all assays were performed at 30°C, under agitation (240 rev min $^{-1}$) and in triplicate.

OTA extraction

OTA extraction from growing culture and adsorption samples. For all samples, after removal of yeasts, 1 ml of supernatant was extracted twice with ethyl acetate (v/v), then evaporated until dry and dissolved in 0·5 ml of methanol before high performance liquid chromatography (HPLC) analysis.

OTA extraction from grape juice. Ten millilitres of grape juice, were diluted with PBS buffer (v/v) after adjusting pH at 7·8 with KOH 2 M. The diluted sample was loaded onto an OchraPrep (Rhône diagnostics Technologies) immuno affinity column operating at a steady flow rate of 2 ml min $^{-1}$. The immunoaffinity column was washed with 20 ml of sterile distilled water, and then dried by pushing air with a vacuum pump. OTA was eluted by applying successively 1·5 ml of methanol/acetic acid (98 : 2) and 1·5 ml of sterile distilled water. The eluted extract was further analysed by HPLC.

Detection and quantification of OTA

Ochratoxin A was detected and quantified by reversed phase HPLC. The HPLC apparatus consisted of a solvent delivery system and fluorescence ($\lambda_{ex} = 332$ nm; $\lambda_{em} = 466$ nm) and u.v. detectors. The analytical column used was a 150 mm × 4·6 mm Uptisphere 5 μm C18 ODB fitted with a guard column of 10 mm × 4 mm. The mobile phase consisted of a mixture of HPLC grade acetonitrile/water/acetic acid (100/99·8/0·2) at a flow rate of 1 ml min $^{-1}$ and the column temperature was at 30°C. Kroma 3000 (Bio Tek, Milan, Italy) was the data acquisition system. Injections were made with an autoinjector (Bio Tek) and the injection volume was of 80 μl. OTA was identified by its retention time (33 min) according to a standard obtained from Sigma (Steinheim, Germany) and quantified by measuring the peak area.

The OTA degradation percentage was calculated according to the following equation: 100×[1− (peak area of OTA/peak area of OTA in control 1)].

Statistical analysis

SPSS, version 11·5.1 (SPSS, Chicago, IL, USA), for windows was used for the statistical analysis of the data. Significant differences in the mean values were reported at $P < 0{\cdot}05$.

RESULTS

Removal of OTA by growing yeasts

Six selected oenological yeasts were tested for their ability to remove OTA in either YPG or SGM media at 30°C, in screw cap tubes and under agitation. A decrease in OTA levels was noted after 6 days of incubation (Fig. 1). The percentage of OTA removal by yeasts was between 11 and 45% in YPG medium and between 1 and 35% in SGM depending on the strain used. Analysis of u.v. and fluorescence HPLC chromatograms (results not shown) showed no degradation products. We can conclude that OTA was not degraded by these micro organisms, and that OTA removal by growing *Saccharomyces* might be a cell binding phenomenon.

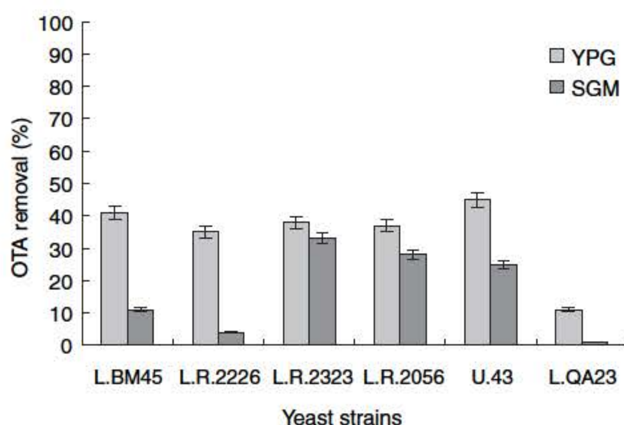

Fig. 1 Removal of OTA (%) by yeast strains during growth: *Saccharomyces cerevisiae* (LALVIN BM45; LALVIN Rhône 2226; UVAFERM 43; LALVIN Rhône 2323 and LALVIN Rhône 2056) and *Saccharomyces bayanus* (LALVIN QA23) in YPG and SGM media, after incubation at 30°C, for 6 days (L, Lalvin; U, Uvaferm; LR, Lalvin Rhône)

