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Claudia Lorena Fernández López, Sandra Beaufort, Cédric Brandam, Patricia Taillandier

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Abstract Traditional tequila fermentation is a complex microbial process performed by different indigenous yeast species. Usually, they are classified in two families: *Saccharomyces* and Non-*Saccharomyces* species. Using mixed starter cultures of several yeasts genera and species is nowadays considered to be beneficial to enhance the sensorial characteristics of the final products (taste, odor). However, microbial interactions occurring in such fermentations need to be better understood to improve the process. In this work, we focussed on a *Saccharomyces cerevisiae/Kluyveromyces marxianus* yeast couple. Indirect interactions due to excreted metabolites, thanks to the use of a specific membrane bioreactor, and direct interaction due to cell-to-cell contact have been explored. Comparison of pure and mixed cultures was done in each case. Mixed cultures in direct contact showed that both yeast were affected but *Saccharomyces* rapidly dominated the cultures whereas *Kluyveromyces* almost disappeared. In mixed cultures with indirect contact the growth of *Kluyveromyces* was decreased compared to its pure culture but its concentration could be maintained whereas the growth of *Saccharomyces* was enhanced. The loss of viability of *Kluyveromyces* could not be attributed only to ethanol. The sugar consumption and ethanol production in both cases were similar. Thus the interaction phenomena between the two yeasts are different in direct and indirect contact, *Kluyveromyces* being always much more affected than *Saccharomyces.*

Keywords Tequila · *Saccharomyces cerevisiae* · *Kluyveromyces marxianus* · Mixed cultures · Yeasts interactions

C. L. F. Lopez · S. Beaufort (✉) · C. Brandam · P. Taillandier
INPT, UPS, Laboratoire de Génie Chimique, Université de Toulouse, 4, Allée Emile Monso, 31030 Toulouse, France
e-mail: sandra.beaufort@ensiacet.fr

P. Taillandier
e-mail: patricia.taillandier@ensiacet.fr

C. L. F. Lopez · S. Beaufort · C. Brandam · P. Taillandier
Laboratoire de Génie Chimique, CNRS, 31030 Toulouse, France

Introduction

Tequila, a distilled beverage obtained from the fermented sugars of cooked agave, has a complex fermentation process performed by different indigenous yeast species. Similar to the wine fermentation process largely studied (Ciani et al. 2006; Fleet et al. 1984), mexican alcoholic and distilled agave beverages involve a complex fermentation in which bacteria (lactic and acetic acid) and yeasts (non-*Saccharomyces* and *Saccharomyces*) are present in stable mixed populations, or succeeding one another (Graciano-Fonseca et al. 2008). The variety of strains present during fermentation is crucial to chemical and volatile compounds formations and so very important to final sensory characteristics of tequila and other agave fermented beverages (Lappe-Oliveras et al. 2008). Using mixed starter cultures of several yeast genera and species is nowadays considered to be beneficial to enhance the sensorial characteristics of final products of wine (Ciani et al. 2010; Romano et al. 2003) or tequila (Lappe-Oliveras et al. 2008). In tequila context, the indigenous yeast community was identified (Lachance 1995). *Candida lusitaniae* and *Metschnikowia agaves* were main species found in fresh agave, whereas during the fermentation step *Saccharomyces cerevisiae* was the dominant strains and *Kluyveromyces marxianus* was among the secondary strains.

The *Kluyveromyces* species yeast has been reported to have biotechnological advantages for its wide variety of substrate type consumption, high ethanol yield and tolerance and also low acetic acid production. They have interest in the bioethanol production but also in fermented beverages elaboration (such as white and red wine, mezcal and tequila) besides they are known for producing aroma compounds to fermented products (Ciani et al. 2006; Graciano-Fonseca et al. 2008; Scharpf et al. 1986).

López-Alvarez et al. (2012) tested *K. marxianus* UMPe-1 for agave must fermentations (in single cultures) and compared them to the fermentation made with *S. cerevisiae* Pan-1 as reference. *K. marxianus* showed higher aroma compounds production and higher ethanol yield compared to the *Saccharomyces* strain. They concluded that *K. marxianus* has industrial potential and that the strain origin is important to its fermentation performance, due to the adaptation to environmental stress (López-Alvarez et al. 2012). Recently others authors (Amaya-Delgado et al. 2013) used non-*Saccharomyces* strains *Pichia kluyveri* GRO3 and *K. marxianus* GRO6, isolated from tequila or mezcal process. Their fermentative profile was compared to the one of *S. cerevisiae* AR5. The non-*Saccharomyces* yeasts showed fermentation efficiency higher than 85 % in agave tequilana juice, and an aroma compounds production higher than *Saccharomyces* yeast.

