Interactions between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* in wine fermentation: influence of inoculation and nitrogen content

Patricia Taillandier · Quoc Phong Lai · Anne Julien-Ortiz · Cédric Brandam

Abstract Alcoholic fermentation by an oenological strain of *Torulaspora delbrueckii* in association with an oenological strain of *Saccharomyces cerevisiae* was studied in mixed and sequential cultures. Experiments were performed in a synthetic grape must medium in a membrane bioreactor, a special tool designed to study indirect interactions between microorganisms. Results showed that the *S. cerevisiae* strain had a negative impact on the *T. delbrueckii* strain, leading to a viability decrease as soon as *S. cerevisiae* was inoculated. Even for high inoculation of *T. delbrueckii* (more than 20× *S. cerevisiae*) in mixed cultures, *T. delbrueckii* growth was inhibited. Substrate competition and cell-to-cell contact mechanism could be eliminated as explanations of the observed interaction, which was probably an inhibition by a metabolite produced by *S. cerevisiae*. *S. cerevisiae* should be inoculated 48 h after *T. delbrueckii* in order to ensure the growth of *T. delbrueckii* and consequently a decrease of volatile acidity and a higher isoamyl acetate production. In this case, in a medium with a high concentration of assimilable nitrogen (324 mg L^{-1}), *S. cerevisiae* growth was not affected by *T. delbrueckii*. But in a sequential fermentation in a medium containing 176 mg L^{-1} initial assimilable nitrogen, *S. cerevisiae* was not able to develop because of nitrogen exhaustion by *T. delbrueckii* growth during the first 48 h, leading to sluggish fermentation.

Keywords *Saccharomyces cerevisiae* · *Torulaspora delbrueckii* · Yeast interactions · Wine fermentation · Co-inoculation

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Introduction

Traditional wine fermentation is a complex microbial process performed by different indigenous yeast species. Usually, they are classified in two groups: *Saccharomyces* and non-*Saccharomyces* species. In wine alcoholic fermentation (AF), the presence of many different non-*Saccharomyces* yeast genera has been reported: *Hanseniaspora* (*Kloeckera*), *Issatchenkia*, *Pichia* and *Metschnikowia* (Fleet and Heard 1993; Ocón et al. 2010), *Schizosaccharomyces*, *Brettanomyces*, *Zygosaccharomyces*, *Kluyveromyces*, *Candida*, *Torulaspora* (Lema et al. 1996; Ciani and Pepe 2002; Xufre et al. 2006; Renouf et al. 2006). Generally, non-*Saccharomyces* are considered to be active in the first part of the fermentation, when the ethanol concentration is not too high. However, compared to *Saccharomyces* species, they are not well adapted to finishing the AF and are rapidly replaced by these more ethanol tolerant yeasts.

As a consequence of the presence of these various indigenous yeast species during the spontaneous AF, it is difficult to control the fermentation process properly and to obtain a final product with constant quality. To remedy this problem, modern wine AF is generally carried out by a massive inoculation of commercial yeast *S. cerevisiae*, which puts that microorganism in a better position to dominate, and so ensures a stable wine quality. However, today,

to obtain more diversity in wines, with more complexity or different aromas, research is being done on inoculations with non-*Saccharomyces* in association with *Saccharomyces*. Ciani et al. (2010) reviewed different associations that have been studied. *Torulaspora delbrueckii* (formerly named *S. rosei*), in particular, is a non-*Saccharomyces* yeast that has recently been studied for this application (Bely et al. 2008; Ciani et al. 2006). This species has many good oenological characteristics, such as a high ethanol concentration tolerance and good glycerol production (Herraiz et al. 1990; Bely et al. 2008), lower formation of acetaldehyde, acetoin and volatile acids and higher production of higher alcohols and fruity esters than *Saccharomyces* (Ciani and Maccarelli 1998; Ciani et al. 2006; Renault et al. 2009). In consequence, some authors have suggested using *T. delbrueckii* and *S. cerevisiae* simultaneously in order to improve the fruity aroma quality and reduce the acetic acid concentration of wines (Bely et al. 2008; Ciani et al. 2006). From a practical point of view, for the winemakers, it is difficult to manage these multistarter fermentations. They must determine the quantities and timing of inoculation for each species in order to optimize the organoleptic characteristics of wines whilst preserving good efficiency of the AF, i.e. rapid and total consumption of sugars. Simultaneous inoculation (Bely et al. 2008) or sequential inoculation with *T. delbrueckii* first has been proposed (Herraiz et al. 1990; Ciani et al. 2006; Pillet et al. 2010). In these cases, *T. delbrueckii* could produce higher alcohols and fruity esters and then *S. cerevisiae* would finish the conversion of sugars into alcohol.

The initial results are promising but interactions have been found to exist between some strains of these species. The growth of *T. delbrueckii* strains is often negatively affected by the presence of *S. cerevisiae*. Indirect interaction i.e. interaction between strains via components of the medium have been demonstrated. Substrate competition or amensalism (production by *S. cerevisiae* of metabolites that inhibit *T. delbrueckii* growth) are examples of the indirect interactions already suggested (Bely et al. 2008; Farkas et al. 2005). Nissen et al. (2003) have also found that direct interaction i.e. interaction that needs a physical contact between strains can explain the behaviour of a pair of strains, *T. delbrueckii* and *S. cerevisiae*. This has been named cell-to-cell contact mechanism.

