Cellular death of two non-\textit{Saccharomyces} wine-related yeasts during mixed fermentations with \textit{Saccharomyces cerevisiae}

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Abstract

The early death of two non-\textit{Saccharomyces} wine strains (\textit{H. guilliermondii} and \textit{H. uvarum}) during mixed fermentations with \textit{S. cerevisiae} was studied under enological growth conditions. Several microvinifications were performed in synthetic grape juice, either with single non-\textit{Saccharomyces} or with mixed \textit{S. cerevisiae}/non-\textit{Saccharomyces} inocula. In all mixed cultures, non-\textit{Saccharomyces} yeasts grew together with \textit{S. cerevisiae} during the first 1–3 days (depending on the initial inoculum concentration) and then, suddenly, non-\textit{Saccharomyces} cells began to die off, regardless of the ethanol concentrations present. Conversely, in both non-\textit{Saccharomyces} single cultures the number of viable cells remained high (ranging $10^7$–$10^8$ CFU ml$^{-1}$) even when cultures reached significant ethanol concentrations (up to 60–70 g l$^{-1}$). Thus, at least for these yeast strains, it seems that ethanol is not the main death-inducing factor. Furthermore, mixed cultures performed with different \textit{S. cerevisiae}/\textit{H. guilliermondii} inoculum ratios (3:1; 1:2; 1:10; 1:100) revealed that \textit{H. guilliermondii} death increases for higher inoculum ratios. In order to investigate if the nature of the yeast–yeast interaction was related or not with a cell–cell contact-mediated mechanism, cell-free supernatants obtained from 3 and 6 day-old mixed cultures were inoculated with \textit{H. guilliermondii} pure cultures. Under these conditions, cells still died and much higher death rates were found for the 6 days than for the 3 day-old supernatants. This strongly indicates that one or more toxic compounds produced by \textit{S. cerevisiae} triggers the early death of the \textit{H. guilliermondii} cells in mixed cultures with \textit{S. cerevisiae}. Finally, although it has not been yet possible to identify the nature of the toxic compounds involved in this phenomenon we must emphasise that the \textit{S. cerevisiae} strain used in the present work is killer sensitive with respect to the classical killer toxins, K1, K2 and K28, whereas the \textit{H. guilliermondii} and \textit{H. uvarum} strains are killer neutral.

Introduction

Spontaneous wine fermentations are carried out by a succession of different yeast populations, the early stages being characterised by the growth of certain non-\textit{Saccharomyces} yeasts belonging to the genera \textit{Candida}, \textit{Kloeckera} and \textit{Hanseniaspora} and the latter stages being invariably dominated by alcohol-tolerant strains of \textit{S. cerevisiae} (Fleet and Heard, 1993; Pretorius, 2000). The growth of some of these non-\textit{Saccharomyces} yeasts is nowadays considered to be beneficial to enhance the wine flavour due to higher production of important metabolites, such as glycerol, esters and higher alcohols (Romano et al., 1997; Egli et al., 1998; Granchi et al., 2002; Fleet, 2003). However, the growth of these non-\textit{Saccharomyces} fermenting yeasts is generally limited to the first two or three days of fermentation, after which they begin to die off, giving way to strains of \textit{S. cerevisiae} which conduct the fermentation exclusively until completion (Constanti et al., 1998; Pretorius, 2000).