Some yeast couples have been studied as multi starter fermentation cultures but only in wine production (Ciani et al. 2006). These authors used mixed cultures of *Hanseniaspora uvarum*, *Torulaspora delbrueckii* and *Lachancea thermotolerans (Kluyveromyces)* together with *S. cerevisiae* in grape musts. They observed that non-*Saccharomyces* species survived during the first stage of fermentation but then, *S. cerevisiae* dominated until the end of fermentation over the non-*Saccharomyces* strains.

In this work, we focussed on the *S. cerevisiae/K. marxianus* yeasts couple isolated from tequila and mezcal fermentation. This couple have not yet been reported for beverage application. Mixed yeast starter was used to performed fermentation in tequila synthetic must medium with goal to evaluate interaction phenomena between *S. cerevisiae* and *K. marxianus.* Both kinds of interaction were studied: Indirect interactions due to excreted metabolites, thanks to the use of a specific membrane bioreactor (MBR) allowing the physical separation of two strains, and direct interaction due to cell-to-cell contact.

Materials and methods

Yeasts and medium

The yeasts used were *K. marxianus DU3* isolated from mezcal artisanal fermentation (Mexico) and *S. cerevisiae* AR5 isolated from tequila fermentation (Arandas, Guadalajara, Mexico). These strains are part of CIATEJ collection strains, in Mexico.

Conservation medium/enumeration of total yeasts population

The yeasts *K. marxianus* and *S. cerevisiae* AR5 were conserved on YPD agar medium. This medium was also used for total yeasts enumeration of the mixed culture experiments performed in flask mixed cultures. The composition was: dextrose or fructose (20 g/L), yeast extract (10 g/L), peptone (20 g/L), agar (20 g/L). The medium was autoclaved for 20 min at 120 °C.

Media used for single and co-culture fermentation

The composition of the synthetic medium named M11 used for fermentation was designed to be close to the agave juice used for mezcal or tequila elaboration in Mexico, and to avoid limitations of carbon, nitrogen, vitamins and mineral elements for the yeast growth. The composition was: fructose (100 g/L), yeast extract (1 g/L), $(NH_4)_2SO_4$ (1 g/L), K_2HPO_4 (2.23 g/L), $MgSO_4{\cdot}7H_2O$ (5.07 g/L), $Ca(NO_3)_2{\cdot}4H_2O$ (4.72 g/L).

The pH of the medium was adjusted to 4.7 with orthophosphoric acid solution (85 % v/v) before autoclaving for 20 min at 120 °C.

Membrane bioreactor

A tool designed specially to study the indirect interactions between two microorganisms was used: a lab-made, two-compartment, MBR. The complete system has been described in detail in Salgado-Manjarrez et al. (2000) and Albasi et al. (2002). It is composed of two jars interconnected by a hollow fiber membrane module immersed in one of the two jars. The membrane fiber diameter of 0.1 μm allows the medium, but not the microorganisms, to pass through the fibers. By applying pressure into the headspace of each of the vessels alternately through sterilised air filters, the medium is allowed to flow and mix, but not the cells. A system of valves controls the admission and expulsion according to the liquid levels into the vessels. Each strain is inoculated into only one compartment, which can be sampled and analysed separately. Hence, the microorganisms grow as if they were in the same liquid medium but they are physically separated, thus allowing the dynamics of each population to be easily followed by microscopic counting (Fig. 1).

