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Mixed culture fermentation using *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* with direct and indirect contact: impact of anaerobic growth factors

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Abstract
The role of the initial concentration of anaerobic growth factors (AGF) on interactions between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* was investigated in strict anaerobiosis. Experiments were performed in a synthetic grape must medium in a membrane bioreactor, a special tool designed for studying direct and indirect interactions between microor-ganisms. In pure culture fermentations, increased AGF concentration had no impact on *S. cerevisiae* behaviour, whereas it induced an extension of *T. delbrueckii* latency. Surprisingly, *T. delbrueckii* used only 75 to 80% of the consumed sugar to produce biomass, glycerol and ethanol. Physical separation influenced the population dynamics of co-fermentations. *S.cerevisiae* dominated the co-cultures having a single dose of AGF as its presence indirectly induced a decrease in numbers of living *T. delbrueckii* cells and physical contact with *T. delbrueckii* stimulated *S.cerevisiae* growth. Increasing the AGF initial concentration completely upset this domination: *S. cerevisiae* growth was not stimulated and *T. delbrueckii* living cells did not decrease. Yeasts incorporate exogenous AGFs, which probably impact their response to competing yeasts. The increase in AGF might have induced changes in the lipid composition of the *T. delbrueckii* membrane, which would hinder its interaction with *S. cerevisiae* antimicrobial peptides. The initial concentration of anaerobic growth factors influenced co-culture fermentation population dynamics tremendously, thus highlighting a new way to monitor population evolution and eventually wine organoleptic properties.

Keywords *S. cerevisiae* · *T. delbrueckii* · Mixed-culture fermentation · Interactions · Growth anaerobic factors increase

Introduction

Wine fermentation is a widely studied process, which can involve pure or mixed yeast cultures. Usually, the yeasts are classified in two groups: *Saccharomyces* and non-*Saccharomyces* species. *Saccharomyces* species are the yeasts most commonly used in winemaking, since they are tolerant of high ethanol levels [1]. The use of non-*Saccharomyces* yeasts has been re-assessed, especially in mixed culture, because of their ability to enhance the analytical and aromatic profile of the wine and to reduce the alcohol content [2–5]. Among the non-*Saccharomyces* yeast species, attention has focused on *Torulaspora delbrueckii*, as this yeast has shown a positive impact on the organoleptic quality of wines and a low production of undesirable compounds, such as acetic acid, ethyl acetate, acetaldehyde, acetoin, hydrogen sulphide and volatile phenols that lessen off-flavours [6–8]. *T. delbrueckii* performs slow and sometimes incomplete alcoholic fermentation under winemaking conditions [4, 9, 10]. Co-fermentation alongside *S. cerevisiae* has been proposed to modulate wine flavour and to ensure complete alcoholic fermentation. Generally, death of *T. delbrueckii* occurs in co-cultures with *S. cerevisiae* [4, 11, 12]. However, the particular *T. delbrueckii* strain Kbarr-1 has been reported to dominate must fermentation in the presence of *S. cerevisiae* [13, 14]. In winemaking

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co-fermentation, population dynamics is influenced by yeast interactions. Three types of interactions have been described: indirect interactions, cell-to-cell contact and competition for space.

There are two sorts of indirect interactions: competition for a substrate and interaction mediated by a secreted component. In co-culture, yeasts spontaneously consume oxygen, sugar, nitrogen, vitamins and lipids, inducing a competition for these substrates. Antimicrobial peptides (AMPs) are secreted to kill a competitor microorganism. *S. cerevisiae* produces killer toxins that are active against other *S. cerevisiae* strains and occasionally non-*Saccharomyces* yeasts but not against *T. delbrueckii* [8, 15]. Some *S. cerevisiae* strains secrete another biocide, called saccharomycin, derived from the glycolytic enzyme GAPDH [16, 17]. This toxin is active against several wine-related non-*Saccharomyces* yeasts such as *T. delbrueckii, H. guilliermondii, K. marxianus* and *K. thermotolerans* GAPDH [16–18]. *T. delbrueckii* Kbarr strains produce a killer toxin (Kbarr-1) that is lethal to *S. cerevisiae* strains and some non-*Saccharomyces* yeasts [22]. In winemaking co-fermentation, non-*Saccharomyces* death is due to yeast interactions rather than nutrient depletion or the presence of toxic compounds [12, 19].

Alcoholic fermentation involves complex mechanisms, which can be affected by several elements of the must, including lipids. The bulk of the lipid in yeast membranes is in the form of fatty acids, phospholipids, sterols and sphingolipids [20]. Sterols and unsaturated fatty acids contribute to plasma membrane integrity, resistance to ethanol and the maintenance of a high ethanol production rate [21, 22]. Tween 80 is frequently used as a source of oleic acid in synthetic media. Under anaerobic conditions, yeasts cannot synthesise sterols or long-chain unsaturated fatty acids. The addition of lipid nutrients containing unsaturated fatty acids and sterols during alcoholic fermentation promotes cell growth in anaerobic conditions [23, 24]. By influencing the lipid composition of yeast cells [25], exogenous lipids affect membrane fluidity, membrane permeability and the activity of membrane-bound proteins [25–28].

Mixed culture fermentation of *S. cerevisiae* alongside *T. delbrueckii* has been the subject of considerable study [4, 11, 19, 29–32]. However, the effects of lipid concentration on interactions occurring between these yeasts have not been investigated. The aim of the present work was to acquire better knowledge about the influence of lipids (anaerobic growth factors) on the behaviour of *S. cerevisiae* and *T. delbrueckii* in mixed culture wine fermentation. Anaerobic co-cultures were undertaken in a double compartment reactor frequently used to study mixed culture fermentation [4, 33–35]. Co-cultures were performed with and without physical separation of the yeasts to evaluate the impact of anaerobic growth factors on indirect and direct interactions.