The objective of this work was to acquire better knowledge of these yeast interactions, in order to better manage the co-inoculation process in alcoholic wine fermentations. Experiments were carried out with two commercial oenological yeast strains of *T. delbrueckii* and *S. cerevisiae* in a synthetic medium. Fermentations with sequential inoculation (first *T. delbrueckii*, then *S. cerevisiae*) were compared to simultaneous inoculation and pure fermentations of each strain. The influence of initial nitrogen content in the medium was also studied.

Materials and methods

Yeasts and medium

The commercial oenological yeasts *T. delbrueckii* NSC123 (commercial name level 2 TD) and *Saccharomyces cerevisiae* QA23 were supplied by Lallemand S.A.S., France, as active dry yeasts.

The composition of the synthetic medium used in this work was designed to be close to that of white grape must and to avoid limitations of carbon, nitrogen, vitamins and mineral elements for the yeast growth. It was a medium regularly used in oenological studies, named MS300. The composition was: glucose (110 g L^{-1}), fructose (110 g L^{-1}), malic acid (6 g L^{-1}), citric acid (6 g L^{-1}), KH_2PO_4 (0.75 g L^{-1}), K_2SO_4 (0.5 g L^{-1}), $MgSO_4{\cdot}7H_2O$ (0.25 g L^{-1}), $CaCl{\cdot}2H_2O$ (0.16 g L^{-1}), NaCl (0.2 g L^{-1}), 1 mL of anaerobic factor stock solution, 1 mL of oligoelement stock solution and 10 mL of vitamin stock solution. The compositions of the stock solutions used to prepare the fermentation medium were:

- Solution of oligoelements (for 1 L of solution): $MnSO_4{\cdot}H_2O$ 4 g, $ZnSO_4{\cdot}7H_2O$ 4 g, $CuSO_4{\cdot}5H_2O$ 1 g, KI 1 g, $CoCl_2{\cdot}6\ H_2O$ 0.4 g, H_3BO_3 1 g, $(NH_4)_6Mo_7O_{24}$ 1 g.
- Solution of vitamins (for 1 L of solution): Myo-Inositol 2 g, Calcium pantothenate 0.15 g, hydrochloride thiamin 0.025 g, nicotinic acid 0.2 g, pyridoxine 0.025 g, biotin 0.0003 g.
- Solution of anaerobic factors (for 1 L of solution): ergosterol 15 g, Oleic acid 5 mL, Tween 80,500 mL, absolute ethanol 500 mL.
- Solution of amino acids (for 1 L of solution): Tyrosine 1.4 g, tryptophan 13.7 g, isoleucine 2.5 g, aspartic acid 3.4 g, glutamic acid 9.2 g, arginine 28.6 g, leucine 3.7 g, threonine 5.8 g, glycine 1.4 g, glutamine 38.6 g, alanine 11.1 g, valine 3.4 g, methionine 2.4 g, phenylalanine 2.9 g, serine 6.0 g, histidine 2.5 g, lysine 1.3 g, cysteine 1.0 g, proline 46.8 g.

The nitrogen source was brought by ammonium ions in NH_4Cl and by the amino acid stock solution. Two different nitrogen concentrations were used (MS300 and MS170). Medium MS300 contained an equivalent of 324 mg N L^{-1} that was assimilable by yeasts (204 mg N L^{-1} from amino acid solution corresponding to 13 mL L^{-1} of amino acid solution and 120 mg N L^{-1} from NH_4Cl corresponding to 0.46 g L^{-1}). Medium MS170 contained only 176 mg N L^{-1} of assimilable nitrogen (108 mg N L^{-1} from amino acid solution corresponding to 7.4 mL L^{-1} and 68 mg N L^{-1} from NH_4Cl corresponding to 0.26 g L^{-1}). The pH of the medium was adjusted to 3.3 with a sodium hydroxide solution (10 N) before autoclaving for 20 min at 120 °C.

Vitamins and oligoelements were added after thermal treatment.

Membrane bioreactor

A tool designed specially to study the indirect interactions between two microorganisms was used: a lab-made two-compartment membrane bioreactor (MBR). The complete system has been described in detail by Salgado et al. (2000) and Albasi et al. (2002). It is composed of two jars interconnected by a hollow fibre membrane module immersed in one of the jars. The membrane fibre diameter of 0.1 μm allows the medium, but not the microorganisms, to pass through the fibres. Each strain is inoculated into only one compartment, which can be sampled and analysed separately. By applying pressure into the headspace of each of the vessels alternately, the medium is made to flow and mix. Compressed, filter-sterilised nitrogen is used to apply the pressure and a system of valves controls its admission and expulsion according to the liquid levels. The time and quantity of liquid inversion is calculated to ensure perfect homogenisation between the two jars. 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. So, this specific system can be used to study indirect interactions without needing a sophisticated method to follow each dynamic population as in other studies (Moreno et al. 1991; Xufre et al. 2006). It is not suitable for direct interactions since the strains are cultivated separately.