Fermentations

Yeast inoculation was performed from yeast grown in YEPD liquid medium overnight. For pure cultures,

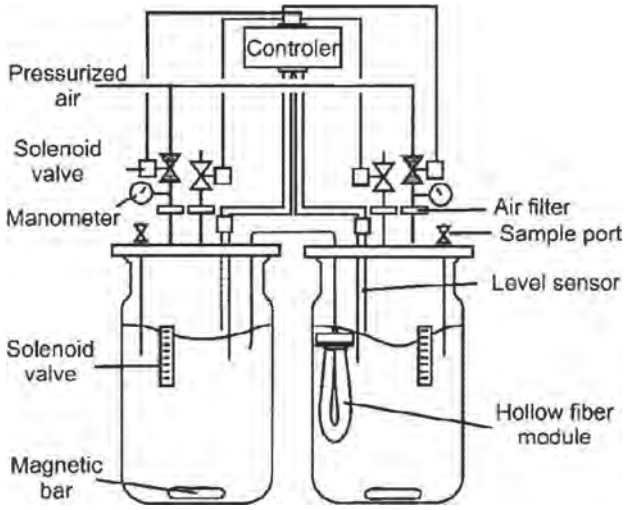

Fig. 1 MBR and control system (Salgado-Manjarrez et al. 2000; Albasi et al. 2002)

5×10^6 viable cells/mL of *K. marxianus* or *S. cerevisiae* were inoculated. Mixed fermentation of *S. cerevisiae* and *K. marxianus* were performed with the inoculation of 5×10^6 total viable cells/mL, 2.5×10^6 viable cells/mL of each one, giving a *K. marxianus* to *S. cerevisiae* ratio of one. Another inoculation level was tested with 5×10^6 viable cells/mL of each strain, but always in a ratio of one, and gave the same results (data not shown).

Mixed fermentations were performed in two ways

- In the MBR with indirect cells contact. *S. cerevisiae* and *K. marxianus* were inoculated in different compartments of 2 L vessels interconnected by the membrane allowing to study indirect interactions between the two yeasts due to metabolites excreted in the medium,
- in flasks where the two strains were inoculated in the same vessel allowing to study at the same time direct and indirect interactions

Pure cultures of each strain performed in the same conditions were used as a control.

Fermentations in MBR were considered in micro-aerobiosis since 0.3 bars of air flux were used for applied pressure in head space vessels to ensure the flow and mix of medium. The temperature was 30 °C and a magnetic stir bar (250 rpm) was used. Cultures samples were taken during the course of fermentation in each vessel to measure each population growth. Liquid was analysed systematically in each vessel to check the homogeneity between the two compartments. For all experiments performed, the differences between the two compartments were always less than the measured precision of the analysis method. So, the system ensured the homogeneity of the liquid between the two jars as already shown by Salgado-Manjarrez et al. (2000).

Direct contact cultures were realized in 500 mL flasks, with 300 mL of the M11 liquid medium. The temperature was 30 °C and the agitation was 100 rpm in an orbital shaker incubator, indeed there were considered as micro-aerobic conditions.

In both types of cultures samples were taken during the course of fermentation and then centrifuged at 11,500 rev/min, at 4 °C, for 10 min. The supernatant was stored in the freezer until substrate and products analyses were made. All experiments were made by duplicate.

Analysis

The ethanol, glycerol and fructose concentrations were analysed by an HPLC-equipped Phenomenex ROA Organic column. The liquid phase was 10 mM of sulfuric acid solution which circulated at 0.170 mL/min at 30 °C. The volume of the injection loop was 25 µL. The peaks of fructose, ethanol and glycerol were detected by infra red detector.

Biomass analysis

The total cell concentration of the two strains was determined using a Thoma hemacytometer chamber, and by determination of dried mass weight. The blue methylene method allowed distinguishing viable and non viable cells. Plate count was also used for mixed cultures to determine viable cells.

For the enumeration of *K. marxianus* during mixed cultures in flasks, WL differential agar medium, containing cyclohexamide (Sigma Aldrich) was used. Indeed, *S. cerevisiae* can not grow on this medium but *K. marxianus* can. So that *S. cerevisiae* was obtained by difference between total cells grown in YPD agar minus *K.marxianus* obtained in the specific medium (WL differential agar medium). Plate count was made by duplicates for each sample. A correlation between the viable cells counted in microscope and the plate count was made for ecah strain, in order to verify the validity of the technique under the different fermentation conditions. A correlation between total cells and the dry mass weight was made. With this correlation and viability, it is possible to have concentration of viable cells express in g/L. Biomass was presented with this unit in all graphs to be homogene.