Materials and methods

Microorganisms and inoculum cultures

Two commercial oenological yeasts were used in this study. *Torulaspora delbrueckii* Zymaflore alpha was supplied by Laffort S.A.S., France, and *Saccharomyces cerevisiae* QA23 was supplied by Lallemand S.A.S., France. The yeasts were maintained on sterile YPD agar slant [yeast extract 1% (w/v), peptone 1% (w/v), glucose 2% (w/v) and agar 2% (w/v)] at 4 °C. When the inoculation cultures were prepared, each yeast was transferred from the agar slant to a flask containing 50 mL of YPD [yeast extract 1% (w/v), peptone 1% (w/v), glucose 2% (w/v)]. The yeasts were incubated at 25 °C with agitation (130 rpm) for 11 h. Subsequently, they were transferred to an Erlenmeyer flask containing 300 mL of YPD to give an initial concentration of 5.10^6 cells/mL and incubated with agitation (130 rpm) at 25 °C for 16 h.

Medium

Two synthetic media, MS300 and MS300M were used in this study. This type of medium is regularly used in oenological studies [4, 36]. Their composition was designed to be close to that of white grape must and to avoid limitations of carbon, nitrogen, vitamins and mineral elements during the yeast growth (the number 300 represents the approximated concentration in mg/L of assimilable nitrogen). They contained (per litre): glucose, 110 g; fructose, 110 g; L-malic acid, 6 g; citric acid, 6 g; mineral salts (KH_2PO_4, 750 mg; K_2SO_4, 500 mg; $MgSO_4{\cdot}7H_2O$, 250 mg; $CaCl{\cdot}2H_2O$, 155 mg; NaCl, 200 mg; $MnSO_4{\cdot}H_2O$, 4 mg; $ZnSO_4{\cdot}7H_2O$ 4 mg; $CuSO_4{\cdot}5H_2O$, 1 mg; KI, 1 mg; $CoCl_2{\cdot}6H_2O$, 0.4 mg; H_3BO_3, 1 mg; and $(NH_4)_6Mo_7O_{24}$, 1 mg); vitamins (myo-inositol, 20 mg; nicotinic acid, 2 mg; calcium pantothenate, 1.5 mg; thiamin-HCl, 0.25 mg; pyridoxine–HCl, 0.25 mg; and biotin, 0.003 mg). The nitrogen was brought by ammonium ions, NH_4Cl, and a concentrated solution of 19 α-amino acids. The composition of the concentrated amino acid solution was (per litre 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, and proline 46.8 g. The ammonium salts and α-amino acids (all amino acids except proline) in the medium were considered as assimilable nitrogen [36]. Both media contained the equivalent of 324 mg N/L that was assimilable by yeasts (204 mg N/L

from the amino acid solution corresponding to 13.09 mL/L of amino acid solution, and 120 mg N/L from NH_4Cl, corresponding to 0.46 g/L). Medium MS300 contained anaerobic growth factors [ergosterol (15 mg/L), oleic acid (0.5 µL/L) and 1 mL/L of a Tween80/ethanol solution (1:1, v/v)]. The concentration of anaerobic growth factors (AGF) was twice as high in MS300M. The pH of each medium was adjusted to 3.3 before autoclaving for 15 min at 121 °C. Vitamins were filtered and added after the thermal treatment.

Fermentation

Single-culture fermentation (SCF), mixed-culture fermentation (MCF) and compartmented-culture fermentation (CCF) were performed in a two-compartment membrane bioreactor (MBR) with *S. cerevisiae* and *T. delbrueckii*. The system was 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 allowed the medium, but not the microorganisms, to pass through the fibres. By applying pressure in the headspace of each of the vessels alternately, the medium was made to flow and mix. Compressed, filter-sterilized nitrogen was used to apply the pressure and a system of valves controlled its admission and expulsion according to the liquid levels. The time and quantity of liquid inversion were calculated to ensure perfect homogenization between the two jars. The complete MBR system is described in detail in [34, 37]. The inoculation volume was calculated to initially reach 5×10^5 and 10^7 cells/mL, respectively, for *S. cerevisiae* and *T. delbrueckii* in the MBR. In CCF, the membrane was used and each strain was inoculated into only one compartment. In SCF and MCF, the membrane was removed, allowing microorganisms to pass into the two compartments, and the inoculated volume was equitably distributed between the two jars. All fermentations were carried out at 20 °C under magnetic agitation (250 rpm). The nitrogen pressure was applied alternately 16 h before the inoculation, ensuring that all the dissolved oxygen was removed from the medium and thus providing strict anaerobic conditions. All fermentations were performed in duplicate. The time of fermentation corresponded to the time needed to consume 97% of the sugar initially present.

Analytical methods

Analysis of growth

Growth of the yeasts was determined by cell counting, using a Thoma haemocytometer. Samples were withdrawn throughout the fermentations and diluted appropriately in NaCl solution 0.9% (w/v). The percentage of living cells was observed by means of methylene blue staining. Growth of the yeasts was also determined by plate counting. Wallerstein laboratory nutrient agar (WLN from Sigma–Aldrich) was used for global counting. WLD (WLN + cycloheximide 2 $mg.L^{-1}$) was used to count only *T. delbrueckii. S. cerevisiae* colony-forming units (CFUs) were obtained by subtracting *T. delbrueckii* CFUs from global CFUs. The plates were incubated at 27 °C and CFU were counted after 2 and 4 days for WLN and WLD, respectively. A correlation between cells and CFUs was established for each yeast in SCFs and CCFs, and was used to estimate the cell counts in MCFs. Cell dry weight was determined by filtering 10 mL culture samples through pre-weighed 0.45 µm cellulose acetate filters. After being washed with 0.9% NaCl, the filters were dried to constant weight at 105 °C under vacuum.

