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Interactions Between Industrial Yeasts and Chemical Contaminants in Grape Juice Affect Wine Composition Profile

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Summary

The interaction between four industrial wine yeast strains and grape juice chemical contaminants during alcoholic fermentation was studied. Industrial strains of *Saccharomyces cerevisiae* (AWRI 0838), *S. cerevisiae* mutant with low H_2S production phenotype (AWRI 1640), interspecies hybrid of *S. cerevisiae* and *S. kudriavzevii* (AWRI 1539) and a hybrid of AWRI 1640 and AWRI 1539 (AWRI 1810) were exposed separately to fungicides pyrimethanil (Pyr, 10 mg/L) and fenhexamid (Fhx, 10 mg/L), as well as to the most common toxin produced by moulds on grapes, ochratoxin A (OTA, 5 μg/L), during alcoholic fermentation of *Vitis vinifera* L. cv. Sauvignon blanc juice. Contaminants were found to strongly impair fermentation performance and metabolic activity of all yeast strains studied. The chemical profile of wine was analyzed by HPLC (volatile acidity, concentrations of ethanol, fructose, glucose, glycerol and organic acids) and the aromatic profile was analyzed using a stable isotope dilution technique using GC/MS (ethyl esters, acetates and aromatic alcohols) and Kitagawa tubes (H_2S). The chemical composition of wine with added contaminants was in all cases significantly different from the control. Of particular note is that the quantity of aromatic compounds produced by yeast was significantly lower. Yeast's capacity to remove contaminants from wine at the end of the alcoholic fermentation, and after extended contact (7 days) was determined. All the strains were able to remove contaminants from the media, moreover, after extended contact, the concentration of contaminants was in most cases lower.

Key words: wine fermentation, *Saccharomyces* spp., interspecies hybrids, pyrimethanil, fenhexamid, ochratoxin A, aromatic profile, fermentation kinetics, H_2S, Sauvignon blanc

Introduction

The chemical composition of grape juice is mainly a consequence of vine physiological processes (*1*). However, grapes may undergo microbiological spoilage by moulds among which some *Aspergillus* and *Penicillium* species, especially *A. carbonarius*, are producers of ochratoxin A (OTA) (*2*). Many studies have investigated its removal from grape juice, wine and other media, to reduce its negative impact on human health (reviewed by Amézqueta *et al.* (*3*)). It was shown that yeasts are able to reduce OTA concentration by its adsorption on their cell wall during alcoholic fermentation; predominantly by mannoproteins, which are released from the yeast cell wall after the end of alcoholic fermentation (*4–6*). The effect of yeasts on the concentration of OTA in the fermen-

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tation media was widely studied, which is not the case with the influence of OTA on yeast metabolism in fermentation media. In a previous study (*4*) we showed that OTA at higher concentrations (approx. 5 μg/L) impaired the yeast fermentative capacities and induced a higher volatile acidity during alcoholic fermentation in synthetic media.

To prevent microbiological spoilage of grapes, many fungicides and other phytopharmaceuticals are used. However, in years when the climate conditions for *Botrytis* infection are favourable, such control measures may cause maximum permitted residue levels to be exceeded (*7*), even though the concentrations of fungicides are significantly reduced during processing of grapes into wine (*8,9*). It has been demonstrated that some fungicides are able to affect the ecology and kinetics of alcoholic fermentations (*10,11*). Additionally, it was found that the aromatic compound production of yeasts is negatively affected (*12–14*).

In recent years the trend has been to use selected yeasts for alcoholic fermentation, because this can guarantee the smooth development of the process, avoid the production of off-flavours and generate positive aroma that improves the sensory properties of the wine (*15*). In order to improve wine aromatic composition, other alternative techniques of inoculation have been adopted, especially mixed/sequenced inocula of selected non-*Saccharomyces* yeasts (*16,17*) and the use of interspecies hybrids of *Saccharomyces* yeasts, *i.e.* hybrids of *S. cerevisiae* and *S. kudriavzevii*, which were found to have good aromatic potential (*18*).

The aim of this study is to uncover the interaction of four genetically different industrial wine yeast strains: *S. cerevisiae* (AWRI 0838), *S. cerevisiae* mutant with low H_2S production phenotype (AWRI 1640), an interspecies hybrid of *S. cerevisiae* and *S. kudriavzevii* (AWRI 1539) and the triple hybrid of the last two strains (AWRI 1810) with the mycotoxin OTA and fungicides pyrimethanil (Pyr) and fenhexamid (Fhx). We have tried to answer the question whether the abusive use of fungicides affects more negatively the final product than the presence of OTA during alcoholic fermentation by determining the removal potential of the strains with different genomic background and the potential of contaminants to affect yeast metabolism (fermentation kinetics and aromatic compound production).

Materials and Methods

Yeast strains

The yeast strains used in this study were all industrial wine yeasts: *Saccharomyces cerevisiae* AWRI 0838, the mutant *Saccharomyces cerevisiae* AWRI 1640 with low H_2S phenotype (*19*), an interspecies hybrid of *Saccharomyces cerevisiae* and *Saccharomyces kudriavzevii*, AWRI 1539, and the hybrid of AWRI 1640 and AWRI 1539, AWRI 1810 (*18*). Three-day-old cultures grown on yeast extract, peptone, dextrose (YPD) agar plates (2 % D-glucose, 1 % yeast extract, 1 % peptone and 2 % agar) were inoculated into 8 mL of sterile YPD broth incubated for 24 h at 28 °C (in 12-mL sterile Falcon tubes), and later transferred into 16 mL of sterile chemically defined must (CDM) (*20*) and incubated for 24 h (in sterile 50-mL Falcon tubes). The concentrations of yeast cells for the inocula were counted by haemocytometer.

Fermentation assays

Alcoholic fermentations were carried out in sterile 250-mL fermentation flasks (Schott, Mainz, Germany) containing 200 mL of Sauvignon blanc 2005 (SB05) grape juice (128.5 g/L of reducible sugars, titratable acidity (pH=8.2) 5.1 g/L, pH=3.19, SO_2 (free) 10 mg/L, SO_2 (total) 19 mg/L, yeast assimilable nitrogen (YAN) 235 mg/L) sterilized by filtering through 0.65- and 0.45-μm cartridge filters (Sartorius, Bohemia, NY, USA). The grape juice with low concentration of reducible sugars and high amount of YAN was chosen to make sure that H_2S production will not be affected by high osmotic pressure and low amount of YAN (*19*). Each of the four strains was inoculated at the final concentration of 10^6 cells per mL (all in triplicate). Four types of media were prepared: (*i*) control, which was composed of SB05 and 1 mL of 80 % ethanol; (*ii*) Pyr or (*iii*) Fhx, which were composed of SB05 and 10 mg of pyrimethanil or fenhexamid suspended in 1 mL of 80 % ethanol, respectively, and (*iv*) OTA, composed of SB05 and 5.0 μg/L of OTA suspended in 1 mL of 80 % (by volume) Et-OH (*4*). All contaminants were obtained as analytical standards from Sigma-Aldrich (St. Louis, MO, USA). The selection of concentrations was done considering previous research on synthetic media (*4,10*). The fermentation flasks were equipped with precision gas detector tubes (Kitagawa, Hiroshima, Japan) and a trap-based method for H_2S quantification during fermentation was used (*18*).