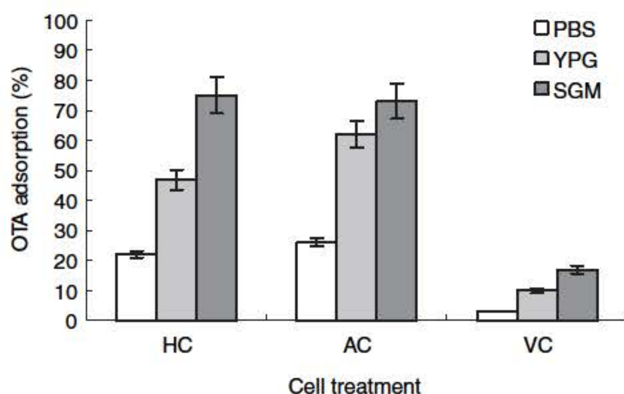

Fig. 2 OTA adsorption in different media (PBS, YPG and SGM) with different treated *Saccharomyces cerevisiae* LALVIN Rhône 2056 cells [heat treated cells (HC); acid treated cells (AC) and viable cells (VC)], at 30°C, during 2 h of incubation and at a concentration of 6·7 g l^{-1}

Adsorption assays

OTA adsorption on yeasts in laboratory medium. *S. cerevisiae* LALVIN Rhône 2056 was inoculated as viable cells (VC), heat (HC) and acid (AC) treated ones, at a concentration of 6·7 g l ^{1} in three media (PBS buffer, YPG and SGM) initially contaminated with 2 μg ml ^{1} OTA. Figure 2 shows that after 2 h of incubation in the presence of HC or AC, the adsorption of OTA was significantly enhanced in comparison with VC whatever the medium used, and that there is no significant difference between the two types of dead cells (heat or acid treated ones). In SGM medium, for example, the percentage of OTA adsorption was of 75, 73 and 17% for HC, AC and VC, respectively. It was also observed that OTA binding depends on the type of medium whatever the cell treatment. OTA adsorption was higher in the SGM medium, followed by YPG with an average of 74 and 54·5%, respectively (the average values were calculated with all cell treatments for each medium). OTA adsorption reached the lowest value in the PBS buffer with a maximum of 26% on AC.

Table 1 OTA removal by *Saccharomyces cerevisiae* LALVIN Rhône 2056 cells at two different concentrations at 30°C, during 2 h in SGM medium

	% adsorption of OTA from *S. cerevisiae* cells (average ± S.D.)	
Yeast concentrations	Viable cells	Heat treated cells
6·7 g l^{-1}	17·0^{a} ± 2·1	75·0^{c} ± 5·0
27·4 g l^{-1}	35·0^{b} ± 3·0	90·8^{d} ± 4·2

a,b,c,d: Values with different superscripts differ at $P < 0.05$.

Effect of cell concentration. VC and HC *S. cerevisiae* LALVIN Rhône 2056, were inoculated at two concentra tions (6·7 and 27·4 g l ^{1}) in SGM medium contaminated with 2 μg l ^{1} OTA during 2 h, at 30°C.

Table 1 shows that the increase in cell concentration significantly enhanced OTA adsorption by 100 and 21% for VC and HC, respectively. It also confirmed the higher capacity of HC to adsorb OTA compared with viable cells.

Effect of time. HC *S. cerevisiae* LALVIN Rhône 2056 and HC *S. bayanus* QA 23 were inoculated at a concentration of 27·4 g l ^{1}, in SGM medium at 30°C and during 72 h. Nearly 90%±0·72 of OTA was bound after 5 min of incubation, no release was observed after 2 h (Fig. 3) and up to 72 h (result not shown), and no significant difference was noted for the two yeast species.

In red grape juice. HC *S. cerevisiae* LALVIN Rhône 2056 and commercial yeast walls additive (YW) were inoculated at concentrations of 6·7 and 0·2 g l ^{1} in a red grape juice contaminated with 10 μg l ^{1} OTA. Figure 4 shows that, whatever the concentration used, OTA removal by HC was rapid and total within 5 min and up to 2 h. For YW, OTA adsorption was total after 15 min and up to 2 h, and no significant differences were noted between the two concen trations used. HC were more efficient than YW in decreasing OTA.