Metabolites analysis

Samples were filtrated (0.45 µm), diluted ten times and kept at − 20 °C until analysis. Metabolite concentrations were determined using high-performance liquid chromatography (Thermo Scientific, France) with a Rezex ROA-Organic acid H+ (8%), 250 × 4.6 mm phase-reverse column (Phenomenex, France). The column was eluted with a degassed mobile phase containing 10 mM sulfuric acid, at 30 °C and at a flow rate of 0.170 mL/min. The injection loop volume was 25 µL. Two detectors were connected in series. Malic, citric, succinic and acetic acid peaks were detected by an ultraviolet detector (Accela PDA detector, Thermo Scientific, France) located after the column. Glucose, fructose, ethanol and glycerol peaks were detected by a refractive index detector (Finnigan Surveyor RI Plus detector, Thermo Scientific, France) located at the end of the loop. Ethanol yield was defined as the quotient of the mass of ethanol formed divided by the mass of glucose consumed.

Yeast available nitrogen

Amino acid and ammonium chloride were the two sources of yeast assimilable nitrogen (YAN). Amino acid nitrogen was measured using an o-Phthaldialdehyde/N-Acetyl-L-Cysteine assay (K-PANOPA; Megazyme International Ireland Ltd). Free ammonium salts were measured by an enzymatic assay (K-AMIAR; Megazyme International Ireland Ltd).

Carbon fraction in biomass

Samples were centrifuged for 5 min at 10,000 rpm. The supernatant was thrown away and the precipitate was kept, washed with ultrapure water, mixed and centrifuged. These operations were performed three times and the final precipitate was dried under vacuum in an oven at 100 °C for 2 days. The carbon fraction in the biomass was determined through

dry combustion, using a PERKIN ELMER 2400 element analyser [38].

Carbon balance calculation

The carbon balance was calculated considering carbon in biomass, sugars, ethanol, glycerol and CO_2. CO_2 concentration was estimated by considering that it was produced with the same stoichiometry as ethanol during fermentation. At some stages of some fermentations, the carbon was present in components not detectable by our analytical system, resulting in an undetermined carbon fraction.

Statistical analysis

The data are presented as mean values with their standard deviation. Statistical analyses were performed using the RStudio program [39]. After the verification of variance homogeneity (Fisher test, $p > 0.05$), one-way analysis of variance (ANOVA) ($p < 0.05$) was used to determine statistically significant differences between modalities. In the text, it appears as no significant differences between the mean values compared (if p value is upper than 0.05) or significant differences (if p value is lower than 0.05).

Results

Effect of anaerobic growth factors on single-culture fermentation (SCF)

SCFs were performed in MS300 and MS300M. AGF augmentation did not modify *S. cerevisiae* growth, sugar uptake or ethanol production profiles (Fig. 1a, b). Moreover, the final glycerol concentrations were similar (≈8 g/L). In the range tested, an increase of AGF had no significant impact on *S. cerevisiae* behaviour in anaerobic fermentation.

Unexpectedly, an AGF increase induced an extension of the *T. delbrueckii* latency phase up to 50 h (Fig. 2a). The resulting sugar consumption and ethanol production were slower than those observed in SCF with AGF single dose (Fig. 2b). In MS300, *T. delbrueckii* did not completely consume the YAN. After AGF increase, despite the slow YAN assimilation, no residual nitrogen compound was observed (Fig. 2a). In *T. delbrueckii* SCF, such AGF augmentation induced a significant increase ($p < 0.05$) in the final glycerol concentration from 9.4 to 15.5 g/L.In both media, *S. cerevisiae* fermentation time was shorter than that of *T. delbrueckii*, in accordance with several studies on this specific pair of yeasts [4, 11].

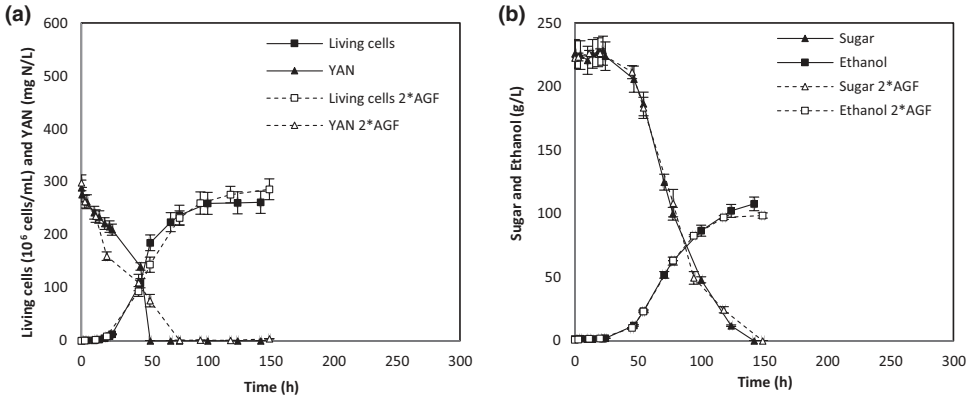

Fig. 1 Effect of increase in anaerobic growth factors on *S. cerevisiae* SCF: **a** Living cells and YAN profiles, **b** sugar and ethanol profiles. Vertical bars represent the standard deviations

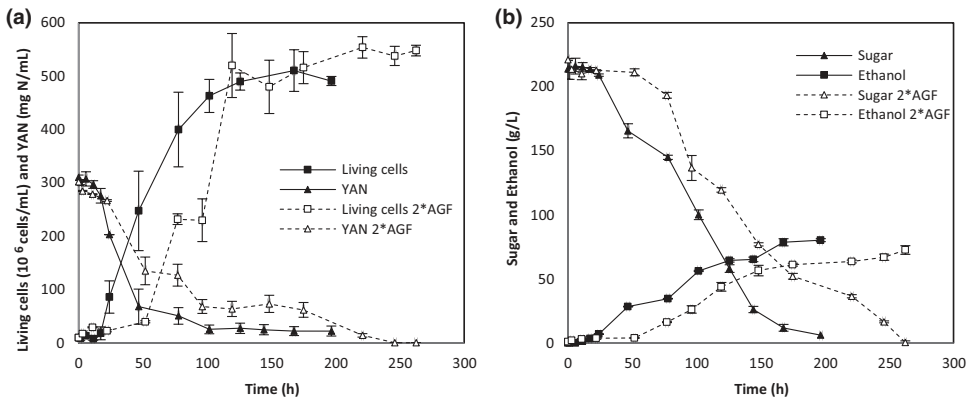

Fig. 2 Effect of growth anaerobic factors increase on *T. delbrueckii* SCF: **a** Living cells and YAN profiles, **b** sugar and ethanol profiles. Vertical bars represent the standard deviations