The assays were performed at 17 °C, with rotary shaking at 150 rpm. The fermentation kinetics was followed by CO_2 measuring the mass loss every 24 h. Fermentations were considered finished when CO_2 release was lower than 0.1 g per 100 mL per day and the concentration of reducible sugars was lower than 2 g/L (Clinitest®, Bayer, Leverkusen, Germany).

After the end of fermentation, samples of wine were taken for the determination of volatile and non-volatile chemical compounds as well as the concentration of contaminants. The samples were taken from homogenized media under aseptic conditions, centrifuged (for 5 min at 11 200×*g*), and the clean supernatant was frozen for analysis.

Extended contact between wine and yeast lees

In order to determine the potential of yeast lees to remove the contaminants, a 7-day extended contact time with daily mixing was performed (*4,10*). After the contact period, the samples were taken from the homogenized media under aseptic conditions, centrifuged (for 5 min at 11 200×*g*), and the clean supernatant was frozen for analysis.

Analysis of the principal chemical compounds in wine

Contents of glucose, fructose, ethanol, glycerol, and acetic, citric, malic and tartaric acids in fermented SB05 wines were analyzed. Their concentration in the media was analyzed by HPLC, using a Bio-Rad HPX-87H column (Bio-Rad, Hercules, CA, USA) as described previously (*19*).

Analysis of fermentation products

Samples of fermented SB05 wines were prepared as follows: from each treatment the same aliquots of the three replicates were taken and mixed together into one sample (from 48 fermentations, 16 final samples). Samples were prepared in 2 dilutions (1/20 and 3/10) with model wine (13.8 % ethanol, 10 % potassium hydrogen tartrate, pH adjusted with tartaric acid to 3.5). Samples were prepared and the content of ethyl esters, acetates and aromatic alcohols was analyzed by Metabolomics Australia (AWRI, Adelaide, Australia) as detailed by Bizaj *et al.* (*18*).

Determination of fungicide residues

Extraction and determination of pyrimethanil and fenhexamid residues in fermented SB05 wines was done using a gas chromatography-mass spectrometry system (GC-MS) and liquid chromatography-tandem mass spectrometry system (LC/MS/MS), respectively, according to the previously described methods (*10*,*21*).

Determination of ochratoxin A residues

The concentration of OTA in the fermented SB05 wines and wine samples of that were collected after the extended contact phase with yeast lees were analyzed by means of immunoaffinity column clean-up and HPLC, as described previously (*22*).

Statistical analysis

Statistical analysis was done using general linear model (SAS software 8.01, SAS Institute Inc., Cary, NC, USA) using the equation:

$$y_{ijk}=\mu+T_i+S_j+T\cdot S_{ij} \qquad /1/$$

where y_{ijk} is the controled value; μ is the average value; T_i is the effect of i-treatment, i=1–8; S_j is the effect of j-strain, j=1–4; and $T\cdot S_{ij}$ is the effect of interaction between i-treatment and j-strain.

The data of fermentation kinetics were statistically analysed by intervals of standard deviation (Microsoft Office Excel 2003, Durham, NC, USA). The statistical level of significance was set at $p\leq 0.05$.

Results and Discussion

Fermentation kinetics

The fermentation kinetics at 17 °C in the SB05 grape juices varied with different yeast strains; the fastest fermentation kinetics in control treatments was performed by AWRI 1539 in 7 days; followed by AWRI 0838 and 1810, which finished the fermentation in 9 days; while AWRI 1640 displayed the slowest fermentation rate, completing it in 12 days (Fig. 1). Differences between the strains were expected in control media (*10*,*23*). More interestingly, all strains responded with significantly slow-