The wine yeast population dynamics is generally ascribed to the higher capacity of \textit{Saccharomyces} to withstand the changing environmental conditions of increasing ethanol and organic acids concentrations, decreasing pH and nutritional depletion.
Saccharomyces, different yeast genera associated with wine-making, including Marquina et al., 2002; Schmitt and Breinig, 2002). Strains yeast strains (sensitive yeasts) (Woods and Bevan, 1968; by certain yeast strains (killer yeasts) that are able to kill other fermentations, is the so-called yeast killer activity (Shimizu, 1993; Another inhibitory mechanism, which can occur in wine strains of Saccharomyces (Ludovico et al., 2001; Fleet, 2003). Another inhibitory mechanism, which can occur in wine fermentations, is the so-called yeast killer activity (Shimizu, 1993; Vadasz et al., 2000; Pérez et al., 2001). This phenomenon consists in the production of specific extracellular glycoproteins by certain yeast strains (killer yeasts) that are able to kill other yeast strains (sensitive yeasts) (Woods and Bevan, 1968; Marquina et al., 2002; Schmitt and Breinig, 2002). Strains exhibiting killer-positive activity have been identified within different yeast genera associated with wine-making, including Saccharomyces, Hanseniaspora, Pichia, Candida, Kluveromyces, Zygosaccharomyces, Metschnikowia and Cryptococcus (Chen et al., 2000; Ciani and Faticalini, 2001).

In fermenting wine musts, oxygen and assimilable nitrogen can be rapidly depleted due to both semi-anaerobic growth conditions and poor initial nitrogen content of the grape juice. Hansen et al. (2001) reported that T. delbrueckii and K. thermotolerans are less tolerant to conditions of low available oxygen than S. cerevisiae. This observation is consistent with their early death during mixed fermentations with S. cerevisiae. On other hand, in wine fermentations where the initial microflora is mainly composed by non-Saccharomyces species, amino acid and vitamin consumption during the first days of fermentation can disable the subsequent growth of S. cerevisiae strains (Fleet, 2003).

In spite of the above-cited reports, the early death of non-Saccharomyces yeasts during mixed fermentations with S. cerevisiae strains has been scarcely described in the literature and usually is straightforward attributed to the lower ethanol tolerance of non-Saccharomyces by comparison to S. cerevisiae. Actually, only few thorough studies have been done aiming to elucidate the causes and the mechanisms underlying this phenomenon (Hansen et al., 2001; Nissen and Arneborg, 2003; Nissen et al., 2003). In the above-mentioned studies, the authors concluded that the early death of two wine-related yeasts (K. thermotolerans and T. delbrueckii) during mixed fermentations with S. cerevisiae was not due to the presence of ethanol or any other toxic compound but instead to a cell–cell contact-mediated mechanism. Pina et al. (2004) also reported that several H. guilliermondii and C. stellata strains could stand much higher ethanol concentrations than previously thought. Additionally, previous work carried out with three non-Saccharomyces wine strains (H. guilliermondii, H. uvarum and C. stellata) revealed that these yeast strains are poorly fermentative due to deficient sugar uptake that is, at least in some extent, related with a nutritional limitation and not to ethanol intolerance (Albergaria et al., 2003a,b).

Aiming to clarify these findings, in the present work the early death of two non-Saccharomyces wine-related yeasts (H. guilliermondii and H. uvarum) under mixed cultures with S. cerevisiae was investigated.

Materials and methods

Yeast strains, growth media and inoculation

Microorganisms

*Hanseniaspora guilliermondii* NYC 2380 (previously isolated from Douro wine region, Portugal), *H. uvarum* PYCC 4193T (Portuguese Yeast Culture Collection, UNL, Lisbon) and *Saccharomyces cerevisiae* CCMI 885 (Culture Collection of Industrial Microorganisms, INETI, Lisbon) were examined in the present study. Strains were routinely maintained at 4 °C on YEPD-agar slants (containing 20 g l⁻¹ glucose, 10 g l⁻¹ yeast extract, 20 g l⁻¹ peptone and 20 g l⁻¹ agar).