Fermentations

Yeast inoculation was performed from active dry yeast rehydrated in liquid medium just before the beginning of the culture. Three types of inoculations were performed: inoculation of only one strain (named pure fermentation), simultaneous inoculation of *T. delbrueckii* and *S. cerevisiae* (named mixed fermentation) and inoculation of *T. delbrueckii* followed 48 h later by the inoculation of *S. cerevisiae* (named sequential fermentation). For all fermentations, 1.0×10^7 viable cells mL^{-1} of *T. delbrueckii* were inoculated. Pure fermentation of *S. cerevisiae* was performed with the inoculation of 5×10^6 viable cells mL^{-1}. For mixed and sequential fermentation, *S. cerevisiae* was also inoculated at 5×10^6 viable cells mL^{-1} giving a *T. delbrueckii* to *S. cerevisiae* ratio of 2, named the low ratio (T/S)L. Another ratio was tested with an experiment performed in the membrane bioreactor with simultaneous inoculation of 1.0×10^7 viable cells mL^{-1} of *T. delbrueckii* and 0.5×10^6 viable cells mL^{-1} of *S. cerevisiae*. In this case, the ratio of *T. delbrueckii* to *S. cerevisiae* was 20, which was named the high ratio (T/S)H.

All fermentations, pure, sequential and mixed, were performed in membrane bioreactor. For sequential and mixed fermentations, *S. cerevisiae* and *T. delbrueckii* were inoculated in different compartment of 2 L interconnected by the membrane whereas, for pure fermentations, the concerned strain was inoculated in only one compartment. Fermentations were considered in anaerobiosis since nitrogen flux was used for applied pressure in headspace vessels to ensure the flow and mix of medium. The temperature was 20 °C and a magnetic stir bar (250 rpm) was used. The samples were taken during the course of fermentation in each vessel to measure each population growth. Samples were then centrifuged at 11,500 rpm, at 4 °C, for 15 min and stored in the freezer at −20 °C until used for composition analyses. The AF was considered to have finished when 97 % of the initial sugar had been consumed.

Liquid was analysed systematically in each vessel to monitor 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. Each experiment was performed in triplicates. The results showed good reproducibility (differences less than the measured precision of the analysis method). Average values are presented in the results section.

Analysis

The number of cell concentrations of the two strains was determined using a Thoma haemocytometer. The percentage of viable cells was observed by means of methylene blue staining. The total sugars (glucose and fructose in this medium) were determined by using α-dinitrosalicylic reagent as described by Miller (1959). The total assimilable nitrogen was defined as the sum of the ammonium and L-α amino acid nitrogen contents analysed by two enzymatic methods: Microdom kit no 1100503700 for NH_4^+ measurement, and Microdom kit no 1101011000 for L-α amino nitrogen measurement. Acetic acid concentrations were also determined by enzymatic method: Boehringer Mannheim kit no 10148261035. The ethanol and glycerol concentrations were analysed by an HPLC-equipped BioRad Aminex HXP-87H column. The liquid phase was 5 mmol of sulphuric acid solution, which circulated at 0.4 mL min^{-1} at 40 °C. The volume of the injection loop was 20 μl. The peaks of ethanol and glycerol were detected by a refractometer.

The ester compounds were analysed by the company Lallemand S.A.S. with the method of Gas Chromatography

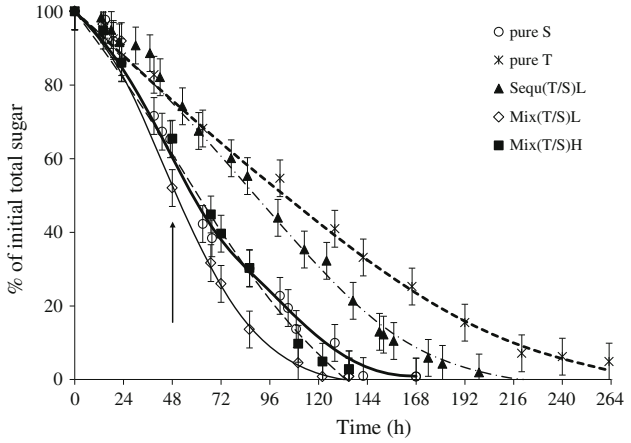

Fig. 1 Residual sugars (in percentage of initial concentration of 220 g L^{-1}) of pure, mixed and sequential cultures of *T. delbrueckii* and *S. cerevisiae* in MS300 at 20 °C. "pure S" is pure *S. cerevisiae* fermentation; "pure T" is pure *T. delbrueckii* fermentation; "Seq(T/S)L" stands for sequential fermentation with the low ratio; "Mix(T/S)L" for mixed fermentation with the low ratio and "Mix(T/S)H" for mixed fermentation with the high ratio of T/S. The *arrow* indicates the moment of inoculation of *S. cerevisiae* in the sequential fermentation

coupled with FID after a solid phase microextraction (SPME). The following compounds were measured: ethyl acetate, ethyl butyrate, isoamyl acetate, ethyl lactate, ethyl octanoate, ethyl decanoate and ethyl hexanoate.