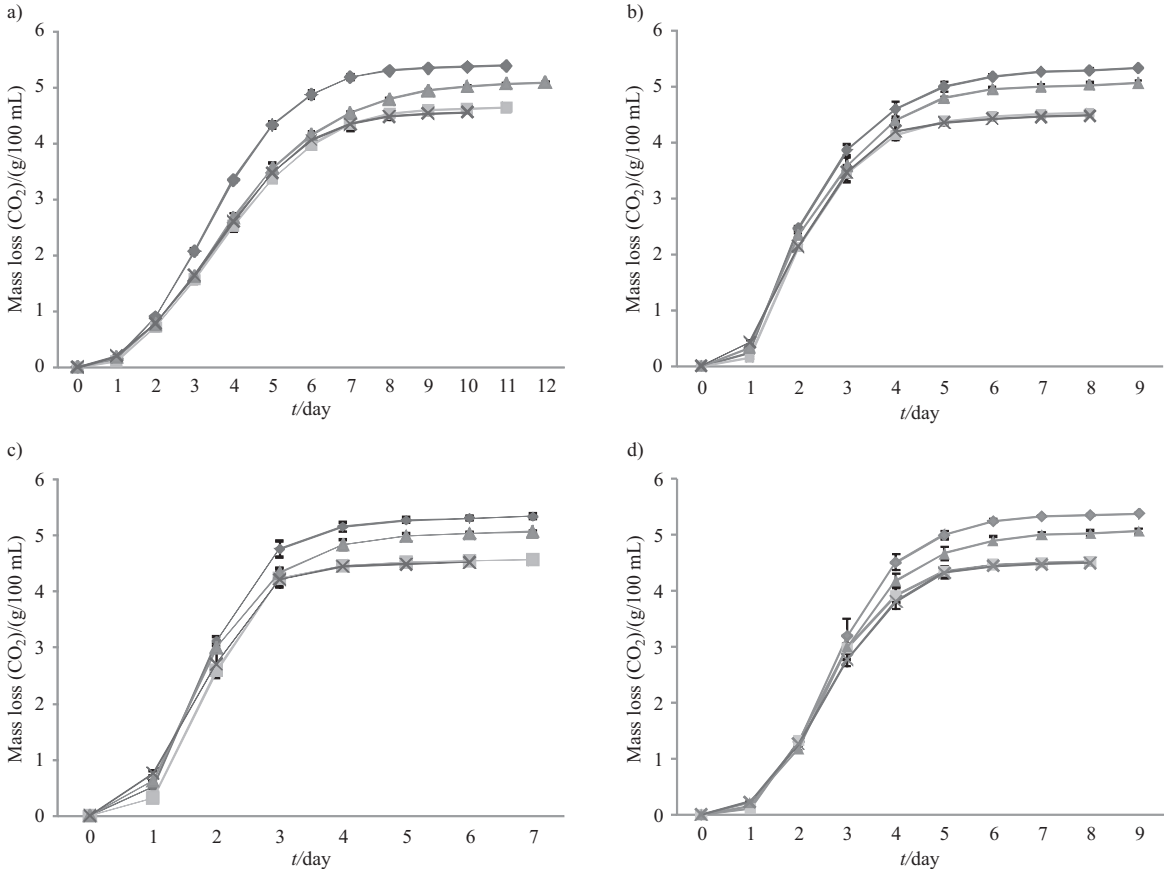

Fig. 1. Fermentation kinetics of the strains: a) AWRI 1640, b) AWRI 0838, c) AWRI 1539 and d) AWRI 1810 with contaminants ochratoxin A (■), fenhexamid (▲), pyrimethanil (×), and control (◆)

er fermentation rates when challenged with contaminants OTA, Pyr and Fhx. However, in Fhx fermentations, the stationary phase was reached earlier, showing that Fhx was less inhibitory if compared to OTA and Pyr. This was also shown by Cabras *et al.* (*24*), who found no negative effect of Fhx on wine yeast fermentation kinetics, even at concentrations greater than that permitted by legislation (3 mg/L). Recently, Bizaj *et al.* (*10*) demonstrated the negative effect of Fhx on the fermentation kinetics at higher concentrations, *i.e.* 10 mg/L, during wine yeast fermentation in synthetic media.

The toxicity of Pyr on alcoholic fermentation was already shown to be greater than the toxicity of Fhx in synthetic media (*10*), but it has now been confirmed for grape juice (Fig. 1). The effect of Pyr was most prominent when using AWRI 1539. Its fermentation rate was initially the fastest, but after the 3rd day of fermentation, a drastic decrease in the fermentation rate can be seen. Similar trend was also observed by Bizaj *et al.* (*10*), where strains exhibiting faster fermentation rates were more affected by the toxicity of contaminants. Moreover, this trend can be strengthened by less negative effect of Pyr on the more slowly fermenting strains, *i.e.* AWRI 1810, 0838 and 1640.

As shown in Fig. 1, OTA had negative effect on fermentation kinetics of all wine yeast strains in grape juice, as determined before for synthetic media (*4*). Moreover, its toxicity seemed to be similar to that of Pyr.

In the assays with OTA and Pyr, all the fermentations tended to be slower, producing less CO_2 in comparison with the control and Fhx fermentations, suggesting their more intense negative effect on all wine yeast strains studied. The only case when fermentation remained stuck was the assay with Pyr and the strain AWRI 1539.

Off-flavour production during alcoholic fermentation

All AWRI 1640 fermentations performed in this study were free from detectable H_2S, as expected (*19*). The three other strains had different capacities for H_2S production: AWRI 1539 was known to be a relatively high H_2S producer (*18*), AWRI 0838, a yeast strain commonly used in commercial fermentations and AWRI 1810, the hybrid between AWRI 1539 and AWRI 1640, were known to be intermediate level producers (*18*). As shown in Table 1 all the strains in control assay produced H_2S. However, the contaminants seem to have different effects on the H_2S production during fermentation. The strain AWRI 0838 produced the highest amount of H_2S in control fermentations, suggesting that all contaminants impaired H_2S production. On the other hand, when using AWRI 1810 the addition of Fhx and Pyr stimulated H_2S production, while in the case of OTA and control assays, the amount of produced H_2S was significantly lower. The highest H_2S producer, AWRI 1539 in the assays with OTA and Pyr produced lower amounts of H_2S than in assays with Fhx or in the control one. In the more slowly fermenting assays and especially in the case of the stuck fermentation when using the combination of Pyr and strain AWRI 1539, the quantities of H_2S produced were significantly lower. These results suggest that there were interactions between the contaminants and the yeast strains with regard to H_2S formation. Fenhexamid, pirymethanil and ochratoxin A had previously been found to have effects on metabolic pathways during alcoholic fermentations (*4,10,25*). Moreover, we have demonstrated here that all of them have the potential to affect H_2S production pathway during alcoholic fermentation of grape juice.

Table 1. Concentration of H_2S produced during alcoholic fermentation of sterile grape juice by strains AWRI 1640, 0838, 1539 and 1810 in assays with contaminants ochratoxin A (OTA), fenhexamid (Fhx) and pyrimethanil (Pyr)

	$\gamma(H_2S)/(mg/L)$			
	Treatment			
Strain	Control	OTA	Fhx	Pyr
AWRI 1640	$(0\pm0)^{aD}$	$(0\pm0)^{aC}$	$(0\pm0)^{aD}$	$(0\pm0)^{aD}$
AWRI 0838	$(19\pm1)^{aB}$	10^{bBn}	6^{bCn}	6^{bCn}
AWRI 1539	365^{aAn}	245^{bAn}	$(347\pm15)^{aA}$	$(153\pm6)^{cA}$
AWRI 1810	13^{aCn}	11^{aBn}	$(20\pm1)^{bB}$	17^{abBn}

Data reported are mean values and standard deviations of three independent experiments carried out under identical conditions.
Mean values with the same lower case letter in the same row do not differ significantly; effect of treatment (significant difference at $p\leq0.05$).
Mean values with the same upper case letter in the same column do not differ significantly; effect of strain (significant difference at $p\leq0.05$).
^{n}N=2 experiments

Principal chemical compounds in wine

The principal chemical compounds analyzed at the end of fermentation were citric, tartaric, malic, succinic, acetic and lactic acids, glycerol, ethanol, glucose and fructose (Table 2). All the strains in all four types of assays were able to consume all the sugars, since no residual sugar was found in the media after the end of alcoholic fermentation.