Co-cultures with a single dose of anaerobic growth factors

SCFs, MCFs and CCFs were performed in MS300. In all conditions, the maximum concentration of living *S. cerevisiae* cells was close to 280 × 10^6 cells/mL (Fig. 3a). The presence of *T. delbrueckii* did not impact the maximum population reached by *S. cerevisiae*. Regarding the first 50 h in SCF and CCF, *S. cerevisiae* growth rates were similar to and lower than those in MCF (Fig. 3a). Consequently, direct contact stimulated *S. cerevisiae* growth whereas indirect contact did not. Compared to SCF, the maximum population and the final viability of *T. delbrueckii* were lower in CCF. In CCF, between 50 and 100 h, the number of *T. delbrueckii* living cells decreased by 48% (Fig. 3a). *S. cerevisiae*, therefore, had an indirect toxic action on *T. delbrueckii*. Such a

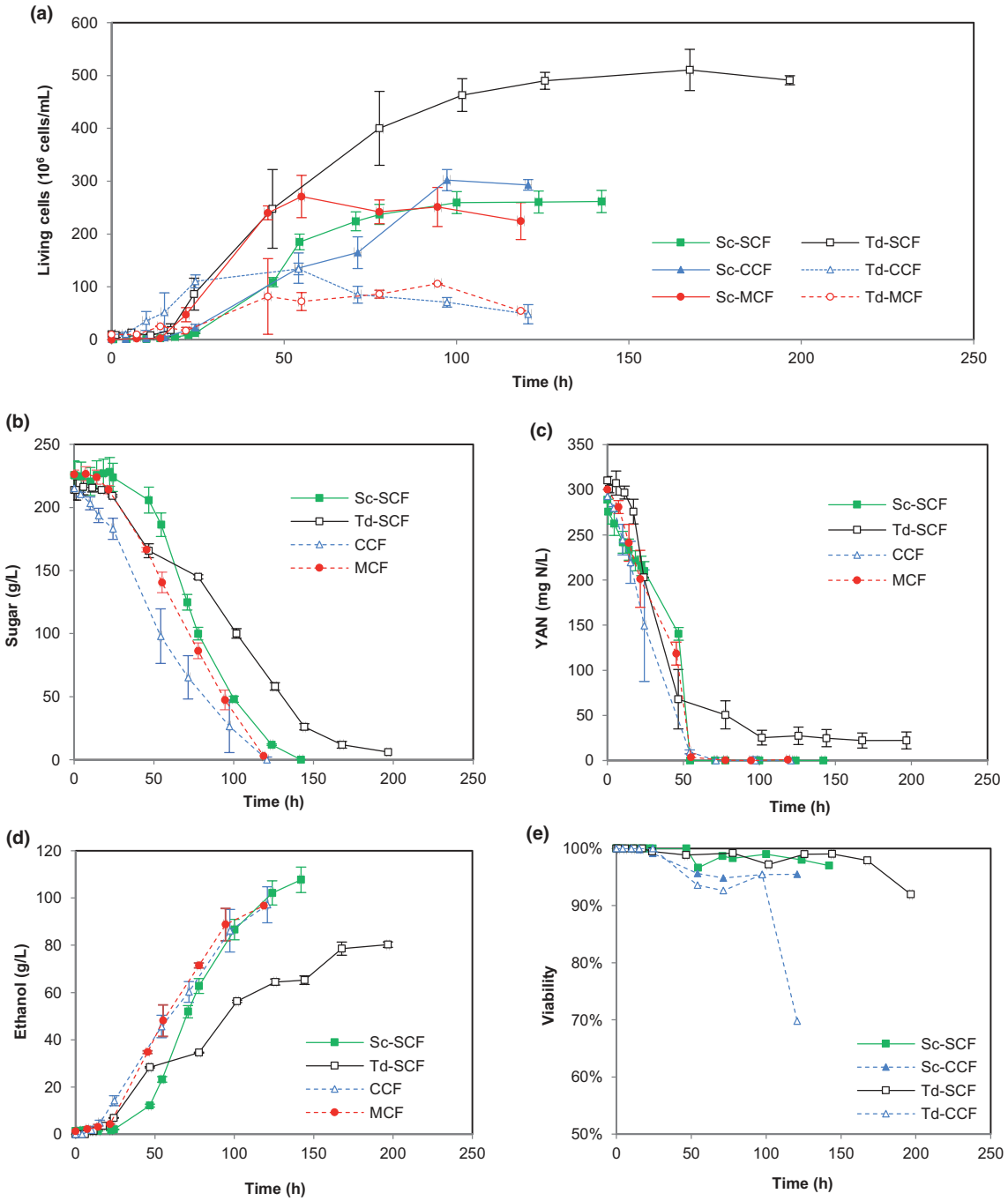

Fig. 3 Living cells (**a**), sugar (**b**), YAN (**c**), ethanol (**d**), and yeasts viability (**e**) profiles in SCF, CCF and MCF performed with MS300. Data are mean values of two fermentations. Vertical bars represent the standard deviations

significant decrease ($p < 0.05$) was associated with a stable viability (Fig. 3e).

The maximum number of living *T. delbrueckii* cells reached 510, 133 and 106 × 10^6 cells/mL in SCF, CCF and MCF, respectively (Table 1). *S. cerevisiae*'s presence, therefore, induced a drastic reduction of the *T. delbrueckii* maximum population. Nevertheless, no significant differences were observed between direct and indirect contact ($p > 0.05$). Consequently, *S. cerevisiae* did not perform any cell-to-cell contact. Despite the initial *T. delbrueckii* population being 20 times higher, *S. cerevisiae* living cells became predominant after 55 and 21 h in CCF and MCF, respectively. Physical contact thus promoted *S. cerevisiae* domination in MS300.