Results

Pure fermentations of *T. delbrueckii* and *S. cerevisiae* in MS300

The kinetics of total sugar consumption expressed in % of the initial weighed total sugars (220 g L^{-1}) in Fig. 1 show that the two yeasts finished their fermentation well since the final concentrations were less than 3 % of the initial sugars (criterion for stopping the experiment) i.e. residual sugars less than 6 g L^{-1} for all fermentations. Nevertheless, *S. cerevisiae* presented a better speed of fermentation since it needed only 145 h (6 days) compared to 258 h (11 days) for *T. delbrueckii*. The viable cell kinetics of *S. cerevisiae* and *T. delbrueckii* are presented in Fig. 2a, b, respectively. *S. cerevisiae* reached the stationary phase after 2 days of fermentation whereas it was 4 days for *T. delbrueckii*. *S. cerevisiae* attained 300×10^6 viable cells mL^{-1}, against 4.3×10^8 viable cells mL^{-1} for *T. delbrueckii* (Table 1). The viability remained constant around 90 % for the two yeasts during the fermentation (data not shown). Ethanol production was approximately identical between the two yeasts with ethanol yields of 0.48

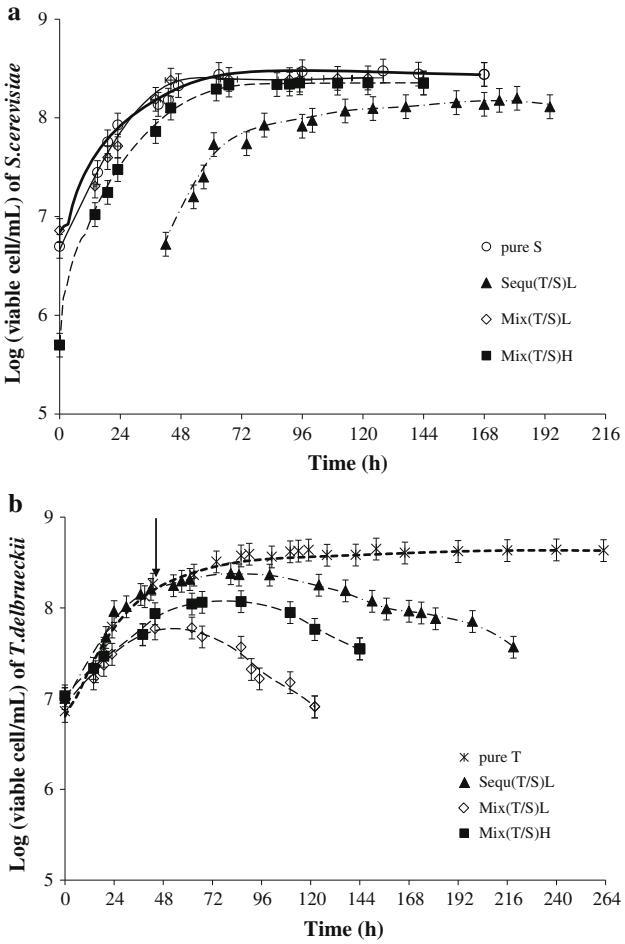

Fig. 2 Cell growth kinetics of viable *S. cerevisiae* (**a**) and *T. delbrueckii* (**b**) in their pure, mixed and sequential cultures in MS300 at 20 °C. "pure S" stands fr pure *S. cerevisiae* fermentation; "pure T" for pure *T. delbrueckii* fermentation; "Seq(T/S)L" for sequential fermentation with low ratio; "Mix(T/S)L" for mixed fermentation with low ratio and "Mix(T/S)H" for mixed fermentation with high ratio of T/S, and the *arrow* indicates the moment of inoculation of *S. cerevisiae* in the sequential fermentation

(13.3 vol%) and 0.49 g g^{-1} of sugars (13.6 vol%) for *S. cerevisiae* and *T. delbrueckii*, respectively. So, this strain of *S. cerevisiae* was more efficient for AF than the strain of *T. delbrueckii* since it produced the same quantity of products in a shorter time.

Final concentration of acetic acid which is highly correlated to volatile acidity in wine was lower with *T. delbrueckii* (0.1 g L^{-1}) than with *S. cerevisiae* (0.58 g L^{-1}). Concerning ester production, Table 2 shows that *T. delbrueckii*, compared to *S. cerevisiae*, produced more ethyl acetate, ethyl lactate and isoamyl acetate but less ethyl octanoate and ethyl decanoate. For ethyl butyrate and ethyl hexanoate, differences between the two strains were not significant since they are less than standard deviation.