Cultivation media

Inoculum cultures were done in YEPD medium (containing 10 g l⁻¹ yeast extract, 20 g l⁻¹ peptone and 20 g l⁻¹ glucose). The single- and mixed-cultures performed with an initial sugar concentration of 200 g l⁻¹ were cultivated in a modified synthetic grape juice media (SGJ), previously described by Ciani and Ferraro (1996). Each litre of SGJ was composed of three different solutions: solution A (500 ml), solution B (250 ml), and solution C (250 ml). The final composition of SGJ was as follows (per litre): (from solution A) D-glucose, 110 g; D-fructose, 110 g; (from solution B) L-(1)-tartaric acid, 6.0 g; L-(2)-malic acid, 3.0 g; citric acid, 0.5 g; (from solution C) YNB (yeast nitrogen base without amino acids and ammonium sulfate) (Difco), 1.7 g; CAA (vitamin-free Casamino Acids) (Difco), 2.0 g; CaCl₂, 0.2 g; arginine–HCl, 0.8 g; L-(2)-proline, 1.0 g; L-(2)-tryptophan, 0.1 g. Solutions B and C were buffered at pH 3.5 with NH₄OH and H₂PO₄, respectively. The three solutions were sterilized separately at 121 °C for 20 min and then combined aseptically. At last, the medium was supplemented with 3 g l⁻¹ of both sterile yeast extract and peptone.

The mixed cultures performed with *H. guilliermondii* and *S. cerevisiae* at an initial sugar (fructose + glucose) concentration of 100 g l⁻¹ were cultivated in the same medium described above but using in *solution A* only half of both sugar concentration.

Inoculation cultures

In preparing the inoculum cultures of *H. guilliermondii*, *H. uvarum* and *S. cerevisiae*, cells from one YEPD-agar slant (pre-
grown for 48 h at 30 °C) were transferred to 50 ml YEPD medium in 100 ml shake flasks and incubated at 30 °C with 150 rpm of agitation for 16 h.

Single and mixed fermentations performed with 200 g l\(^{-1}\) of initial sugar

All mixed and single cultures were performed under semi-aerobic growth conditions, in 2 l-erlenmeyers containing 1.5 l of synthetic grape juice (SGJ) at 18 °C without agitation. All single fermentations, *H. guilliermondii*, *H. uvarum* and *S. cerevisiae*, were inoculated at an initial cell concentration of 10\(^6\) CFU ml\(^{-1}\). The mixed fermentation of *H. uvarum* with *S. cerevisiae* was inoculated with 7 × 10\(^5\) and 3 × 10\(^6\) CFU ml\(^{-1}\), respectively. The mixed fermentations of *H. guilliermondii* (Hg) with *S. cerevisiae* (Sc) were inoculated with initial cell concentration of (Sc/Hg): 1.4 × 10\(^6\)/5.4 × 10\(^5\) CFU ml\(^{-1}\), 2.2 × 10\(^6\)/4.1 × 10\(^6\) CFU ml\(^{-1}\), 1.0 × 10\(^6\)/3.8 × 10\(^5\) CFU ml\(^{-1}\), 6.0 × 10\(^5\)/5.7 × 10\(^5\) CFU ml\(^{-1}\). Samples were taken daily for viable cell counts and quantification of glucose, fructose and ethanol concentrations.

Mixed fermentations performed with 100 g l\(^{-1}\) of initial sugar

Two mixed cultures of *H. guilliermondii* (Hg) with *S. cerevisiae* (Sc) were carried out in the same growth conditions as described for the 200 g l\(^{-1}\) single and mixed fermentations, with an initial sugar concentration of 100 g l\(^{-1}\). One of the cultures was performed at an initial (Sc/Hg) cell ratio of 1.5 × 10\(^6\)/2.8 × 10\(^6\) CFU ml\(^{-1}\) and the other at 1.0 × 10\(^6\), 2.9 × 10\(^6\) CFU ml\(^{-1}\), respectively. Samples were taken daily for viable cell counts and quantification of glucose, fructose and ethanol concentrations.