DISCUSSION

The present study demonstrates that the oenological yeast strains tested were able to remove OTA in growing culture from laboratory medium (YPG) and from SGM.

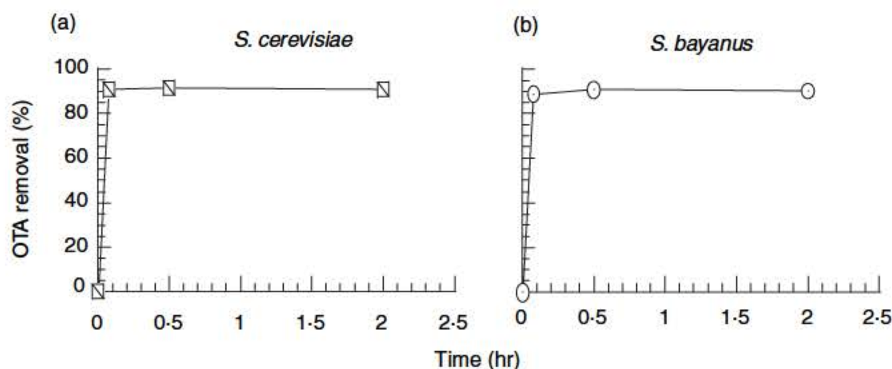

Fig. 3 Kinetics of OTA removal (%) for two *Saccharomyces* heat treated cells (*S. cerevisiae* LALVIN Rhône 2056 and *S. bayanus* LALVIN QA23), at 30°C, at a concentration of 27·4 g l^{-1} and in SGM medium

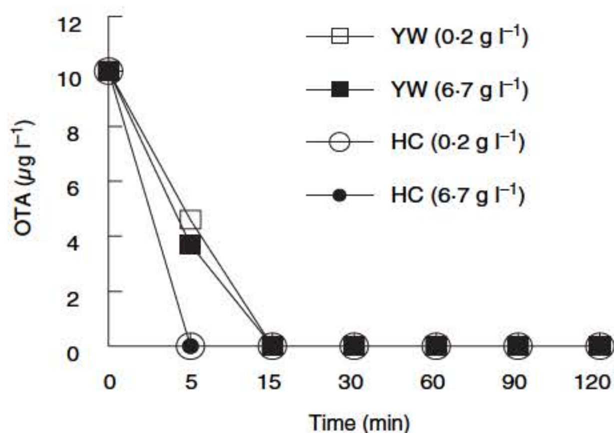

Fig. 4 The OTA removal in commercial grape juice (contaminated with OTA at a concentration of 10 μg l^{-1}) with HC *Saccharomyces cerevisiae* LALVIN Rhône 2056 and yeast walls (YW) at concentrations of 0·2 and 6·7 g l^{-1}

In our study, HPLC chromatograms of samples from growing culture were analysed in order to distinguish between biodegradation and adsorption of OTA. The absence of degradation products suggested that adsorption rather than catabolism might be involved. The OTA adsorption, compared with its metabolization, can be a promising decontamination process because it does not generate any degradation metabolites. These metabolites could be more toxic than the mycotoxin itself, as has been reported for zearalenone (El Sharkawy *et al.* 1991).

Adsorption assays were performed with the three kinds of cells. VC, HC and AC *S. cerevisiae* LALVIN Rhône 2056 at a concentration of 6·7 g l^{-1} were able to remove OTA (2 μg ml^{-1}) from PBS buffer, YPG and SGM media after 2 h of incubation at 30°C. OTA removal by viable cells (VC) reached a maximum of 17% in SGM medium. This adsorption can probably be carried out by cell walls. Those, harbouring polysaccharides (glucan, mannan), proteins and lipids, exhibit numerous different and easily accessible adsorption sites including different adsorption mechanisms, e.g. hydrogen bonding, ionic or hydrophobic interactions (Huwig *et al.* 2001). In another study, adsorption of mutagens on *Streptococcus* and *Lactobacillus* was carried out mainly by polysaccharides and peptidoglycans (Zhang and Ohta 1993; Rajendran and Ohta 1998). The adsorption mechanism could probably not be specific as it is shown for yeast killer toxins bound by polysaccharides and not by proteins, fatty acids (Radler and Schitt 1987; Santos *et al.* 2000), cellulose or glycogen (Radler and Schitt 1987) of sensitive yeasts.