Table 1 Principal characteristics of fermentations performed in membrane bioreactor, at 20 °C, 250 rpm with nitrogen flux in headspace of vessels, in MS300 or MS170 medium

Fermentation	Pure culture of *S. cerevisiae* MS300	Pure culture of *T. delbrueckii* MS300	Sequential fermentation (T + Ss)L MS300	Mixed fermentation with low ratio (T/S)L MS300	Mixed fermentation with high ratio (T/S)H MS300	Pure culture of *T. delbrueckii* MS170	Sequential fermentation (T + Ss)L MS170
Ethanol ($g\ L^{-1}$)	105.0 ± 3.2	108.2 ± 3.3	99.1 ± 3.0	101.1 ± 3.0	107.6 ± 3.2	101.4 ± 3.0	103.9 ± 3.1
Yield of ethanol[a] ($g\ g^{-1}$)	0.48	0.49	0.45	0.46	0.49	0.46	0.47
Maximum viable cells ($10^6\ mL^{-1}$) of *T. delbrueckii*	–	430 ± 52	240 ± 29	59 ± 7	119 ± 14	300 ± 36	115 ± 14
Maximum viable cells ($10^6\ mL^{-1}$) of *S. cerevisiae*	300 ± 36	–	150 ± 18	255 ± 31	226 ± 27	–	23 ± 3
Time of fermentation (h)[b]	145	258	192	114	128	295	402
Mean of sugar consumption rate ($g\ L^{-1}\ h^{-1}$)	1.52 ± 0.08	0.85 ± 0.04	1.15 ± 0.06	1.93 ± 0.1	1.72 ± 0.09	0.75 ± 0.04	0.55 ± 0.03
Residual nitrogen ($mg\ L^{-1}$)	0	25 ± 3	0	0	0	0	0
Final acetic acid ($g\ L^{-1}$)	0.58 ± 0.03	0.10 ± 0.01	0.26 ± 0.02	0.52 ± 0.03	0.45 ± 0.03	0.15 ± 0.01	0.31 ± 0.02

Values are means of three experiments ± standard deviations

[a] Gram of ethanol produced per gram of sugar consumed

[b] Time to consume 97 % of initial sugar in AF

Table 2 Final ester composition of fermentations performed in membrane bioreactor, at 20 °C, 250 rpm with nitrogen flux in headspace of vessels, in MS300 medium

Final concentration ($mg\ L^{-1}$)	Pure culture of *S. cerevisiae*	Pure culture of *T. delbrueckii*	Sequential fermentation (T + Ss)L	Mixed fermentation with low ratio (T/S)L	Mixed fermentation with high ratio (T/S)H	Odor threshold[a]
Ethyl acetate	72 ± 4.7	90 ± 5.8	52.8 ± 3.4	74 ± 4.8	74.5 ± 4.8	12.3
Ethyl butyrate	0.135 ± 0.009	0.135 ± 0.015	0.11 ± 0.012	0.08 ± 0.018	0.10 ± 0.014	0.02
Isoamyl acetate	<0.05	0.96 ± 0.06	0.5 ± 0.03	0.16 ± 0.03	0.26 ± 0.03	0.03
Ethyl lactate	<0.1	4.0 ± 0.3	3.35 ± 0.25	4.0 ± 0.26	5.3 ± 0.38	157.8
Ethyl octanoate	0.14 ± 0.009	<0.05	<0.05	<0.05	<0.05	0.005
Ethyl decanaote	2.615 ± 0.17	0.03 ± 0.01	0.055 ± 0.01	0.035 ± 0.01	0.02 ± 0.009	0.2
Ethyl hexanoate	0.05 ± 0.003	<0.05	<0.05	<0.05	<0.05	0.014

Values are means of three experiments ± standard deviations

[a] Values cited in Escudero et al. (2004)

Comparison between fermentations with the two strains in the same vessel and in separate vessels

The membrane bioreactor was designed to study indirect interactions between microorganisms since they were grown in separate vessels so that the dynamics of each population could be followed easily by microscopic counting. Nevertheless it was also possible to perform an experiment with inoculation of the two microorganisms in the same vessel and compare it with the separate-vessel experiment. In this case, *S. cerevisiae* and *T. delbrueckii* yeast cannot be distinguished by microscopic counting, only total cell population can be determined. The results obtained in terms of the total sugar consumption kinetics and total cell population are compared in Fig. 3 for the experiments performed with inoculation of the two yeasts in the same vessel and in separate vessels. Differences between the two experiments were always smaller than the

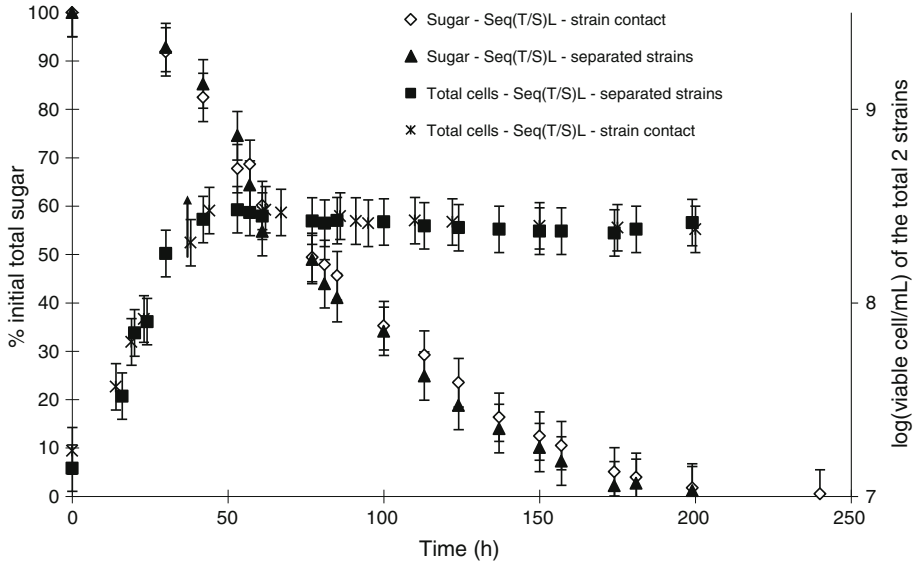

Fig. 3 Total sugar consumption and total cell growth kinetics in sequential fermentation with low ratio performed in MS300, at 20 °C, in BRM with inoculation of the two strains in the same vessel and in separate vessels