Results

Cultures pures in flasks

Saccharomyces cerevisiae and *K. marxianus* behaviours in single cultures were first studied in flask cultures and are

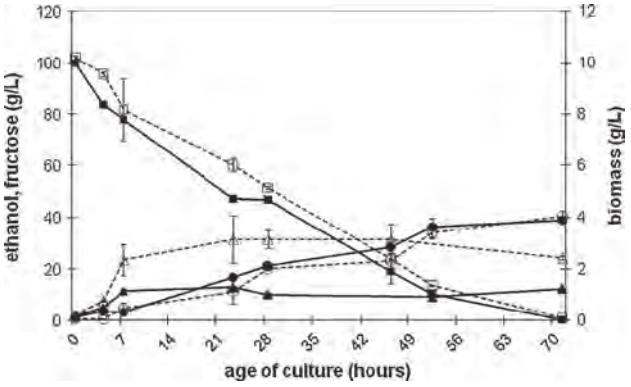

Fig. 2 Evolution of substrate (fructose—*squares*), ethanol (*circle*) and viable biomass (*triangles*) during *S. cerevisiae* AR5 (*lines—full symbols*) or *K. marxianus* DU3 cultures (*dot—empty symbols*) in pure flask cultures. *Error bars* corresponds to experiment duplicates

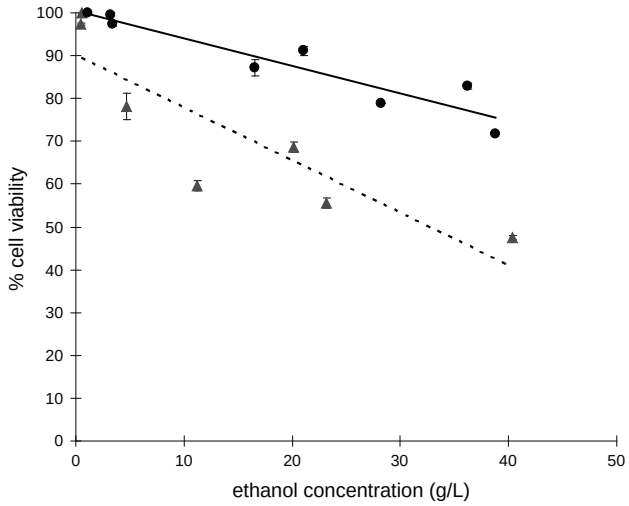

Fig. 3 Viability loss versus ethanol concentration during flasks pure cultures of *S. cerevisiae* (*line with circle* symbol) or *K. marxianus* (*dots* and *triangle* symbols)

shown in Fig. 2. Fructose consumption was similar for *S. cerevisiae* and *K. marxianus,* they both consume all sugars in about 65–70 h with the same rate. Ethanol production reached also a similar final value, and for glycerol production there was a small difference: 4.84 g/L for *K. marxianus* and 3.64 g/L for *S. cerevisiae*. Viable biomass production was higher for *K. marxianus* (6.6×10^7 cell/mL compared to *S. cerevisiae* (3.82×10^7 cell/mL) which correspond to 2.4 and 1.3 g/L respectively.

Consequently, yields obtained for ethanol was similar, there was a small difference for glycerol, and for the biomass it was nearly twice for *K. marxianus* compared to *S. cerevisiae* (Table 1).

Another difference concerned the viablility loss of each strain. Indeed, the viability of each strain was near 100 % at the begining, but it decreased to 87.2 and 59.6 % after 23 h and only reached 71.7 and 47.5 % at the end of the fermentation respectively for *S.cerevisiae* strain and *K. marxianus* (Table 1).

The percentage of cell viability versus ethanol concentration for each strain were plotted (Fig. 3).

Even if it could not been established that ethanol is the only metabolite explaining the loss of viability, good correlations were obtained between cell viability loss and ethanol concentration. The loss of viability is quicker for *K. marxianus* than to *S. cerevisiae.*

Mixed cultures in flasks—direct contact comparison to single cultures

In the second part of this study, mixed cultures of *S. cerevisiae* and *K. marxianus* were performed, and compared with pure cultures, in order to put in evidence interactions occurring between them (Fig. 4).

In the mixed culture with direct contact, the growth of *S. cerevisiae* was slightly decreased since the final viable biomass reached maximum 1 g/L (±0.05) in mixed cultures and was 1.3 g/L (±0.05) in pure culture.