Except for the strain AWRI 1640, all strains degraded more malic acid if a contaminant was present in the media. The highest concentrations of malic acid were found in all assays when AWRI 1640 was used, suggesting that, since this strain was produced by random chemical mutagenesis (*19*), malic degradation enzyme might have been affected in the way of reducing the malate degradation by malo-ethanolic fermentation (*26*). Interestingly, higher concentration of malic acid was characteristic also for a triple hybrid AWRI 1810, which showed intermediate phenotype, suggesting that it has inherited part of AWRI 1640 genome background. Similar trend was observed also for citric acid in terms of contaminant effect; on the other hand, there were not any particular trends present between different strains. Succinic acid is considered to be particularly important for sensory wine quality (*1*). The production of succinic acid was significantly lower in all assays where the contaminants were present, meaning that their presence in the media negatively affected its production. Strain dependency was evident again; the inherent ability to degrade malic acid as well as to produce succinic acid was present. The highest production by AWRI 0838 and 1640 was observed, the lowest by

Table 2. Concentration of principal chemical compounds in finished wines fermented by strains AWRI 1640, 0838, 1539 and 1810 with contaminants ochratoxin A (OTA), fenhexamid (Fhx) and pyrimethanil (Pyr)

		γ/(g/L)				
		Strain				
Compound	Treatment	AWRI 1640	AWRI 0838	AWRI 1539	AWRI 1810	
citric acid	control	$(0.181\pm0.009)^{bA}$	$(0.210\pm0.007)^{aA}$	$(0.21\pm0.01)^{aA}$	$(0.195\pm0.006)^{abA}$	$p_T<0.001$
	OTA	$(0.17\pm0.02)^{bA}$	$(0.190\pm0.004)^{aB}$	$(0.182\pm0.004)^{abB}$	$(0.171\pm0.006)^{bB}$	$p_S<0.001$
	Fhx	$(0.18\pm0.01)^{aA}$	$(0.211\pm0.006)^{bA}$	$(0.204\pm0.002)^{aAB}$	$(0.197\pm0.003)^{aA}$	$p_{T\cdot S}<0.001$
	Pyr	$(0.16\pm0.01)^{bA}$	$(0.197\pm0.002)^{aAB}$	$(0.146\pm0.009)^{cC}$	$(0.185\pm0.005)^{aA}$	
tartaric acid	control	$(2.04\pm0.06)^{aA}$	$(2.09\pm0.04)^{aA}$	$(1.85\pm0.21)^{aB}$	$(1.9\pm0.1)^{aA}$	$p_T<0.001$
	OTA	$(1.73\pm0.15)^{bB}$	$(1.92\pm0.08)^{aB}$	$(1.99\pm0.06)^{aAB}$	$(1.64\pm0.04)^{bC}$	$p_S<0.0850$
	Fhx	$(1.96\pm0.05)^{bA}$	$(1.76\pm0.07)^{cB}$	$(2.06\pm0.03)^{aA}$	$(1.73\pm0.06)^{cB}$	$p_{T\cdot S}<0.001$
	Pyr	$(1.7\pm0.1)^{bB}$	$(1.87\pm0.10)^{aB}$	$(1.56\pm0.1)^{bC}$	$(1.9\pm0.1)^{aB}$	
malic acid	control	$(3.06\pm0.03)^{aA}$	$(2.349\pm0.008)^{cA}$	$(2.34\pm0.01)^{cA}$	$(2.55\pm0.06)^{bA}$	$p_T<0.001$
	OTA	$(2.9\pm0.2)^{aA}$	$(2.16\pm0.02)^{cB}$	$(2.22\pm0.09)^{bcAB}$	$(2.38\pm0.06)^{bB}$	$p_S<0.001$
	Fhx	$(3.0\pm0.06)^{aA}$	$(2.16\pm0.02)^{cB}$	$(2.20\pm0.02)^{cB}$	$(2.38\pm0.02)^{bB}$	$p_{T\cdot S}<0.001$
	Pyr	$(3.1\pm0.1)^{aA}$	$(2.15\pm0.06)^{cB}$	$(1.8\pm0.1)^{dC}$	$(2.46\pm0.04)^{bAB}$	
succinic acid	control	$(1.14\pm0.03)^{aA}$	$(1.11\pm0.02)^{aA}$	$(0.93\pm0.03)^{cA}$	$(1.05\pm0.02)^{bA}$	$p_T<0.001$
	OTA	$(0.96\pm0.06)^{abB}$	$(1.00\pm0.01)^{aB}$	$(0.79\pm0.02)^{cB}$	$(0.93\pm0.03)^{bB}$	$p_S<0.001$
	Fhx	$(1.03\pm0.04)^{aB}$	$(0.98\pm0.00)^{bC}$	$(0.821\pm0.007)^{cB}$	$(0.95\pm0.01)^{bB}$	$p_{T\cdot S}<0.001$
	Pyr	$(0.99\pm0.06)^{bB}$	$(1.06\pm0.01)^{aAB}$	$(0.66\pm0.03)^{cC}$	$(1.01\pm0.01)^{abAB}$	
lactic acid	control	$(0.059\pm0.003)^{dA}$	$(0.103\pm0.004)^{aA}$	$(0.081\pm0.006)^{cA}$	$(0.091\pm0.002)^{bA}$	$p_T<0.001$
	OTA	$(0.043\pm0.005)^{bBC}$	$(0.11\pm0.05)^{aA}$	$(0.063\pm0.009)^{bAB}$	$(0.09\pm0.01)^{abA}$	$p_S<0.001$
	Fhx	$(0.049\pm0.008)^{cB}$	$(0.096\pm0.004)^{aA}$	$(0.080\pm0.004)^{bA}$	$(0.091\pm0.000)^{aA}$	$p_{T\cdot S}<0.0097$
	Pyr	$(0.037\pm0.003)^{cC}$	$(0.069\pm0.002)^{aB}$	$(0.049\pm0.005)^{bB}$	$(0.075\pm0.007)^{aA}$	
acetic acid	control	$(0.044\pm0.006)^{bA}$	$(0.024\pm0.006)^{bA}$	$(0.12\pm0.02)^{aA}$	$(0.02\pm0.01)^{bA}$	$p_T<0.001$
	OTA	$(0.026\pm0.004)^{bB}$	$(0.024\pm0.006)^{bA}$	$(0.14\pm0.01)^{aA}$	$(0.02\pm0.01)^{bA}$	$p_S<0.001$
	Fhx	$(0.026\pm0.007)^{bB}$	$(0.018\pm0.001)^{bA}$	$(0.13\pm0.01)^{aA}$	$(0.027\pm0.003)^{bA}$	$p_{T\cdot S}<0.001$
	Pyr	$(0.006\pm0.000)^{cC}$	$(0.03\pm0.01)^{bA}$	$(0.095\pm0.006)^{aA}$	$(0.030\pm0.008)^{bA}$	
glycerol	control	$(3.6\pm0.1)^{cA}$	$(3.94\pm0.07)^{bA}$	$(4.19\pm0.08)^{aA}$	$(3.52\pm0.04)^{cAB}$	$p_T<0.001$
	OTA	$(3.0\pm0.2)^{cB}$	$(3.70\pm0.08)^{aAB}$	$(3.76\pm0.06)^{aB}$	$(3.34\pm0.06)^{bB}$	$p_S<0.001$
	Fhx	$(3.1\pm0.1)^{dB}$	$(3.66\pm0.02)^{bB}$	$(3.84\pm0.06)^{aB}$	$(3.49\pm0.04)^{cAB}$	$p_{T\cdot S}<0.001$
	Pyr	$(3.2\pm0.2)^{cB}$	$(4.1\pm0.02)^{aA}$	$(3.2\pm0.1)^{cC}$	$(3.71\pm0.06)^{bA}$	
ethanol	control	$(66.5\pm0.7)^{aA}$	$(65.5\pm0.9)^{aA}$	$(66\pm2)^{aA}$	$(65\pm2)^{aA}$	$p_T<0.001$
	OTA	$(56\pm3)^{aB}$	$(56.03\pm0.08)^{aB}$	$(56.6\pm0.5)^{aC}$	$(56.2\pm0.6)^{aB}$	$p_S<0.0025$
	Fhx	$(59\pm1)^{aB}$	$(58.8\pm0.7)^{aB}$	$(60\pm1)^{aB}$	$(59.2\pm0.6)^{aB}$	$p_{T\cdot S}<0.001$
	Pyr	$(51\pm3)^{bC}$	$(55\pm0.5)^{aB}$	$(42\pm1)^{cD}$	$(55,4\pm0.3)^{aB}$	