At the very end of the fermentation, with high ethanol level and sugar impoverishment, in CCF, *T. delbrueckii* viability fell sharply to 70% (Fig. 3e), that of *S. cerevisiae* remained high (95%), and only in MCF were aggregated stained cells observed. The aggregated stained cells were smaller than the scattered, unstained cells.

Compared to SCFs, sugar was consumed faster in co-culture (Fig. 3b; Table 1). Fermentation time observed in CCF and MCF were not significantly different ($p > 0.05$). Except in *T. delbrueckii* SCF, YAN was completely consumed in less than 60 h (Fig. 3c). Ethanol concentrations reached in CCF, MCF and *S. cerevisiae* SCF were similar (≈ 100 g/L) and significantly higher ($p < 0.05$) than that in *T. delbrueckii* SCF (80 g/L) (Fig. 3d). The characteristics of all fermentations performed in MS300 are summed up in Table 1.

Co-cultures with a double dose of anaerobic growth factors

In MS300M, the maximum number of living *S. cerevisiae* cells reached 286, 129 and 61 × 10^6 cells/mL for SCF, CCF and MCF, respectively (Fig. 4a). *T. delbrueckii*'s presence thus considerably reduced the *S. cerevisiae* maximum population. *S. cerevisiae* growth was more impacted when both yeasts where in direct contact. Unlike in SCF, *T. delbrueckii* did not present any extended latency phase in co-cultures with a double dose of AGF. Compared to SCF, the maximum concentration of *T. delbrueckii* living cells was lower in co-cultures. It can thus be concluded that the presence of *S. cerevisiae* hindered *T. delbrueckii* growth. Regarding co-cultures, when the two yeasts were not separated, *T. delbrueckii* reached a higher maximum population and, in parallel, *S. cerevisiae* reached a lower maximum population (Fig. 4a). Moreover, for both yeasts, before 50 h of fermentation, the growth rate observed in CCF was not significantly different from that observed in MCF. Each yeast had a different maximum population, although YAN profiles in CCF and MCF were similar (Fig. 4c).

S. cerevisiae completed SCF faster than *T. delbrueckii*. Paradoxically, MCF, in which *T. delbrueckii* was preponderant, presented a higher mean sugar consumption rate than CCF (Fig. 4b). Direct contact thus enhanced the ability of the pair of yeasts to consume sugar. At the end of the CCF, *T. delbrueckii* viability suddenly fell and *S. cerevisiae* viability remained high—in the same way for CCF in MS300 (Figs. 3e, 4e). Nevertheless, no aggregation was observed in MCF in MS300M, unlike in MS300. The characteristics of all fermentations performed in MS300M are summed up in Table 2.

Carbon balance

It should be stressed that CO_2 was not measured but its production was estimated by assuming the same stoichiometry as that of ethanol during fermentation. This assumption was adequate to describe co-cultures and

Table 1 Final parameters of fermentations performed in membrane bioreactor for single-culture fermentation (SCF) of *S. cerevisiae* and *T. delbrueckii*, mixed-culture fermentation (MCF) and compartmented-culture fermentation (CCF)

	SCF		CCF	MCF
	S. cerevisiae	*T. delbrueckii*		
Maximum living cells of *T. delbrueckii* (10^6 cells/mL)	–	510 ± 9	133 ± 11	106 ± 1
Maximum living cells of *S. cerevisiae* (10^6 cells/mL)	262 ± 27	–	302 ± 20	271 ± 40
Time of fermentation (h)	142 ± 7	197 ± 10	121 ± 7	118 ± 8
Initial sugar (g/L)	225 ± 4	222 ± 4	217 ± 3	226 ± 3
Mean sugar consumption rate (g/L/h)	1.58 ± 0.03	1.12 ± 0.02	1.79 ± 0.02	1.89 ± 0.03
Final ethanol (g/L)	107.7 ± 1.3	80.2 ± 1.2	97.1 ± 5.9	102.7 ± 0.8
Ethanol yield (g/g)	0.48 ± 0.01	0.35 ± 0.02	0.45 ± 0.02	0.46 ± 0.01
Glycerol (g/L)	7.9 ± 0.3	9.4 ± 0.2	7.9 ± 0.7	7.9 ± 0.5
Final undetermined carbon fraction	1%	20%	5%	4%
Residual nitrogen (mg N/L)	0	22.3 ± 10	0	0

Cultures were performed at 20 °C, 250 rpm with nitrogen flux in headspace of vessels, in MS300 medium