measurement error of the analysis method. So the behaviour was the same with an inoculation of *T. delbrueckii* and *S. cerevisiae* in the same vessel or in two separate vessels of the BRM.

Comparison of pure, mixed and sequential fermentations with low T/S ratio in MS300

The growth kinetics and the number of viable cells of *S. cerevisiae* reached in the stationary phase (Fig. 2a) were approximately the same for pure (3×10^8 viable cells mL^{-1}) and mixed (T/S)L fermentation (2.55×10^8 viable cells mL^{-1}) since the difference of 15 % is close to the maximal deviation of 12 % observed on the counting method. In sequential fermentation, the growth of *S. cerevisiae* occurred with a 48 h delay, due to the sequential inoculation, and the cell concentration attained was lower (1.5×10^8 viable cells mL^{-1}).

On the other hand, the concentration of viable cells of *T. delbrueckii* was affected by the presence of *Saccharomyces* (Fig. 2b) whereas it was constant in pure fermentation. In sequential fermentation, the viability of *T. delbrueckii* decreased rapidly when *S. cerevisiae* was inoculated after 48 h. After reaching 2.4×10^8 viable cells mL^{-1} at 48 h as in its pure culture, the number of viable cells decreased regularly to become only 5.0×10^7 viable cells mL^{-1} at the end of AF. In mixed (T/S)L fermentation, the maximum population of *T. delbrueckii* was only 5.9×10^7 viable cells mL^{-1} after 70 h and a decrease of the viability was then also observed.

The duration of the mixed (T/S)L AF (114 h) was logically shorter than that of the pure *S. cerevisiae* fermentation (145 h) since the *S. cerevisiae* concentration was the same as in the pure fermentation but the action of *T. delbrueckii* was added. The length of the sequential fermentation (192 h) was between the two pure fermentations (between 145 h and 258 h) since the action of *T. delbrueckii* was the same during the first 48 h, but the AF was then accelerated by the growth of inoculated *S. cerevisiae*. Thus, the time taken to reach 97 % sugar consumption in Fig. 1 was consistent with the observed populations.

Sequential fermentation presented a low acetic acid concentration compared to mixed fermentation. It was coherent with pure fermentations since *T. delbrueckii* reduced volatile acidity compared to *S. cerevisiae*. The ester composition obtained at the end of AF was different for the two experiments. Isoamyl acetate and ethyl decanoate concentrations were higher (×2 and ×1.5) in sequential fermentation, where *T. delbrueckii* grew before *S. cerevisiae* inoculation, than in the mixed fermentation. On the other hand, ethyl acetate concentrations were lower but differences between other products were not significant since they are inferior to standard deviation.

Influence of *T. delbrueckii* to *S. cerevisiae* inoculation ratio in MS300

To simulate the inoculation of *T. delbrueckii* in a real case where the grape must is not sterile, an experiment was carried out with a high inoculation ratio (20:1) of *Torulaspora/Saccharomyces*. The initial *S. cerevisiae* population of only 0.5×10^6 viable cells mL^{-1} represented the indigenous microorganism presence that can be found in natural grape must, even though the indigenous population can also be smaller in real cases. Results for fermentation kinetics and population growths are presented in Figs. 1 and 2. Sugar consumption occurred in 128 h, compared to 114 h for the experiment with the low *Torulaspora:Saccharomyces* ratio

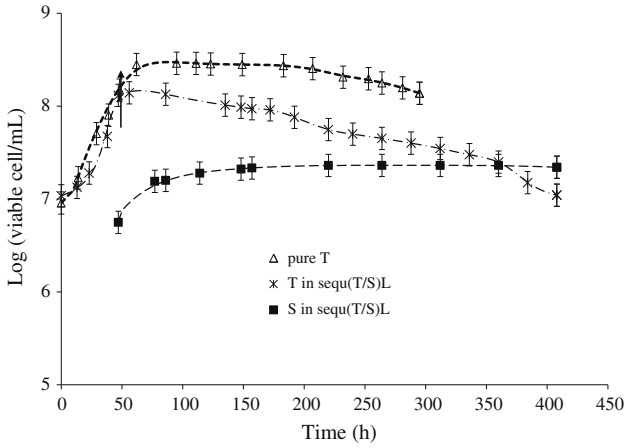

Fig. 4 Viable cell growth of pure and sequential cultures *T. delbrueckii* and *S. cerevisiae* in MS170 at 20 °C. "pure T" stands for pure *T. delbrueckii* fermentation; "T in Seq(T/S)L" for *T. delbrueckii* in sequential fermentation with low ratio; "S in Seq(T/S)L" for *S. cerevisiae* in sequential fermentation with low ratio; and the *arrow* indicates the moment of inoculation of *S. cerevisiae* in the sequential fermentation