Data reported are mean values and standard deviations of three independent experiments carried out under identical conditions. Mean values with the same lower case letter in the same row do not differ significantly; effect of strain (significant difference at $p\leq0.05$). Mean values with the same upper case letter in the same column do not differ significantly; effect of treatment (significant difference at $p\leq0.05$).
T=treatment
S=strain

AWRI 1539 and intermediate by AWRI 1810. Strain dependency was also observed to be more important in the case of lactic and acetic acid production. AWRI 0838 was the highest producer of lactic acid, followed by AWRI 1810, 1539 and 1640 in control assays; on the other hand, a particular trend of the effect of contaminant on its production was not present, except when AWRI 1640 was used, in which case contaminants negatively affected lactic acid production. AWRI 1539 is known for its high potential for acetic acid production (*18*), which can also be seen in this study; but none of the contaminants were able to affect acetic acid production. Similarly to lactic acid, AWRI 1640 was strongly affected by the presence of contaminants, suggesting that metabolic pathways after the synthesis of pyruvate are particularly susceptible to the contaminants in this strain.

Glycerol production during alcoholic fermentation was affected when the contaminant was present in the medium. The ability of AWRI 1640 and 1539 strains to produce glycerol was clearly impaired by all three contaminants, especially the latter in the assay with Pyr when the fermentation remained stuck. On the other hand, AWRI 0838 and 1810 strains were overall less sensitive; moreover, Pyr seemed to stimulate their glycerol production. The production of ethanol seemed to be the most negatively affected. Even though all the reducible sugars are consumed by yeasts during fermentation, it seems that due to the effect of contamination on metabolic pathway and the increasing concentration of ethanol in media, yeasts were not able to convert sugars to ethanol. Yeasts in assays with contaminants produced roughly 14 % less ethanol. Again, Pyr was found to be the most toxic contaminant during fermentation. Its effect was the most negative on AWRI 1539, where a 35.8 % decrease in ethanol production was observed. However, this was expected from the fermentation kinetics (Fig. 1c). Similar data were found for AWRI 1640, where pyrimethanil was found to be the most toxic. On the other hand, we could not observe any significant differences between assays with contaminants when using AWRI 0838 or 1640 strain. The primary fermentation product concentrations were significantly lower in the case of slower fermentation kinetics and especially in the case when the fermentation remained stuck.

Volatile fermentation products in wine

In Table 3 the production of aromatic compounds by the yeast strains in fermentative assays with and without contaminants is presented.

Ethyl acetate was the highest produced ethyl ester by all strains. This compound imparts pleasant smell to wine when present in concentrations lower than 80 mg/L (*1*), which is the case in all our assays. Its production was impaired in all assays using contaminants, especially when pyrimethanil was present (AWRI 1640 and 1539), when the fermentation kinetics was slow or remained stuck for AWRI 1539. Strain-related sensitivity can be seen as well. Interestingly, in the case of *S. cerevisiae* AWRI 0838, ethyl acetate production was negatively affected by all contaminants to a similar degree, suggesting high sensitivity of the strain to the three chemical compounds. This is in contrast to the studies of García *et al.* (*13*), where the non-hybrid *S. cerevisiae* strain was found to be the most resistant to pesticides, including pyrimethanil.

Ethyl hexanoate is well known for its important and positive effect on the aroma, especially of young wines (*27*). In all our assays, the presence of contaminants in the media negatively affected its production, which is in accordance with the results obtained by García *et al.* (*13*). Moreover, the final concentration in the media was shown to be strain dependent.

Ethyl propanoate and ethyl butanoate were all produced below the perception threshold (*28*). Ethyl butanoate production was in all assays negatively affected by the contaminants, and showed a strong strain dependency. Interestingly, strain dependency was also present in the production of ethyl propanoate, but except for AWRI 1810, in all other assays the contaminants stimulated its production.

Ethyl 2-methylpropanoate, ethyl 2-methylbutanoate and ethyl 3-methylbutanoate are derivates of acids considered as indicators of lower quality of wine (*29*). Strains AWRI 0838 and 1810 were the highest producers of ethyl 2-methylpropanoate. Interestingly, contaminants positively affected its production, especially Pyr (AWRI 1640 and 0838) and OTA (AWRI 1539 and 1810), which may have the effect of lowering the quality of wines. Ethyl 2-methylbutanoate was found in traces and ethyl 3-methylbutanoate could not be detected at all in all assays.

2-Phenyl ethyl acetate, which confers the flavour of roses and violets to young wines, was found to be below the perception thresholds in all our assays. Strains AWRI 1539 and 1810 were high producers of 2-phenyl ethyl acetate. When contaminants were added, the production was significantly lower in most of the cases, especially when Pyr was added in the assays with AWRI 0838, where 2-phenyl ethyl acetate was not detected at all. Interestingly, in the assays with AWRI 1640, no 2-phenyl ethyl acetate was detected. This data suggest that during its production by mutagenesis (*19*), a mutation occurred in genes involving enzymes for 2-phenyl ethyl acetate production. However, this negative trait was not conferred to AWRI 1810 (*18*).

The concentration of hexyl acetate, giving the flavour of cherries or pears, in all assays was below the perception threshold. Strain-dependent production can be observed; the order from the highest to the lowest producer was as follows: AWRI 1810>1539>0838>1640. In all assays where contaminants were added, they had a negative effect on the hexyl acetate production; the only exception was OTA used with strain AWRI 1539.