Our results showed that treatments of yeasts by heating and acidity enhanced significantly OTA removal from liquid media (75%) compared with viable cells but no significant difference was noted between those two treatments. Polysaccharides and peptidoglycans are both expected to be affected by heat and acid treatments. Heating may cause denaturation of proteins or formation of Maillard reaction products. Acidic conditions could affect polysaccharides by releasing monomers, further fragmented into aldehydes after the breaking down of glycosidic linkages. These released products could offer more adsorption sites than viable cells and may increase surfaces for OTA binding. Moreover, the decrease in wall thickness of peptidoglycan and/or the increase in its pore size under heat and acid treatments could probably make available other sites from yeast cells for OTA adsorption (El Nezami *et al.* 2002). The efficient OTA removal by nonviable cells, observed in our work, confirmed the adsorption mechanism. Other previous works have shown that nonviable lactic acid bacteria (HC and AC) are able to adsorb zearalenone, α zearalenol, aflatoxins and trichotecenes, (El Nezami *et al.* 1998, 2002).

In our study, the best adsorption level was noted first in the SGM medium (pH 3·8) and then in the YPG one (pH 5) and was, respectively, three times and twice more important than in PBS buffer (pH 7). The acidity could probably improve the OTA adsorption mechanism. In other conditions, previous studies have shown an OTA absorption from animal stomach as a result of its acidic properties [pK_a (OTA) = 7·1] (Galtier 1978; Roth *et al.* 1988). In our experiment, the increase of OTA adsorption in acid

conditions could be explained by the amino group ionization of the OTA molecule, which is enhanced in the acid media used (SGM and YPG). Consequently, ionic properties of the OTA molecule as well as wall yeast membrane state, were significant factors in improving OTA adsorption.

Biomass concentration was also a significant factor, playing an important role in OTA adsorption. A fourfold increase in yeast concentration enhanced significantly OTA adsorption by about 100% for VC and 21% for HC but in this last case the saturation level (90%) was reached. This has already been observed in a previous study of zearalenone and α zearalenol binding (El Nezami *et al.* 2002).

Kinetic studies of OTA adsorption by HC *S. cerevisiae* and HC *S. bayanus* at 27·4 g l^{-1} concentration, have shown first that OTA binding was a rapid reaction reaching 90% after 5 min of incubation and equilibrium was still main tained until 72 h and secondly, there was no difference between the two species behaviour in OTA removal process. In a previous study (El Nezami *et al.* 2002), zearalenone and α zearalenol were first instantly adsorbed at 60%, then released in the MRS liquid medium and finally rebound by the bacteria up to 72 h. In another study, the adsorption of killer toxins by sensitive yeasts was also rapid and complete within 2 min (Santos *et al.* 2000). HC *S. cerevisiae* LALVIN Rhône 2056 were used to test the efficiency of OTA adsorption in a contaminated red grape juice in comparison with commercial yeast walls additive (YW) commonly used in oenology as an activator of alcoholic fermentations. The concentrations tested 0·2 and 6·7 g l^{-1} correspond, respect ively, to the highest authorized concentration for the YW and to living yeast concentration at the end of alcoholic fermentations (generally comprised between 4 and 7 g l^{-1}). The rapid and total OTA adsorption by HC and YW whatever the concentration used shows their high decon taminating efficiency. Moreover the adsorption by HC cells was quicker. For a oenological use, according to some of our experiments, living yeasts concentration at the end of an alcoholic fermentation could reduce OTA through adsorp tion, although HC could be more efficient.

In conclusion, OTA removal by *Saccharomyces* strains corresponds to an adsorption mechanism confirmed by both absence of degradation products during growing culture and ability of dead cells (HC and AC) to reduce OTA concen tration during the adsorption assays. Ionic properties of the OTA molecule, yeast membrane state and biomass concen tration were the major factors affecting the OTA adsorption phenomenon. SGM associated with heat treated cells gave the best results whatever the *Saccharomyces* species used.

The preliminary study on a naturally contaminated grape juice has confirmed the rapid adsorption of OTA. These results give new hope for OTA decontamination in natural grape juice (as the cells are dead and so no risk of fermentation is involved) or in wine.

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