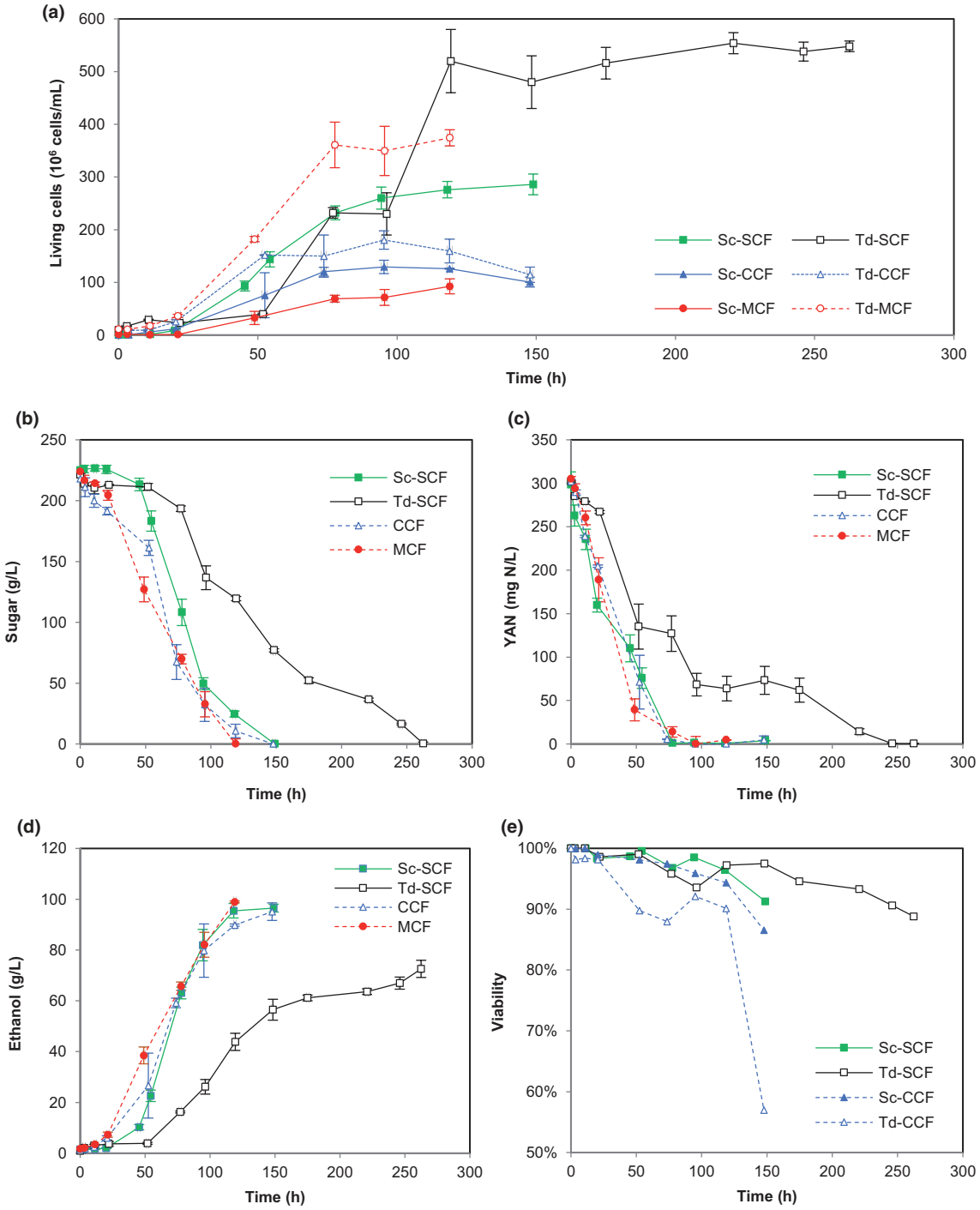

Fig. 4 Living cells (**a**), sugar (**b**), YAN (**c**), ethanol (**d**), and yeasts viability (**e**) profiles in SCF, CCF and MCF performed with MS300M. Data are mean values of two fermentations. Vertical bars represent the standard deviations

S. cerevisiae SCFs (Fig. 5a, b). In *T. delbrueckii* SCFs, no unbalance was observed during a long fermentation period (≈ 100 h), then the carbon unbalance increased progressively until the end of the fermentation (Fig. 5c, d). Final carbon unbalances higher than 20% were observed

Table 2 Final parameters of fermentations performed in membrane bioreactor for single-culture fermentation (SCF) of *S. cerevisiae* and *T. delbrueckii*, mixed-culture fermentation (MCF) and compartmented-culture fermentation (CCF)

	SCF		CCF	MCF
	S. cerevisiae	*T. delbrueckii*		
Maximum living cells of *T. delbrueckii* (10^6 cells/mL)	–	554 ± 20	181 ± 17	374 ± 16
Maximum living cells of *S. cerevisiae* (10^6 cells/mL)	286 ± 19	–	129 ± 2	61 ± 15
Time of fermentation (h)	149 ± 8	263 ± 8	148 ± 1	119 ± 7
Initial sugar (g/L)	223 ± 3	221 ± 1	218 ± 3	224 ± 1
Mean sugar consumption rate (g/L/h)	1.50 ± 0.02	0.84 ± 0.01	1.47 ± 0.02	1.88 ± 0.01
Final ethanol (g/L)	98.6 ± 2.1	72.6 ± 3.4	95.1 ± 3.5	99.0 ± 0.4
Ethanol yield (g/g)	0.44 ± 0.01	0.32 ± 0.02	0.44 ± 0.02	0.44 ± 0.01
Glycerol (g/L)	8.2 ± 0.3	15.5 ± 0.4	10.0 ± 0.6	11.6 ± 0.5
Final undetermined carbon fraction	7%	25%	8%	6%
Residual nitrogen (mg N/L)	0	0	4.7 ± 4.5	0

Cultures were performed at 20 °C, 250 rpm with nitrogen flux in headspace of vessels, in MS300M medium