3-Methyl butyl acetate, 2-methyl butyl acetate and 2-methyl propyl acetate are very important in determining young wine flavour, conferring banana or fruity flavours in white wines. In all assays where contaminants were present their negative effect can be observed, especially that of pyrimethanil. This is opposite to what was observed by García *et al.* (*13*), where its production was stimulated. However, a different yeast strain was used in their study.

Higher alcohols are compounds that are produced by yeasts during alcoholic fermentation, and their concentrations below 300 mg/L positively affect wine flavour. Two isoamyl alcohols were analyzed, 2-methyl butanol and 3-methyl butanol, which are considered to be among the major volatiles that confer intensity of fruity flavour to wine (*30*). Their perception threshold was exceeded in all assays and their concentration was highly dependent on yeast strain, with interspecies hybrids being higher producers. The presence of contaminants in the media had a significant effect on the concentration of produced amyl alcohols; the production of 3-methyl butanol was negatively affected in all cases, on the other hand the production of 2-methyl butanol in assays with yeasts AWRI 0838 and 1810 was positively affected by Pyr and Fhx, respectively. Productions of 2-methyl propanol, butanol and hexanol were negatively affected by contaminants in all our assays.

Table 3. Concentration of the fermentation products in finished wines obtained by strains AWRI 1640, 0838, 1539 and 1810 with contaminants ochratoxin A (OTA), fenhexamid (Fhx) and pyrimethanil (Pyr)

		γ/(mg/L)				
		Strain				
Compound	Treatment	AWRI 1640	AWRI 0838	AWRI 1539	AWRI 1810	
ethyl acetate	control	$(28871\pm1216)^{aA}$	$(20292\pm854)^{cA}$	$(23451\pm987)^{bA}$	$(27464\pm1156)^{aA}$	$p_T<0.0001$
	OTA	$(21213\pm893)^{aC}$	$(15247\pm642)^{bB}$	$(21936\pm924)^{aA}$	$(13436\pm566)^{cC}$	$p_S<0.0001$
	Fhx	$(24078\pm1014)^{aB}$	$(15292\pm644)^{cB}$	$(16804\pm708)^{cB}$	$(21142\pm890)^{bB}$	$p_{T\cdot S}<0.0001$
	Pyr	$(14405\pm607)^{aD}$	$(14451\pm608)^{aB}$	$(13218\pm557)^{bC}$	$(13549\pm571)^{abC}$	
ethyl propanoate	control	$(294\pm12)^{aB}$	$(117\pm5)^{cB}$	$(61\pm2)^{dC}$	$(216\pm9)^{bA}$	$p_T<0.0001$
	OTA	$(302\pm12)^{aB}$	$(117\pm5)^{bB}$	$(63\pm3)^{cC}$	$(45\pm2)^{dD}$	$p_S<0.0001$
	Fhx	$(248\pm10)^{aC}$	$(99\pm4)^{cC}$	$(118\pm5)^{bA}$	$(66\pm3)^{dC}$	$p_{T\cdot S}<0.0001$
	Pyr	$(334\pm13)^{aA}$	$(129\pm5)^{cA}$	$(107\pm4)^{dB}$	$(151\pm6)^{bB}$	
ethyl 2-methyl-propanoate	control	$(15\pm1)^{cB}$	$(20\pm1)^{bB}$	$(30\pm2)^{aB}$	$(17\pm1)^{cC}$	$p_T<0.0001$
	OTA	$(15\pm1)^{dB}$	$(21\pm1)^{cB}$	$(34\pm2)^{aA}$	$(28\pm2)^{bA}$	$p_S<0.0001$
	Fhx	$(14\pm1)^{cB}$	$(16\pm1)^{bC}$	$(13\pm1)^{cC}$	$(25\pm1)^{aB}$	$p_{T\cdot S}<0.0001$
	Pyr	$(18\pm1)^{bA}$	$(26\pm1)^{aA}$	$(14\pm1)^{cC}$	$(17\pm1)^{bC}$	
ethyl butanoate	control	$(69\pm4)^{cA}$	$(179\pm11)^{aA}$	$(132\pm8)^{bA}$	$(142\pm9)^{bA}$	$p_T<0.0001$
	OTA	$(61\pm4)^{dB}$	$(145\pm9)^{aB}$	$(124\pm7)^{bA}$	$(74\pm4)^{cC}$	$p_S<0.0001$
	Fhx	$(59\pm4)^{dB}$	$(133\pm8)^{aB}$	$(88\pm5)^{cB}$	$(117\pm7)^{bB}$	$p_{T\cdot S}<0.0001$
	Pyr	$(43\pm3)^{cC}$	$(139\pm8)^{aB}$	$(80\pm5)^{bB}$	$(81\pm5)^{bC}$	
ethyl 2-methyl-butanoate	control	n.d.	$(5\pm0)^{aA}$	$(5\pm0)^{aA}$	n.d.	$p_T<0.0001$
	OTA	n.d.	$(5\pm0)^{aA}$	$(5\pm0)^{aA}$	$(5\pm0)^{a}$	$p_S<0.0001$
	Fhx	n.d.	$(5\pm0)^{A}$	n.d.	n.d.	$p_{T\cdot S}<0.0001$
	Pyr	n.d.	n.d.	n.d.	n.d.	
ethyl 3-methyl-butanoate	control	n.d.	n.d.	n.d.	n.d.	$p_T>1.000$
	OTA	n.d.	n.d.	n.d.	n.d.	$p_S>1.000$
	Fhx	n.d.	n.d.	n.d.	n.d.	$p_{T\cdot S}>1.000$
	Pyr	n.d.	n.d.	n.d.	n.d.	
ethyl hexanoate	control	$(209\pm13)^{cA}$	$(420\pm25)^{aA}$	$(317\pm19)^{bA}$	$(420\pm25)^{aA}$	$p_T<0.0001$
	OTA	$(197\pm12)^{cAB}$	$(361\pm22)^{aB}$	$(311\pm19)^{bA}$	$(193\pm12)^{cD}$	$p_S<0.0001$
	Fhx	$(181\pm11)^{cB}$	$(343\pm21)^{aB}$	$(268\pm16)^{bB}$	$(283\pm17)^{bB}$	$p_{T\cdot S}<0.0001$
	Pyr	$(154\pm9)^{cC}$	$(327\pm20)^{aB}$	$(236\pm14)^{bB}$	$(249\pm15)^{bC}$	
2-methylpropyl acetate	control	$(19\pm1)^{dA}$	$(31\pm2)^{cA}$	$(60\pm3)^{aA}$	$(41\pm2)^{bB}$	$p_T<0.0001$
	OTA	$(17\pm1)^{dB}$	$(26\pm1)^{cB}$	$(57\pm3)^{aA}$	$(37\pm2)^{bB}$	$p_S<0.0001$
	Fhx	$(15\pm1)^{cC}$	$(27\pm1)^{bB}$	$(30\pm2)^{bB}$	$(56\pm3)^{aA}$	$p_{T\cdot S}<0.0001$
	Pyr	$(14\pm1)^{bC}$	$(26\pm1)^{aB}$	$(25\pm1)^{aC}$	$(27\pm1)^{aC}$	
2-methylbutyl acetate	control	$(32\pm1)^{dA}$	$(60\pm3)^{cA}$	$(71\pm3)^{aA}$	$(65\pm3)^{bA}$	$p_T<0.0001$
	OTA	$(29\pm1)^{dB}$	$(44\pm2)^{cC}$	$(55\pm2)^{aB}$	$(49\pm2)^{bB}$	$p_S<0.0001$
	Fhx	$(30\pm1)^{cAB}$	$(53\pm2)^{bB}$	$(54\pm2)^{bB}$	$(68\pm3)^{aA}$	$p_{T\cdot S}<0.0001$
	Pyr	$(25\pm1)^{cC}$	$(42\pm2)^{bC}$	n.d.	$(45\pm2)^{aB}$	
3-methylbutyl acetate	control	$(195\pm11)^{cA}$	$(838\pm47)^{bA}$	$(833\pm47)^{bA}$	$(1013\pm57)^{aA}$	$p_T<0.0001$
	OTA	$(135\pm8)^{dB}$	$(537\pm30)^{bBC}$	$(651\pm37)^{aB}$	$(425\pm24)^{cC}$	$p_S<0.0001$
	Fhx	$(109\pm6)^{cC}$	$(588\pm33)^{bB}$	$(540\pm31)^{bC}$	$(769\pm44)^{aB}$	$p_{T\cdot S}<0.0001$
	Pyr	$(59\pm3)^{cD}$	$(513\pm29)^{aC}$	$(417\pm24)^{bD}$	$(393\pm22)^{bC}$	
2-phenylethyl acetate	control	n.d.	$(376\pm27)^{bA}$	$(603\pm43)^{aA}$	$(591\pm42)^{aA}$	$p_T<0.0001$
	OTA	n.d.	$(315\pm22)^{cB}$	$(542\pm29)^{aA}$	$(468\pm33)^{bB}$	$p_S<0.0001$
	Fhx	n.d.	$(314\pm22)^{cB}$	$(384\pm27)^{bB}$	$(612\pm44)^{aA}$	$p_{T\cdot S}<0.0001$
	Pyr	n.d.	n.d.	$(323\pm23)^{aB}$	$(303\pm22)^{aC}$	

