Appl Microbiol Biotechnol (2017) 101:7603–7620
DOI 10.1007/s00253-017-8492-1

Combined effect of the *Saccharomyces cerevisiae* lag phase and the non-*Saccharomyces* consortium to enhance wine fruitiness and complexity

Warren Albertin[1,2] · **Adrien Zimmer**[1,3] · **Cécile Miot-Sertier**[1,4] · **Margaux Bernard**[1,3] · **Joana Coulon**[3] · **Virginie Moine**[3] · **Benoit Colonna-Ceccaldi**[5] · **Marina Bely**[1] · **Philippe Marullo**[1,3] · **Isabelle Masneuf-Pomarede**[1,6]

Received: 31 March 2017 / Revised: 23 July 2017 / Accepted: 30 July 2017 / Published online: 14 September 2017

Abstract Non-*Saccharomyces* (NS) species that are either naturally present in grape must or added in mixed fermentation with *S. cerevisiae* may impact the wine's chemical composition and sensory properties. NS yeasts are prevailing during prefermentation and early stages of alcoholic fermentation. However, obtaining the correct balance between *S. cerevisiae* and NS species is still a critical issue: if *S. cerevisiae* outcompetes the non-*Saccharomyces*, it may minimize their impact, while conversely if NS take over *S. cerevisiae*, it may result in stuck or sluggish fermentations. Here, we propose an original strategy to promote the non-*Saccharomyces* consortium during the prefermentation stage while securing fermentation completion: the use of a long lag phase *S. cerevisiae*. Various fermentations in a Sauvignon Blanc with near isogenic *S. cerevisiae* displaying short or long lag phase were compared. Fermentations were performed with or without a consortium of five non-*Saccharomyces* yeasts (*Hanseniaspora uvarum*, *Candida zemplinina*, *Metschnikowia* spp., *Torulaspora delbrueckii*, and *Pichia kluyveri*), mimicking the composition of natural NS community in grape must. The sensorial analysis highlighted the positive impact of the long lag phase on the wine fruitiness and complexity. Surprisingly, the presence of NS modified only marginally the wine composition but significantly impacted the lag phase of *S. cerevisiae*. The underlying mechanisms are still unclear, but it is the first time that a study suggests that the wine composition can be affected by the lag phase duration per se. Further experiments should address the suitability of the use of long lag phase *S. cerevisiae* in winemaking.

Keywords Non-conventional yeast · Prefermentation stage · Wine · Lag phase

Introduction

Grape musts contain naturally complex and diverse microbial community. The alcoholic fermentation process is mainly conducted by *Saccharomyces cerevisiae* with either inoculated or indigenous strains. However, many other yeast species and genera (referred to here as non-*Saccharomyces* yeasts) can persist and survive at significant levels during the fermentation process, even at the late stages of fermentation (Andorra et al. 2011; David et al. 2014; Wang et al. 2014; Zott et al. 2008). Before active CO_2 release (prefermentation) and during the early stages of alcoholic fermentation, these species are predominant with populations that may reach up to 10^5–10^7 cells/mL (Wang et al. 2015; Zott et al. 2010). The population dynamics of yeast species during the fermentation is complex and mainly due to competition or other interactions among yeast species. It is noteworthy that *S. cerevisiae* produces

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00253-017-8492-1) contains supplementary material, which is available to authorized users.

✉ Warren Albertin
warren.albertin@u-bordeaux.fr

[1] Univ. Bordeaux, ISVV, Unité de Recherche Œnologie EA 4577, USC 1366 INRA,, Bordeaux INP, 33140 Villenave d'Ornon, France

[2] ENSCBP, Bordeaux INP, 33600 Pessac, France

[3] Biolaffort, 33100 Bordeaux, France

[4] INRA, ISVV, Unité de Recherche Œnologie EA 4577, USC 1366 INRA, Bordeaux INP, 33140 Villenave d'Ornon, France