of 2:1, and the ethanol production, acetic acid concentration, ester composition and *Saccharomyces* population were very close in the two experiments (Tables 1, 2). Besides, the difference between *T. delbrueckii* cell concentrations was significant since the maximum viable population attained 59×10^6 cells mL^{-1} for the low ratio compared to 1.19×10^8 cells mL^{-1} for high-ratio experiments. Thus, even with a high *T. delbrueckii* to *S. cerevisiae* inoculation ratio, the *T. delbrueckii* growth was weaker than in the pure culture.

Influence of nitrogen content of the grape must on sequential fermentation

The residual assimilable nitrogen concentrations measured at the end of all fermentations are presented in Table 1. For the experiment carried out on the MS300 medium, nitrogen was totally exhausted for the pure *S. cerevisiae* culture, the mixed and the sequential fermentation. Only for the pure *T. delbrueckii* culture was nitrogen not totally exhausted since 25 mg L^{-1} remained at the end of the AF. In the sequential fermentation, after 48 h, when *S. cerevisiae* was inoculated, the nitrogen concentration measured was 87 mg L^{-1} in MS300 and only 14 mg L^{-1} in MS170.

The fermentation with pure *T. delbrueckii* in MS170 was 37 h longer than for pure *T. delbrueckii* in MS300 (Table 1). This seems logical since the maximum population attained was only 3×10^8 cells mL^{-1}, as against 4.3×10^8 cells mL^{-1} for the MS300.

In sequential inoculation in MS170 experiments, the *S. cerevisiae* population attained only 2.3×10^7 cells mL^{-1}. For *T. delbrueckii*, growth was identical between pure *T. delbrueckii* fermentation and sequential fermentation for the first 48 h (Fig. 4). However, when *S. cerevisiae* was inoculated, even when its growth was very weak, the viability of *T. delbrueckii* fell from 1.15×10^8 cells mL^{-1} to 1.5×10^7 cells mL^{-1} in 400 h. Consequently, the AF time was longer than with pure *T. delbrueckii* in MS170, since it only finished after 400 h.

Discussion

In our study, with an initial sugar concentration of 220 g L^{-1}, *T. delbrueckii* could bring the fermentation to an end 11 days after its inoculation into the synthetic medium MS300 and 12 days after its inoculation into MS170. We also checked that the strain was able to exhaust 240 g L^{-1} of sugars (data not shown). So, this strain showed good ethanol tolerance as a non-*Saccharomyces* starter species, unlike what is reported in the literature for other *T. delbrueckii* strains with similar or higher sugar concentrations. For example, in a comparison of 21 strains, Renault et al. (2009) found that none of them were able to exhaust 240 g L^{-1} of sugars in similar conditions. Ciani et al. (2006) found incomplete fermentation by a *T. delbrueckii* strain on grape must containing 270 g L^{-1} of total sugars, with about 50 % of residual sugar.

In a medium with reduced nitrogen content (MS170), the duration of *T. delbrueckii* fermentation in pure culture was increased by 37 h compared to MS300 due to the presence of a lower viable biomass during the stationary phase although the rate growth was similar during the first 48 h. In MS300, the nitrogen consumption in the pure cultures was higher for *Saccharomyces* than for *T. delbrueckii*. This observation has also been reported by Bely et al. (2008) for other strains of *T. delbrueckii*, which always consumed less nitrogen than the *S. cerevisiae* strains tested.

As it has been observed by Ciani et al. (2006), *T. delbrueckii* is non-*Saccharomyces* yeast with lower volatile acidity production compared to *S. cerevisiae*. Moreover, *T. delbrueckii* produced more isoamyl acetate (banana, pear-drop aromas) than *S. cerevisiae*. It also produced more ethyl lactate (strawberry) but at a weak level compared to the odour threshold (Table 2). Production of ethyl octanoate, decanoate and hexanoate was insignificant as has already been described for this yeast by Renault et al. (2009).

In this study, the two yeasts were not affected in the same manner when they grew together. In mixed cultures, *Saccharomyces* could develop practically as well as in its pure culture, giving similar kinetics whatever the inoculation ratio, whereas *T. delbrueckii* growth was reduced by a

factor of four for the high ratio of the non-*Saccharomyces* strain and eight for the low ratio. After 24 h, *S. cerevisiae* became the dominant strain in both cases. Moreover, during the stationary phase, the viability of *S. cerevisiae* remained constant whereas that of *T. delbrueckii* decreased regularly. This was observed even when the *T. delbrueckii* strain was dominant at the outset: the high T/S ratio corresponded to 95 % of non-*Saccharomyces* yeast. In sequential fermentation MS300, *Saccharomyces* growth was lower than in pure *Saccharomyces* fermentation. A hypothesis is that, when *S. cerevisiae* was inoculated 48 h after *T. delbrueckii*, the nitrogen content of the must was not sufficient for the normal growth of *Saccharomyces*. In MS170, the sequential fermentation was twice as long as that in MS300. This rate decrease can be explained by the weak growth of *S. cerevisiae* compared to pure culture since only 2.3×10^7 cells mL^{-1} were produced. The inoculation of *Saccharomyces* took place after 48 h and the assimilable nitrogen was nearly exhausted (only 14 mg L^{-1}). So, the nitrogen content in MS170 was insufficient to reach the population obtained in MS300 but no decrease of viability for *S. cerevisiae* was observed. Another reason for the longer fermentation time in sequential MS300 or MS170 was that the viability of *T. delbrueckii* decreased strongly in the stationary phase of the mixed culture as soon as the *S. cerevisiae* were inoculated.