Table 3. – continued

		γ/(mg/L)				
		Strain				
Compound	Treatment	AWRI 1640	AWRI 0838	AWRI 1539	AWRI 1810	
hexyl acetate	control	$(29\pm2)^{dA}$	$(78\pm5)^{cA}$	$(99\pm6)^{bA}$	$(125\pm7)^{aA}$	$p_T<0.0001$
	OTA	$(22\pm1)^{dB}$	$(53\pm3)^{cC}$	$(93\pm5)^{aA}$	$(67\pm4)^{bC}$	$p_S<0.0001$
	Fhx	$(19\pm1)^{dC}$	$(62\pm4)^{cB}$	$(74\pm4)^{bB}$	$(83\pm5)^{aB}$	$p_{T\cdot S}<0.0001$
	Pyr	$(16\pm1)^{cD}$	$(52\pm3)^{bC}$	$(63\pm4)^{aC}$	$(65\pm4)^{aC}$	
2-methyl propanol	control	$(451\pm13)^{bA}$	$(471\pm14)^{abA}$	$(421\pm12)^{cA}$	$(494\pm14)^{aA}$	$p_T<0.0001$
	OTA	$(434\pm13)^{aA}$	$(443\pm13)^{aB}$	$(402\pm13)^{bB}$	$(388\pm111)^{bD}$	$p_S<0.0001$
	Fhx	$(440\pm13)^{bA}$	$(472\pm14)^{aA}$	$(460\pm12)^{abB}$	$(460\pm13)^{abB}$	$p_{T\cdot S}<0.0001$
	Pyr	$(392\pm11)^{cB}$	$(455\pm13)^{aAB}$	$(417\pm13)^{bB}$	$(420\pm12)^{bC}$	
butanol	control	$(15476\pm427)^{dA}$	$(19637\pm542)^{bB}$	$(21647\pm597)^{aA}$	$(16972\pm468)^{cB}$	$p_T<0.0001$
	OTA	$(13967\pm385)^{cB}$	$(18798\pm519)^{aB}$	$(19100\pm527)^{aB}$	$(16423\pm453)^{bB}$	$p_S<0.0001$
	Fhx	$(14367\pm396)^{cB}$	$(17045\pm470)^{bC}$	$(14515\pm400)^{cC}$	$(20871\pm576)^{aA}$	$p_{T\cdot S}<0.0001$
	Pyr	$(10396\pm287)^{cC}$	$(20798\pm574)^{aA}$	$(14209\pm392)^{bC}$	$(14996\pm414)^{bC}$	
2-methylbutanol	control	$(44961\pm1630)^{dA}$	$(55416\pm2009)^{bAB}$	$(61366\pm2224)^{aA}$	$(48855\pm1771)^{cB}$	$p_T<0.0001$
	OTA	$(38196\pm1385^{cB}$	$(52898\pm1917)^{aB}$	$(52762\pm1912)^{aB}$	$(44958\pm1630)^{bC}$	$p_S<0.0001$
	Fhx	$(40593\pm1471)^{cB}$	$(47583\pm1725)^{bC}$	$(40825\pm1480)^{cC}$	$(60208\pm2182)^{aA}$	$p_{T\cdot S}<0.0001$
	Pyr	$(27140\pm984)^{cC}$	$(58008\pm2103)^{aA}$	$(39144\pm1419)^{bC}$	$(39744\pm1441)^{bD}$	
3-methylbutanol	control	$(209\pm6)^{cA}$	$(420\pm12)^{aA}$	$(317\pm9)^{bA}$	$(420\pm12)^{aA}$	$p_T<0.0001$
	OTA	$(197\pm6)^{cB}$	$(361\pm11)^{aB}$	$(311\pm9)^{bA}$	$(193\pm6)^{cD}$	$p_S<0.0001$
	Fhx	$(181\pm5)^{cC}$	$(343\pm10)^{aBC}$	$(268\pm8)^{bB}$	$(283\pm8)^{bB}$	$p_{T\cdot S}<0.0001$
	Pyr	$(154\pm5)^{cD}$	$(327\pm10)^{aC}$	$(236\pm7)^{bC}$	$(249\pm7)^{bC}$	
hexanol	control	$(1796\pm56)^{aA}$	$(1599\pm49)^{bcA}$	$(1525\pm47)^{cA}$	$(1681\pm52)^{bA}$	$p_T<0.0001$
	OTA	$(1777\pm55)^{aA}$	$(1585\pm47)^{bB}$	$(1510\pm47)^{bA}$	$(1328\pm41)^{cC}$	$p_S<0.0001$
	Fhx	$(1815\pm46)^{aA}$	$(1531\pm53)^{bB}$	$(1454\pm45)^{bAB}$	$(1469\pm45)^{bB}$	$p_{T\cdot S}<0.0001$
	Pyr	$(1498\pm49)^{cB}$	$(1720\pm47)^{aB}$	$(1411\pm44)^{bcB}$	$(1553\pm48)^{bB}$	