*H. guilliermondii* cultivation on cell-free supernatants

Cell-free supernatants were obtained from sterile filtration (0.22 µm Millipore membranes) of one Hg single-culture, previously fermented during 8 days, and two Sc/Hg mixed cultures fermented for 3 and 6 days, respectively. Supernatants were supplemented with nitrogen source ([NH\(_4\)\(_2\)HPO\(_4\)]) vitamins, salts and yeast extract to avoid nutritional limitations. All cell-free supernatants were inoculated with 10\(^6\) CFU ml\(^{-1}\) of pure Hg cultures (pre-grown for 16 h at 30 °C and 150 rpm) and cultivated in the same growth conditions used for single and mixed fermentations.

Analysis of growth

Cell-free samples (filtration through 0.45 µm Millipore membranes) were used to analyse different substrates and metabolites of all fermentations. Ethanol, glucose and fructose were determined by HPLC (Merck Hitachi, Darmstadt, Germany) using a Sugar-Pak™ column (Waters, Milford, USA) connected to a RI detector (L-7490, Merck Hitachi, Darmstadt, Germany). The column was eluted at 70 °C with a degassed aqueous mobile phase containing 50 mg l\(^{-1}\) CaEDTA, at a flow rate of 0.5 ml min\(^{-1}\). All samples were analysed in duplicate.

Killer activity strain screenings

For killer activity screening several *S. cerevisiae* reference strains for killer and sensitive traits were used: K1 killer strain (JCR2, obtained from Dr. R.B. Wickner, Section of Genetics of Simple Eukaryotes, National Institute of Diabetes, Digestive and Kidney Diseases, NIH Bethesda, MD 20892, USA); K2 killer (EX85, *S. cerevisiae* industrial wine strain isolated and selected by Regodón et al., 1997), K28 killer (GY-2-3a obtained from Manfred Schmitt, Angewandte Molekularbiologie, Universität des Saarlandes, Im Sadtwald, Gebäude 2, D-66123 Saarbrücken, Germany); killer sensitive (EX33, *S. cerevisiae* industrial wine strain, isolated and selected by Regodón et al., 1997).

The tests were performed as described by Boone et al. (1990). Plates of MB (0.5% yeast extract, 1% peptone, 2% glucose, 2% agar, 0.003% methylene blue, 0.1 M sodium citrate) with different pH values (pH 3.5, 3.7, 4.0, 4.3, 4.7) were seeded with killer-sensitive strains (pre-grown for 48 h on YEPD-agar slants). Strains to be tested for killer activity were loaded onto the seeded agar. Colonies exhibiting clear halos on the sensitive lawns after 3–7 days of incubation at 20 °C were considered to be killer positive. To test killer-sensitive traits, MB plates were seeded with the strains to be tested and overlaid with K2, K1 and K28 killer strains. Strains exhibiting clear halos on the plates were considered to be killer sensitive towards the respective reference killer strains (K1, K2 and K28).
Results and discussion

Early death of *H. guilliermondii* and *H. uvarum* in mixed fermentations with *S. cerevisiae*

Single cultures of *H. guilliermondii*, *H. uvarum* and *S. cerevisiae* and mixed cultures of *S. cerevisiae/H. guilliermondii* and *S. cerevisiae/H. uvarum* were performed in synthetic grape juice (SGJ) under semi-anaerobic growth conditions. Viable cells, sugar consumption and ethanol production profiles of single and mixed cultures are compared in Figs. 1 and 2. The early death of *H. guilliermondii* and *H. uvarum* was observed at the beginning of both fermentations. In the mixed culture of *S. cerevisiae/H. guilliermondii*, both yeasts grew together during the first day of fermentation with similar maximum specific growth rates, attaining $10^7$ CFU ml$^{-1}$ (Fig. 1-C). Then, *H. guilliermondii* cells began to die off and death was complete (less than 1 CFU ml$^{-1}$) within 7–8 days of fermentation. In the mixed culture of *S. cerevisiae/H. uvarum*, *H. uvarum* was unable to grow and the onset of death was observed immediately after the first day of fermentation, being complete within 2–3 days (Fig. 2-C). This faster death effect was possibly due to the higher *S. cerevisiae/H. uvarum* inoculum ratio in comparison with the one used for *S. cerevisiae/guilliermondii* mixed culture. Conversely, in the single cultures of these non-*Saccharomyces* yeasts (Figs. 1-A and 2-A) the number of viable cells remained at concentrations close to $10^7$–$10^8$ CFU ml$^{-1}$ during a much longer fermentation time (20 days in the *H. guilliermondii* culture and 15 days in the *H. uvarum* culture).