For *K. marxianus*, both the growth rate and the biomass obtained were different comparing to the pure culture. The maximum viable biomass obtained at 7 h was 0.5 g/L, while in pure culture, the maximum viable biomass obtained was near 3 g/L, six times higher. Moreover, an ascendancy of *S. cerevisiae* growth over *K. marxianus* was observed during mixed fermentation in direct contact, especially after the first 24 h, since at that point the concentration of *K. marxianus* declined, and *S. cerevisiae* dominated. The global viability decreased to 78.9 %, and *K. marxianus* nearly disappeared from the culture. Nevertheless fermentation time corresponding to total consumption of substrate,

Table 1 Yields for ethanol, glycerol, biomass production from fructose, and viability, in flasks pure cultures

	Ethanol/fructose	Glycerol/fructose	Biomass/fructose	% Viabilty after 23 h	% Viabilty after 71 h
Saccharomyces cerevisiae	0.38 g/g (±0.01 g/g)	0.037 g/g (±0.001 g/g)	0.016 g/g (±0.001 g/g)	87.2 (±1.8)	71.7 (ND)
Kluyveromyces marxianus	0.38 g/g (±0.02 g/g)	0.046 g/g (±0.002)	0.048 g/g (±0.002 g/g)	59.6 (±1.2)	47.5 (±0.5)

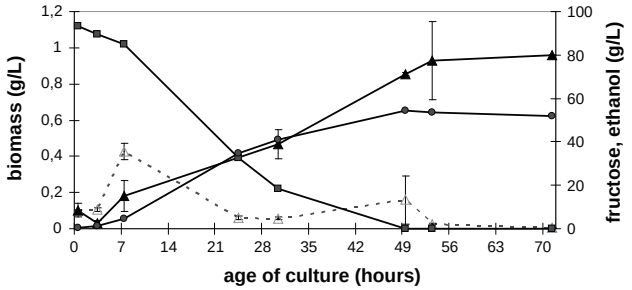

Fig. 4 Evolution of substrate (fructose—*squares*), products (ethanol—*circle*) and viable biomass (*triangles*) during *S. cerevisiae* AR5 (*lines—full symbols*) and *K. marxianus* DU3 (*dot—empty symbols*) in mixed cultures cell-to-cell contact. *Error bars* on biomass corresponds to experiment duplicates

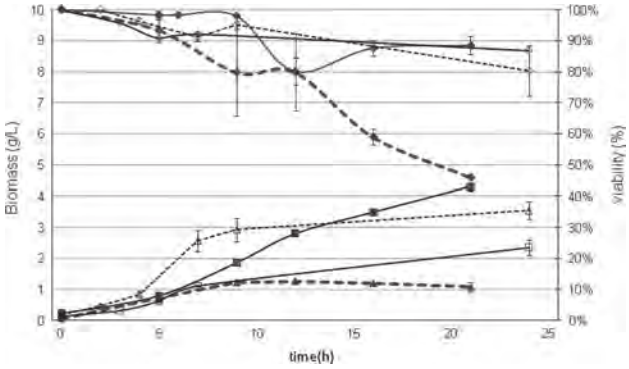

Fig. 5 Evolution of total biomass *S. cerevisiae* AR5 (*lines*) or *K. marxianus* DU3 (*dots*) in pure (*empty symbols*) and mixed cultures (*full symbols*) in the MBR. Biomass symbols correspond to *triangles* for *K. marxianus*, *squares* for *S. cerevisiae,* and viability corresponds to *diamonds*, with *empty symbol* for pure cultures, *full symbol* for mixed culture

decreased from 60 to 71 h in the single culture to 49 h in mixed culture in direct contact, which is an important advantage cost in industrial level. The ethanol production was around 50 g/L, higher than in the single culture of both strains. Final glycerol concentration of 3.3 g/L was obtained in mixed cultures. Maximum specific growth rate was similar for *S. cerevisiae* and *K. marxianus* (0.38 and 0.33 h^{-1}), however the cell growth decrease (growth arrest) of *K. marxianus* after 10 h suggests that there was something affecting its growth.