The difference of fermentation rate could be one of the reasons for the antagonism against *T. delbrueckii. Saccharomyces* grew more rapidly than *T. delbrueckii*, which had no time to increase its population. So competition could exist for various substrates: sugars, nitrogen and oxygen. Mauricio et al. (1998) and Hansen et al. (2001) suggested that the death of *T. delbrueckii* in mixed cultures at low available oxygen levels was due to the lack of this substance. Nevertheless, in our work all experiments were performed under nitrogen flow and anaerobiosis could not be incriminated in the decrease of *T. delbrueckii* viability during mixed and sequential culture since the viability always remained at a high level for the pure fermentations due to the presence of anaerobiosis factors. Moreover in a previous work we showed that this strain compensated the lack of oxygen when grown under nitrogen by producing more glycerol (Brandam et al. 2013).

However, in our case, the hypothesis of substrate competition cannot explain the phenomenon of *T. delbrueckii* death in sequential fermentation. The production of an unknown metabolite by *S. cerevisiae* that affected the viability of *T. delbrueckii* seems to be most probable. Our data seem to indicate a phenomenon of amensalism exerted by *Saccharomyces* towards *T. delbrueckii*. This kind of interaction was previously suggested by Farkas et al. (2005). Ciani et al. (2010) reported that some negative interactions between *Saccharomyces* and non-*Saccharomyces* could be due to acetaldehyde or acetoin. Some other authors suggest that killer toxin factors are responsible for amensalism towards non-*Saccharomyces* (Jacobs and Van Vuuren 1991; Zagorc et al. 2001; Pérez et al. 2006). Nevertheless, Renault et al. (2009) showed no inhibition effect of K2 toxins secreted by *S. cerevisiae* on any of the 17 *T. delbrueckii* wine-related strains tested and it is admitted that killer toxin is not active against *T. delbrueckii*. Recently, Albergaria et al. (2010) found some 2–10 kDa peptides produced by *S. cerevisiae* that inhibited the growth of *T. delbrueckii* wine-related strains. Consequently, the effect of antimicrobial peptides on *T. delbrueckii* development in the mixed or sequential cultures with *S. cerevisiae* need to be further investigated.

Nissen et al. (2003) proposed another mechanism to explain the *T. delbrueckii* growth arrest in mixed cultures with *S. cerevisiae*: the negative effect of cell-to-cell contact due to high space competition. In our case, since the two yeasts were separated by a hollow fibre membrane in the membrane bioreactor, this hypothesis was not plausible. Figure 3 clearly shows that the behaviour was the same with or without contact between the two different strains. So, a cell-to-cell contact mechanism cannot be responsible for the interactions observed.

In sequential cultures, only isoamyl acetate was increased with respect to the *S. cerevisiae* culture, whereas all esters of fatty acids were reduced. In mixed cultures, despite the low presence of *T. delbrueckii,* the same compounds were affected and isoamyl acetate production seemed to be proportional to the presence of *T. delbrueckii*. Moreno et al. (1991); Rojas et al. (2001) and Moreira et al. (2008) have previously shown more isoamyl acetate production in co-inoculation of *S. cerevisiae* with a non-*Saccharomyces* in wine fermentations. For ethyl acetate, concentrations below 70 mg L^{-1} are considered positive for the wine aroma (Rapp et al. 1992), but above 150 mg L^{-1}, it can create negative aromas, such as solvent-like ones (Jackson 1994)]. In our work, all of the fermented wines had the ethyl acetate at an acceptable level. Our results show that *T. delbrueckii* had an influence on ester production in mixed and especially sequential fermentations. Nevertheless, it is difficult to conclude on the benefits of sequential inoculation compared to co-inoculation as far as ester production is concerned since our work has been done in synthetic medium. Many investigations with sensorial analysis on grape musts will be necessary. On the other hand, for the growth and presence of each strain, it is clear that sequential inoculation is better than co-inoculation.

In conclusion, in mixed cultures or in the case of inoculation of *T. delbrueckii* in a grape must containing *S. cerevisiae* indigenous populations, the non-*Saccharomyces* yeast should always be overcome by *S. cerevisiae*, thus

decreasing the impact of *T. delbrueckii* on the fermented wine. Sequential inoculation guarantees the development of *T. delbrueckii* prior to the inoculation by *S. cerevisiae*. Nevertheless, in this case, the medium should contain enough assimilable nitrogen to allow the development of *S. cerevisiae* and a good fermentation rate.

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