It is generally accepted that non-*Saccharomyces* yeasts begin to die off during the early stages of spontaneous wine fermentations due to their inability to tolerate the increasing ethanol concentrations present in the must medium (Egli et al., 1998; Cocolin et al., 2000; Fleet, 2003). However, recent
studies have demonstrated that *H. guilliermondii* is able to stand much higher ethanol concentrations than previously thought (Pina et al., 2004). Explanations for the early death of non-*Saccharomyces* yeasts in mixed cultures with *Saccharomyces* spp., could rely upon several of factors: competition for sugar uptake, oxygen availability, nutrient limitation, presence of toxic compounds, cell–cell contact, quorum sensing (Hansen et al., 2001; Fleet, 2003; Nissen and Arneborg, 2003; Nissen et al., 2003).

The sugar and ethanol concentrations present in the *S. cerevisiae/H. guilliermondii* mixed fermentation at the onset of the non-*Saccharomyces* death (Fig. 1-F) were 177 and 12 g l\(^{-1}\), respectively, and in the *S. cerevisiae/H. uvarum* mixed fermentation (Fig. 2-F) were 195 and 6.6 g l\(^{-1}\), respectively. In the single cultures, however, *H. guilliermondii* and *H. uvarum* viable cell concentrations remained at high values (ranging 10\(^7\)–10\(^8\) CFU ml\(^{-1}\)) during 15–20 days of fermentation, even when ethanol had reached relatively high concentrations (60–70 g l\(^{-1}\)) (Figs. 1-D and 2-D). These results seem to indicate that the early death of non-*Saccharomyces* in mixed growth with *S. cerevisiae* was not caused by nutrient limitation or high ethanol concentrations in the culture medium. Rojas et al. (2003) found that, for mixed cultures of *H. guilliermondii* and/or *P. anomala* with *S. cerevisiae*, the onset of the non-*Saccharomyces* death always began after the initial 1–2 days of fermentation, which agrees well with our results. Moreover, Nissen and Arneborg (2003) reported similar results for two non-*Saccharomyces* wine yeasts (*Kluyveromyces thermotolerans* and *Torulospora debruecki*) in mixed cultures with *S. cerevisiae*. These authors suggested that viable *S. cerevisiae* cells at high concentrations provoke the early death of non-*Saccharomyces*.

Fig. 2. Viable cells of *H. uvarum* (○) and *S. cerevisiae* (○), glucose and fructose consumption (■) and ethanol production (▲) during single cultures of *H. uvarum* (A,D) and *S. cerevisiae* (B,E) and mixed culture of *H. uvarum* with *S. cerevisiae* (C,F).
yeasts in mixed cultures by a cell-to-cell contact-mediated mechanism.

In the current study, the early death of *H. guilliermondii* and *H. uvarum* yeasts in mixed growth with *S. cerevisiae* was also confirmed under different cultivation conditions, namely aerobic growth and different culture media, and tested against several *S. cerevisiae* strains (data not shown). These findings led us to conclude that this early death is not dependent of the growth conditions or of a specific *S. cerevisiae* strains but it is rather related to the physical presence of *S. cerevisiae* cells and/

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**Fig. 3.** Viable cells of *H. guilliermondii* (Hg) (○) and *S. cerevisiae* (Sc) (⊙), glucose and fructose consumption (□) and ethanol production (▵), during the mixed cultures performed with different (Sc/Hg) inoculum ratios: A, A′—(3:1); B, B′—(1:2); C, C′—(1:10); D, D′—(1:100).
or toxic agents produced by them against other non-Saccharomyces yeasts.