Mixed cultures in MBR—indirect contact, comparison to single culture

The MBR lets the strains to shear the medium and produce metabolites without cell to cell contact, so the effects due to the cell to cell contact are avoided. This allows observing if the strain behavior is the same in indirect contact than in pure culture. Pure cultures of *S. cerevisiae* and *K. marxianus* were realized in duplicate in the BRM as a control, with the liquid M11 medium. They were compared to mixed culture in indirect contact in Fig. 5.

Fouling occurred after 21 h and so the media transport from to the other compartment was stopped, even when the pressure was increased from 0.3 to 0.5 bars. The results showed a difference in the growth of both strains in single culture compared to mixed culture until 21 h, even if there wasn't a cell to cell contact. For the *S. cerevisiae* strain, the biomass obtained for mixed culture was almost twice to that obtained in pure culture. For *K. marxianus,* the contrary was observed, with a biomass higher in pure culture than in mixed culture.

In MBR mixed culture, there was a similar behavior between the two strains in growth in the first 10 h but there was difference in the μ_{max} values of 0.17 and 0.25 h^{-1}, for *K. marxianus* DU3 and *S. cerevisiae* respectively. Between 10 and 15 h of fermentation *K. marxianus* loosed viability to around 60 % while *S cerevisiae* maintained its viability around 90 % until the end of fermentation. In the mixed fermentation the population of *K. marxianus* at 21 h of fermentation was 1.42×10^7 cell/mL, and the *S. cerevisiae* concentration was 1.08×10^8 cell/mL this means 0.5 g/L to *K. marxianus* and 2.8 g/L to *S. cerevisiae* respectively.

In mixed culture in MBR the substrate consumption, in this first 16 h reached 27.5 g/L, then at 21 h when the flocculation started, sugar consumed were 39.4 g/L. Values were similar to that consumed at the same time in single culture (47.2 g/L) of *S. cerevisiae* and of *K. marxianus* DU (33.2 g/L).

Ethanol production in the mixed cultures in MBR at 21 h was of 22.2 g/L. The viability obtained at 21 h (45 %) for *K. marxianus* couldn't be due only to the ethanol concentration in the medium. As shown in Fig. 6, the viability was of 60 % at 20–25 g/L of ethanol (Fig. 6) in pure culture in the MBR of *K. marxianus.*

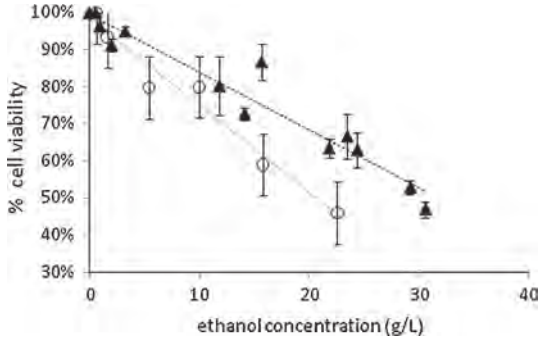

Fig. 6 Viability loss versus ethanol concentration during *K. marxianus* pure culture in MBR (*full triangles*), and in mixed culture in MBR (*empty circles*)