Data reported are mean values and standard deviations of three independent experiments carried out under identical conditions. Mean values with the same lower case letter in the same row do not differ significantly; effect of strain (significant difference at $p\leq0.05$). Mean values with the same upper case letter in the same column do not differ significantly; effect of treatment (significant difference at $p\leq0.05$).
T=treatment
S=strain
n.d.=not determined

The production of secondary metabolites was significantly affected by the presence of contaminants in the media; however, no direct link to the fermentation kinetics was found.

Removal of contaminants during alcoholic fermentation and after extended contact with wine yeasts

Yeast strains were found to remove contaminants from grape juice as well as synthetic media in fermentative and stationary assays (*4,5,10,31*). In previous studies, it was demonstrated that Pyr, Fhx and OTA can be removed only by the adsorption of the cell wall mannoproteins (*4,6,10,32*) and not by degradation in synthetic media and in grape juice, in contrast to some other pesticides (*33*).

The capacity of four yeast strains to remove Pyr, Fhx and OTA from Sauvignon blanc must is presented in Table 4. The adsorption potential was evaluated after alcoholic fermentation, and after the extended contact between yeasts and media containing contaminants. Nunez *et al.* (*6*) and Bizaj *et al.* (*10*) demonstrated that the main release of mannoproteins from the yeast cell wall occurs within seven days after the end of fermentation, when they also adsorb a fraction of contaminants. Our results confirmed this, as the amount of removed OTA was significantly higher after the extended contact in all assays, except for strain AWRI 1640. The fractions of adsorbed contaminants were higher in natural wine than in synthetic media (*4,10*). Additionally, the work of Bejaoui *et al.* (*32*) showed that contaminants such as OTA can be released back into the synthetic media after being adsorbed onto yeast components. This did not happen in our work, where real grape must was used. In this way we confirmed the importance of environmental conditions for the adsorption capacity of yeast cell wall, espe-

Table 4. The potential of strains AWRI 1640, 0838, 1539 and 1810 to remove contaminants fenhexamid (Fhx), pyrimethanil (Pyr) (mg/L) and ochratoxin A (OTA) (μg/L) from grape juice. The potential was evaluated after the fermentative stage (EF) and after prolonged contact (7 days) between yeast lees and media (PCL)

Strain	Contaminant	Mass concentration at different stages of fermentation		*w*(contaminant removed)/%	
		EF	PCL	EF	PCL
AWRI 1640	Fhx	7.7^{aAn}	7.4^{bAn}	23.3	25.8
AWRI 0838	Fhx	$(7.4\pm0.1)^{aA}$	6.2^{bBn}	25.6	37.5
AWRI 1539	Fhx	$(7.3\pm0.4)^{aAB}$	$(6.4\pm0.1)^{Bb}$	27.4	36.0
AWRI 1810	Fhx	$(7.00\pm0.05)^{aB}$	6.4^{bBn}	30.0	35.7
AWRI 1640	Pyr	$(6.8\pm0.5)^{aA}$	$(5.5\pm0.5)^{bA}$	31.5	45.2
AWRI 0838	Pyr	$(8.6\pm0.5)^{aB}$	$(5.5\pm0.2)^{bA}$	13.8	46.3
AWRI 1539	Pyr	$(9.0\pm0.1)^{aB}$	$(7.54\pm0.6)^{bB}$	10.0	24.6
AWRI 1810	Pyr	$(7.82\pm0.05)^{aC}$	$(6.13\pm0.06)^{bC}$	21.8	38.7
AWRI 1640	OTA	3.5^{aAn}	$(3.4\pm0.3)^{aA}$	29.4	32.4
AWRI 0838	OTA	$(3.1\pm0.1)^{aB}$	$(1.85\pm0.02)^{bB}$	39.0	63.0
AWRI 1539	OTA	$(2.5\pm0.2)^{aC}$	$(1.5\pm0.2)^{bC}$	50.8	70.8
AWRI 1810	OTA	$(2.3\pm0.1)^{aC}$	$(1.6\pm0.1)^{bC}$	53.6	67.2

Data reported are mean values and standard deviations of three independent experiments.
Mean values with the same lower case letter in the same row do not differ significantly (significant difference at $p\leq0.05$).
Mean values with the same upper case letter in the same column (each treatment separately) do not differ significantly; effect of strain (significant difference at $p\leq0.05$).
^{n}N=2 experiments

cially pH, which determines the charge of functional groups on mannoproteins and binding contaminants (*34*). The importance of the final amount of produced biomass in the media is crucial for the removal potential (*32*), and this was found to be dependent on the fermentation kinetics.

From the results shown in Table 4, it is also evident that the removal potential of yeast strains in fermentative assays is strain and species dependent, as probably different genetic background of the four strains and the induced mutations in AWRI 1640 define yeast cells morphologically, chemically and metabolically.

Conclusion

In this study we highlighted the complexity of interactions of genetically different industrial wine yeasts and their contaminants, originating on the one hand from natural spoilage mycobiota on grape berries (ochratoxin A), and on the other from fungicides (pyrimethanil and fenhexamid), working antagonistically against spoilage mycobiota. It was demonstrated for the first time that OTA, Fhx and Pyr negatively affect fermentation kinetics of industrial yeast cells in natural grape juice. However, their intensiveness was dependent on the genetic background of the yeast strain. In all assays the contaminants affected metabolic pathways that dictate the aromatic and basic composition of wines. Moreover, metabolic pathways were found to be affected differently by the same contaminant. This suggests that these interactions define the composition of the final product.

Furthermore, the final composition of wines was affected by the ability of yeasts to remove contaminants. A significant part was removed already during alcoholic fermentation, and not only after the extended contact of yeast lees with wine.

None of the three contaminants was found to increase the concentration of any compounds known to confer desirable sensory characteristics, but on the other hand, they were found to increase the concentration of undesirable compounds.

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