Effect of S. cerevisiae viable cells concentration on the cell death process

In order to evaluate the influence of S. cerevisiae cell density on the non-Saccharomyces death process, several mixed cultures were performed using different S. cerevisiae/H. guilliermondii (Sc/Hg) inoculum ratios (3:1; 1:2; 1:10; 1:100).

Viable cell concentrations of H. guilliermondii and S. cerevisiae of these mixed cultures and the corresponding sugars and ethanol profiles, throughout the cultivation period, are represented on Fig. 3. Plots A–D show the relationship between death kinetics and initial Sc/Hg cell concentration ratio. In all mixed cultures, Saccharomyces became dominant at the end of the fermentation but H. guilliermondii death began, and was completed, at varying stages of the fermentation depending on the initial Sc/Hg cell concentration ratio (Fig. 3). Results show that death rates of H. guilliermondii in the mixed cultures increased for higher Sc/Hg inoculum ratios and death process began sooner: at the first day of fermentation for the initial inoculum ratio of (3:1) and after 15 days for the initial inoculum ratio of (1:100).

Comparing S. cerevisiae with H. guilliermondii cell profiles (Fig. 3, plots A–D) it can be observed that the onset of the non-Saccharomyces death depends, not only from the initial relative proportions of S. cerevisiae/H. guilliermondii but also from the S. cerevisiae cell density value present in the mixed cultures. In fact, H. guilliermondii death in the mixed cultures occurred only when S. cerevisiae cell density reached values close to $10^7$ CFU ml$^{-1}$. The initial number of viable cells, the initial day of H. guilliermondii death process and the corresponding S. cerevisiae cell density in the mixed cultures are summarised in Table 1.

Once again, it is clear that the sugars and ethanol concentrations were not the primary cause of H. guilliermondii death (Fig. 3, plots A’–D’). In fact, at the onset of Hg death the sugars and ethanol concentrations were 187 g l$^{-1}$ and 0 g l$^{-1}$, respectively, in the (3:1) Sc/Hg mixed culture (Fig. 3-A’) and 52 g l$^{-1}$ and 72 g l$^{-1}$, respectively, in the (1:100) Sc/Hg mixed culture (Fig. 3-D’). These results prove that the early death of H. guilliermondii is highly dependent of the S. cerevisiae concentration and two explanations suggest themselves: production of toxic compounds, other than ethanol, by S. cerevisiae, or cell-

### Table 1

Relation between the initial Sc/Hg inoculum ratio and the onset of the Hg cell death in each Sc/Hg mixed culture plotted in Fig. 3

<table>
<thead>
<tr>
<th>Plots</th>
<th>Initial cell density (CFU ml$^{-1}$)</th>
<th>Initial day of Hg cell death</th>
<th>S. cerevisiae cell density at initial day of death (CFU ml$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>$1.4 \times 10^6$</td>
<td>1</td>
<td>$6.6 \times 10^6$</td>
</tr>
<tr>
<td>B</td>
<td>$2.2 \times 10^6$</td>
<td>2</td>
<td>$5.1 \times 10^6$</td>
</tr>
<tr>
<td>C</td>
<td>$1.0 \times 10^4$</td>
<td>3</td>
<td>$6.9 \times 10^6$</td>
</tr>
<tr>
<td>D</td>
<td>$6.0 \times 10^3$</td>
<td>15</td>
<td>$3.6 \times 10^6$</td>
</tr>
</tbody>
</table>

![Fig. 4](image-url) Viable cells of H. guilliermondii (○) and S. cerevisiae (■), glucose and fructose consumption (—) and ethanol production (△) during the mixed cultures performed with 100 g l$^{-1}$ of initial total sugars at different (Sc/Hg) inoculums ratios: A, A’—(1:2); B, B’—(1:10).
to-cell contact, as previously reported by Nissen et al. (2003) and Nissen and Arneborg (2003).