Discussion

Recently, Ciani et al. (2010) have reported that mixed cultures of *Saccharomyces* and non-*Saccharomyces* strains can produce unexpected compounds during mixed fermentation, which is interesting in fermented beverages like wine and tequila since it could allow the modification of aromatic profile of the final product. To our knowledge, this work is one of the first that showed the existence of interaction phenomena between *S. cerevisiae* AR5 and *K. marxianus* DU3, strains isolated from mezcal and tequila fermentation in Mexico. Differences in mixed cultures compared to pure cultures were observed, whatever the mixed culture conditions were. These differences were in kinetics and in final biomass obtained, but mainly in the viability loss of *K. marxianus*. This yeast seems to be more affected by the mixed culture conditions than *S. cerevisiae* despite the fact, it growths and ferments in pure culture as well as or better than *S. cerevisiae*. Some authors (Lane and Morrissey 2010) have reported before the high specific growth rate of *Kluyveromyces* strains over *S. cerevisiae* strains. In our work *S. cerevisiae* was negatively affected in direct contact mixed culture but positively affected in indirect contact mixed culture. Ciani et al. (2010) reported interactions between *Saccharomyces* and non-*Saccharomyces* strains, and focused on the survival ability of *Saccharomyces* yeasts. In other works the early death of non-*Saccharomyces* yeasts was also observed by Pérez-Nevado et al. (2006) without being explained. In other works the reason that caused the interaction was searched, like in Pérez-Nevado et al. (2006), who used two wine strains *H. guilliermondii* and *H. uvarum* in mixed fermentations with *S. cerevisiae*. They reported the death of non-*Saccharomyces* yeast to different inoculum ratios and even when the *Saccharomyces* strains were killer neutral. Other changes were reported in the degree of flocculation in the co-culture of *K. apiculate* and *S. cerevisiae* strains (Sosa et al. 2008), these interactions were a result of mixed culture, besides there were changes in cell growth and cell viability. Nissen and Arneborg (2003) have repported the early death of non-*Saccharomyces* cells in a defined grape medium.When they were in mixed cultures with *S. cerevisiae*, an early arrest of growth occured after 30 h for *L. thermotolerans (other name of Kluyveromyces thermotolerans)* and after 21 h for *T. delbrueckii*. It must be noted that in their case the inoculum and media conditions were not the same but the inoculum ratio was. So it can be assumed that the negative effect of *S. cerevisiae* on non-*Saccharomyces* is general and also that the time of growth arrest is strain dependent. In the same work, the authors also showed that for the couple *K. thermotolerans* and *S. cerevisiae*, fermentation had a lower ethanol concentration in the stationary phase (30 g/L) than in our work (50 g/L). There was not complete substrate consumption: from 200 it went to 100 g/L, and at the growth arrest time glucose was around 176 g/L and ethanol 7.1 g/L. (fructose was 84.9 g/L and ethanol was around 4.5 g/L for growth arrest in our conditions). This low ethanol concentration confirms that the growth arrest of *K. marxianus* isn't only due to a low ethanol tolerance. Authors reported ethanol tolerance of *K. marxianus* is between 39 and 79 g/L (Graciano-Fonseca et al. 2008). In all this works the fermentations were carried out with direct contact of the cells. In our work, *K. marxianus* appeared to be less tolerant to ethanol than *S. cerevisiae,* as several non-*Saccharomyces* yeasts are. The sensibility depends on the strain used. Indeed, in tequila fermentation at industrial level, authors founded a *K. marxianus* strain isolated from tequila with a higher osmotic and ethanol tolerance (10 % v/v) than a *S. cerevisiae* strain (López-Alvarez et al. 2012). Others authors (Fiore et al. 2005) showed that non-*Saccharomyces* yeasts isolated of tequila had better stress tolerance to ethanol and SO_2 than wine non-*Saccharomyces* yeasts.

The experiment in MBR allowed to conclude that there was not only a direct interaction due to cell-to-cell contact, and that metabolites excreted other than ethanol are also involved in the interaction between *S. cerevisiae* AR5 and *K. marxianus* DU3. There is a negative effect on *K. marxianus* growth and a positive effect on *S. cerevisiae*. Nissen and Arneborg (2003) also reported that cellular death of non-*Saccharomyces* species was not due to killer toxins presence excreted by *S. cerevisiae* which have been widely reported as involved in interactions between yeasts of the same species. Also they discarded ethanol concentration reached or apoptosis, and suggested that the cell density in cell to cell mixed culture could be implicated in the early cell arrest of non-*Saccharomyces*–*Saccharomyces* species but the implicated interaction mechanisms are still unknown. Abranches et al. (1997) have reported the killer toxins production by *Kluyveromyces* strains, despite the fact that *Saccharomyces* killer toxins have stronger activity than other non-*Saccharomyces* killer toxins. However this work didn't check the killer toxins activity.

Strains interactions could be different depending on the couple tested. Some of them compete for space and reach similar general biomass and ethanol product in single culture than in mixed culture, and others present amensalism phenomenon or changes in aromatic profile of final beverage.

As we can see in fermentation, it is interesting to use mixed culture of two strains, however this implies difficulties to perform fermentation, because of unexpected behavior of mixed fermentation. In industrial tequila fermentation, using mixed starter cultures of several yeast genera and species is nowadays considered to be beneficial to enhance the sensorial characteristics of the final

products. But the fermentation process results of both individual and mixed behaviors due to potential interactions between the yeasts used. Further experimentations studies should be realized to precise the mechanism of cell-to-cell contact interactions. Moreover, the compounds implied in the indirect interaction have also to be identified.

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