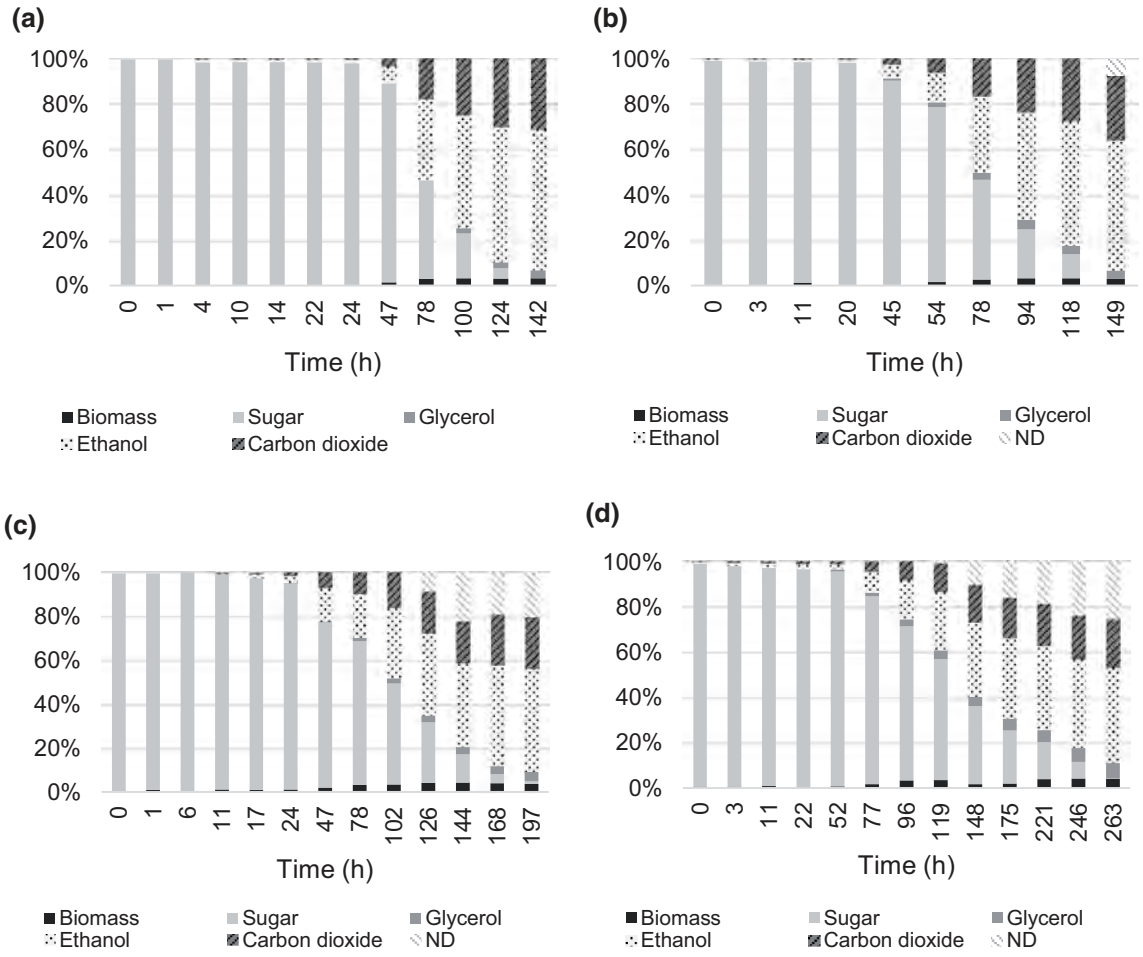

Fig. 5 Carbon mass partition in single culture fermentations. *S. cerevisiae* in MS300 (**a**) and MS300M (**b**) and *T. delbrueckii* in MS300 (**c**) and MS300M (**d**). ND represents the carbon fraction not determined

in *T. delbrueckii* SCFs, with no significant differences observed between AGF doses ($p > 0.05$).

Discussion

In this work, SCF, CCF and MCF of *S. cerevisiae* and *T. delbrueckii* were undertaken with different concentrations of anaerobic growth factors to evaluate their effect on interactions between yeasts. By doubling the AGF dose, ergosterol, oleic acid and Tween 80 concentrations rose from 15 to 30 mg/L, from 0.5 to 1 µL/L and from 0.5 to 1 mL/L, respectively.

T. delbrueckii latency phase extension induced by AGF

A residual YAN concentration subsisted in *T. delbrueckii* SCF with a single dose of AGF. Since we suspected that a component in the medium was limiting, the dose of AGF was doubled to see whether *T. delbrueckii* could consume all the YAN available. After the increase of AGF initial concentration, *T. delbrueckii* could consume all the YAN available. However, *T. delbrueckii* displayed an extended latency phase after the AGF increase, suggesting that the raised AGF concentration was toxic for *T. delbrueckii* growth. The 65% increase in the final glycerol concentration confirms that *T. delbrueckii* cells were more stressed after the AGF concentration was doubled. Unlike SCF, *T. delbrueckii* did not display any extended latency phase in co-cultures with a double dose of AGF. This result strongly suggests that *T. delbrueckii* absorbed a lower amount of AGF than the toxic dose when competing with *S. cerevisiae* present in the medium. By competing with *S. cerevisiae* for AGF, *T. delbrueckii* incorporates less AGF and the AGF concentration did not reach a level that was toxic for *T. delbrueckii*. Ergosterol is the predominant sterol in yeast cell membranes [40]. It is implicated in endocytosis and membrane curvature formation [41, 42]. Ergosterol influences the diffusion of proteins through the yeast plasma membrane [43]. Incorporation of exogenous lipids inside the membranes is expected to modify the hydrophobic lipid–lipid and lipid–protein interactions. Therefore, the spatial organization of membranes and the biological function of membrane-bound enzymes, such as those involved in the transport systems, could be indirectly affected [44]. An upset of the transport systems is consistent with the low YAN uptakes observed during the *T. delbrueckii* extended latency phase (Fig. 4c in comparison with Fig. 3c).

T. delbrueckii sugar metabolism in strict anaerobiosis

In all fermentations, nitrogen pressure was applied alternately starting 16 h before the inoculation and continuing throughout the fermentation, to ensure that no dissolved oxygen remained in the medium and thus that conditions were strictly anaerobic. In *T. delbrueckii* SCFs, the carbon balance showed that between 75 and 80% of the sugar consumed was used for biomass, glycerol and ethanol production. In conditions that were not strictly anaerobic, with a single dose of AGF, *T. delbrueckii* rapidly consumed the available dissolved oxygen; sugar was completely consumed and entirely used for biomass, glycerol and ethanol production (data not shown). The same *T. delbrueckii* strain growing, in non-strict anaerobiosis, in Sauvignon Blanc grape must, did not show any carbon unbalance [45]. Consequently, carbon unbalance occurs only in strict anaerobiosis. It indicates the existence of a biochemical reaction using sugar as the substrate and producing CO_2 or a metabolite different from ethanol or glycerol. This reaction is triggered after a long fermentation period and does not depend on the initial concentration of AGF. Must needs to be investigated to identify this biochemical reaction. However, such a reaction does not lead to malic, citric, acetic or succinic acid production and is absent in co-culture.