The amount of sugar metabolised by S. cerevisiae had a pronounced effect on the death rate of H. guilliermondii in mixed cultures. In fact, for the mixed culture performed with 100 g l\(^{-1}\) of initial sugars (Sc/Hg inoculum ratio ≈ 1:10), the viable cells concentrations of H. guilliermondii remained at values close to 10\(^6\) CFU ml\(^{-1}\) until the 7th day of fermentation and death was not completed, at least, during the first 34 days of fermentation (Fig. 3-B). Whereas in the mixed culture performed with 200 g l\(^{-1}\) of initial sugars (similar inoculum ratio) H. guilliermondii death began on the 4th day of fermentation and was completed within 12–13 days (Fig. 3-C). A similar delay on the onset of cellular death was observed when a mixed culture was grown at (1:2) inoculum ratio (Sc/Hg) using 100 g l\(^{-1}\) instead of 200 g l\(^{-1}\) of initial total sugars (Fig. 4-A compares with Fig. 3-B), although S. cerevisiae caused the total death of H. guilliermondii cells in both situations. Cell densities achieved by S. cerevisiae in the mixed fermentations performed with 100 g l\(^{-1}\) of initial sugars were similar to those found in the mixed fermentations performed with 200 g l\(^{-1}\) (above 10\(^7\) CFU ml\(^{-1}\)), but H. guilliermondii death was retarded in the former case when compared to the latter. Thus, apparently, death of H. guilliermondii depends not only on the S. cerevisiae cell density but also on the amount of sugar metabolised. These findings suggest that one or more toxic compounds produced by S. cerevisiae is/are the cause of the early death of H. guilliermondii during mixed growths with S. cerevisiae.

It should be mentioned that the overall behaviour of the different yeast species in the mixed fermentations performed agree well with the yeast population dynamics found in spontaneous and inoculated wine fermentations. Several studies (Constantti et al., 1998; Cocolin et al., 2000; Raspor et al., 2002) report the dominance of non-Saccharomyces yeasts during the first days (3–7 days) of spontaneous vinifications, where the initial yeast population ranges 10\(^4\)–10\(^5\) CFU ml\(^{-1}\) for non-Saccharomyces yeasts and 10\(^2\)–10\(^3\) CFU ml\(^{-1}\) for Saccharomyces yeasts. This performance is similar to the yeast behaviour exhibited in the mixed fermentations represented in Fig. 3-C and D. Conversely, in vinifications where S. cerevisiae starts

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**Table 2**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Killer activity at different pH</th>
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<tbody>
<tr>
<td></td>
<td>3.5</td>
</tr>
<tr>
<td>H. guilliermondii</td>
<td>N1</td>
</tr>
<tr>
<td></td>
<td>N2</td>
</tr>
<tr>
<td></td>
<td>N28</td>
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<tr>
<td></td>
<td>N33</td>
</tr>
<tr>
<td>H. uvarum</td>
<td>N1</td>
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<td></td>
<td>N2</td>
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<td></td>
<td>N28</td>
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<td>N33</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>S1</td>
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<td>S2</td>
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<td></td>
<td>S28</td>
</tr>
<tr>
<td></td>
<td>N33</td>
</tr>
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</table>

N1: Killer neutral with respect to K1 killer toxin; N2: Killer neutral with respect to K2 killer toxin; N28: Killer neutral with respect to K28 killer toxin; N33: Killer neutral with respect to killer-sensitive strain EX33; S1: Killer sensitive with respect to K1 killer toxin; S2: Killer sensitive with respect to K2 killer toxin; S28: Killer sensitive with respect to K28 killer toxin.