Effects of lipids on yeast behaviour

In this study, *S. cerevisiae* and *T. delbrueckii* showed different behaviour regarding AGF increase in the medium. AGF augmentation had no impact on *S. cerevisiae* SCF, whereas it induced an extended latency phase during *T. delbrueckii* SCF. Mauricio *et al*., observed that the supplementation of grape must with 25 mg/L of ergosterol and 31 mg/L of oleic acid enhanced the fermentation rate of *S. cerevisiae* E-1 and *T. delbrueckii* M1-20-4 [46]. In the same study, the supplementation of grape must with only 25 mg/L of ergosterol enhanced the fermentation rate of *S. cerevisiae*, whereas it reduced that of *T. delbrueckii*. In our case, 30 mg/L of ergosterol, 1 µl/L of oleic acid and 1 mL/L of tween were added (double dose). Since the initial lipids concentration in the grape must was not mentioned, it is not possible to compare with the lipid concentrations used in the present work. However, it appears that the effects of lipid increase on growth and fermentation performance depends on the nature of the lipid mixture, the yeast genus and the medium composition.

Physical contact and competition

S. cerevisiae dominated all co-cultures with a single dose of AGF. In presence of *T. delbrueckii, S. cerevisiae* reached a maximum population comparable to that observed in SCF.

However, when both yeasts were in direct contact (MCF), *S. cerevisiae* presented an increase of growth rate at the beginning of the fermentation. Recently, Tronchoni et al. analysed the transcriptional response to direct contact co-cultivation of *S. cerevisiae* and *T. delbrueckii* [47]. Early changes in the transcription pattern suggested a stimulation the growth and glycolytic activity of both yeasts as a consequence of the presence of competition in the same medium, with a delayed response of *T. delbrueckii*. Secreted Hsp12, which is involved in the coordinated response of cells in multi-strain cultures of *S. cerevisiae*, was suspected be related to this early response [48]. Since *S. cerevisiae* growth was not stimulated when the yeasts were separated by a membrane in the present study, physical contact is believed to play a role in *S. cerevisiae* response to *T. delbrueckii* presence. Unlike in the last cited work, *T. delbrueckii* early growth was not stimulated by the presence of *S. cerevisiae* in our study. It should be stressed that the inoculation ratio of *T. delbrueckii*/*S. cerevisiae* was 20/1 in the present work, while it was 1/1 in the cited work. The strain used was also different.

S. cerevisiae completed SCF faster than *T. delbrueckii*. In MS300M, paradoxically, MCF, in which *T. delbrueckii* was preponderant, presented a mean sugar consumption rate that was higher than that of CCF. This observation is reminiscent of a stimulation of glycolytic activity observed by Tronchoni et al. when the two yeasts were in physical contact [47].

In a study involving *S. cerevisiae* and *H. guilliermondii* focusing on yeast interactions at molecular level in natural grape must, it was found that *GAP1, AGP1, MEP1* and *MEP2*, encoding amino acid transporters for the first two and ammonium permease for the last two, were more highly expressed in *S. cerevisiae* in the single culture, than in the mixed culture [50]. Yeasts were not separated by a membrane. The reduction of the expression of such genes responsible for YAN assimilation could explain the suspected reduction of YAN assimilation by *S. cerevisiae* in MS300M. This phenomenon appears to be induced by physical contact with a non-*saccharomyces* yeast.

AGF influence on *S. cerevisiae amensalism*

In MS300, *S. cerevisiae* induced death of *T. delbrueckii* by indirect interaction and so dominated co-cultures. The decrease of *T. delbrueckii* concentration while the viability values remained stable led us to suspect that *T. delbrueckii* cells undergoing *S. cerevisiae* amensalism were lysed too rapidly to be coloured by methylene blue. Moreover, at the end of fermentation in MCF, aggregated cells were observed and the aggregated stained cells were smaller than the scattered unstained cells. Since *T. delbrueckii* has a smaller cell size than *S. cerevisiae* [50], we suppose that *T. delbrueckii* dead cells were aggregated and living *S. cerevisiae* were scattered. In consequence, direct contact with *S. cerevisiae* would cause aggregation of dead *T. delbrueckii* cells. Such aggregation was not observed in MS300M.

AGF augmentation completely upset *S. cerevisiae* domination. Several authors have shown that *S. cerevisiae* amensalism against non-*Saccharomyces* yeasts is mediated by AMPs [16, 17, 51]. The toxicity of AMPs depends on their concentration [52]. Albergaria et al. observed that *S. cerevisiae* continuously secretes AMPs from the second day to end of the fermentation [16]. Consequently, we supposed that the AMP concentration was sufficient to induce *T. delbrueckii* death in co-cultures with the double dose of AGF. However, after the AGF increase, *T. delbrueckii* growth was not impeded, pointing out that *S. cerevisiae* AMPs were less efficient.

The death mechanisms induced by these AMPs on sensitive yeasts involve cell membrane permeabilization [51]. Most AMPs induce death of sensitive cells by interacting with cell membranes and permeabilizing them [53, 54]. Interaction between sensitive cell membrane and AMPs is the first step of the toxic activity [55]. The lipid composition of the sensitive cell membrane plays a primordial role in this interaction [56]. Cells that are resistant to permeabilization induced by AMPs have a membrane lipid composition different from that of sensitive cells [57]. Since incorporated exogenous lipids modify membrane lipid composition, we assumed that *T. delbrueckii* growing in MS300 and MS300M had a different membrane lipid composition. We hypothesize that *T. delbrueckii* membrane lipid composition did not allow an efficient interaction with AMPs, thus avoiding their toxic activity during co-cultures in MS300M.

To the best of our knowledge, this is the first time that a modification of a nutrient concentration completely reverses the domination. The initial concentration of anaerobic growth factors greatly influences co-culture fermentation population dynamics, thus highlighting a new means for monitoring population evolution and eventually wine organoleptic properties.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights This article does not contain any studies with human or animal subjects.

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