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**Presence of toxic compounds**

In order to investigate the cause of the non-Saccharomyces early death, cell-free supernatants obtained from 3 and 6 days-old mixed cultures (Hg + Sc) and from an 8 days-old single culture of Hg were inoculated with H. guilliermondii at an initial cell density of 10\(^6\) CFU ml\(^{-1}\). The viable cell profiles of these cultures (Fig. 5) showed that total death of H. guilliermondii was achieved within 5 and 13 days when cultivated in the 6 and 3 day-old mixed culture supernatants, respectively. Conversely, when cultivated in the Hg single-culture supernatant, cell viability remained relatively stable during 13 days, with a slight decrease being observed from an initial cell density of 2 × 10\(^6\) CFU ml\(^{-1}\) to a final cell density of 8 × 10\(^4\) CFU ml\(^{-1}\). The initial content of sugars and ethanol in the mixed cultures supernatants were 138 and 26 g l\(^{-1}\), respectively, in the 3 day-old supernatant and 74 and 58 g l\(^{-1}\), respectively, in the 6 day-old supernatant. It should be pointed out that the latter values compare with those initially present on

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**Fig. 5.** Viable cells profiles of H. guilliermondii inoculated in free-cell supernatants obtained from previous 8 day-old Hg single-culture (○), 3 day-old Sc/Hg mixed culture (□) and 6 day-old Sc/Hg mixed culture (●).

**Fig. 6.** Pictures of the killer activity plate tests performed for S. cerevisiae CCMI 885, H. guilliermondiiNCYC 2380 and H. uvarum PYCC 4193T with the reference strains, K1 killer JCR2, K2 killer EX85, K28 killer GY-2-3a and killer-sensitive EX33.
the Hg single-culture supernatant (initial concentration of sugars and ethanol of 67 and 52 g l⁻¹, respectively). These results sustain the conclusion that the primary cause of the early death of H. guilliermondii in mixed growth with S. cerevisiae is not ethanol or nutrient depletion (all cell-free supernatants were supplemented with nitrogen and yeast extract). Conversely to the results reported by Nissen et al. (2003), where the early death of K. thermotolerans and Torulaspora delbrueckii in mixed cultures with S. cerevisiae suggested a cell–cell contact-mediated mechanism, our results strongly indicate that some toxic compounds produced exclusively by S. cerevisiae are responsible for the early death of H. guilliermondii yeast.

It is well known that during wine fermentations yeasts can produce, besides ethanol, other toxic compounds, namely, killer toxins, short- and medium-chain fatty acids and sulphite, able to induce death of other yeasts — Saccharomyces spp. or non-Saccharomyces spp. (Schmitt and Breinig, 2002; Fleet, 2003). Several killer strains of S. cerevisiae, isolated from wine musts, have been found to kill other sensitive yeasts within the first days of fermentation, although the majority of these toxins are only effective against other S. cerevisiae strains (Shimizu, 1993; Pérez et al., 2001). To assure that classical killer toxins produced by S. cerevisiae were not involved in this early death phenomenon, killer activity of the three yeast strains used in the present work was tested against the classical killer toxins K1, K2, and K28 (at different pH). Tests revealed that S. cerevisiae CCMI 885 is killer-sensitive against the reference killer toxins (K1, K2 and K28) and H. guilliermondii NCYC 2380 as well as H. uvarum PYCC 4193T are killer neutral (Table 2 and Fig. 6). Additionally, none of the strains is killer positive towards the killer-sensitive strain EX33 (Table 2). Although significant, these results do not exclude however the production by S. cerevisiae of any other unknown killer-like toxins (e.g. peptides, proteins or glycoproteins) or even some fermentative metabolites potentially toxic for some non-Saccharomyces strains. To determine the nature of the toxic compounds involved in the early death of these non-Saccharomyces strains, as well as the death mechanism underlying this phenomenon, further investigation will be carried out.

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