[5] Pernod-Ricard, 94000 Créteil, France

[6] Bordeaux Sciences Agro, 33170 Gradignan, France

biomass and consumes sugars and nitrogen more efficiently and rapidly compared to other yeasts. Moreover, the Crabtree effect and heat production are described as a fitness advantage for *S. cerevisiae* niche construction (Goddard 2008; Salvadó et al. 2011). Yeast metabolites such as ethanol, medium-chain fatty acid, killer toxin, and antimicrobial peptides have been also considered to explain yeast interactions (Albergaria et al. 2010; Branco et al. 2015; Cheraiti et al. 2010; Fleet 2003; Wang et al. 2015). Finally, quorum sensing or cell-to-cell contact phenomena were also reported between *S. cerevisiae* and other species (Arneborg et al. 2005; Nissen and Arneborg 2003; Renault et al. 2013) and contribute to explaining the interaction phenomenon. Three main genera (*Hanseniaspora*, *Candida*, and *Metschnikowia*) dominate the yeast consortium during the prefermentation stage. *Hanseniaspora uvarum* has been widely reported as a major non-*Saccharomyces* yeast during the initial stages of wine fermentation (Beltran et al. 2002; Combina et al. 2005; Jolly et al. 2003; Li et al. 2010; Pretorius 2000; Zott et al. 2010). *Candida zemplinina* (synonym *Starmerella bacillaris*) was isolated from grape must by different authors, whatever the wine-producing region or the grape variety considered (Masneuf-Pomarede et al. 2015; Nisiotou and Nychas 2007; Pfliegler et al. 2014; Tofalo et al. 2012; Tristezza et al. 2013; Zott et al. 2008). Finally, *Metschnikowia* spp. was reported at high population levels in grape must (Garofalo et al. 2016; Jolly et al. 2014).

Non-*Saccharomyces* (NS) yeasts may affect the wine fermentation both directly, by producing flavors and indirectly by modulating the growth and the metabolism of *S. cerevisiae*. During fermentation, those yeasts could impact the higher alcohol content of wine. Increased production of 2-phenylethyl alcohol, which is associated with pleasant aromas at moderate concentration, was reported for *Metschnikowia pulcherrima* (Clemente-Jimenez et al. 2004), *Lachancea thermotolerans* (Beckner Whitener et al. 2015), and *C. zemplinina* (Andorra et al. 2010). However, wines fermented by *C. zemplinina* had concentrations of higher alcohols exceeding 400 mg/L thus resulting in a negative effect (Andorra et al. 2010). *Hanseniaspora* is frequently reported to produce fruity acetate esters (Mateo et al. 1991; Rojas et al. 2001; Romano et al. 1997; Viana et al. 2008). In mixed fermentation with *S. cerevisiae*, *Torulaspora delbrueckii* enhances the complexity and fruity notes of wine compared to a pure culture of *S. cerevisiae.* This phenomenon seems to be linked with the enhancement of specific ethyl esters (Renault et al. 2015). Specific enzymatic activities among which glycosidases and carbon-sulfur lyases are produced by non-*Saccharomyces* that may catalyze the release of volatile compounds from a non-volatile precursor, such as terpenols (Garcia et al. 2002; Hu et al. 2016; Rodriguez et al. 2010; Sadoudi et al. 2012) or volatile thiols (Anfang et al. 2009; Zott et al. 2011). The de novo synthesis of monoterpenes by non-*Saccharomyces* was also recently reported (Rossouw and Bauer 2016).

Due to their production of positive volatile compounds, the presence of NS yeast has been associated with higher wine quality and complexity (Anfang et al. 2009; Ciani et al. 2006; Egli et al. 1998; Fleet 2003; Pérez et al. 2011; Rossouw and Bauer 2016; Swiegers and Pretorius 2005). Their dominance during the early stages of winemaking can influence the final composition of the wine (Romano et al. 1997) and some authors suggest that the limitation of the non-*Saccharomyces* population could result in a loss of complexity in wines (Varela et al. 2009). The possibility to enhance wine aromatic complexity using non-*Saccharomyces* species in mixed fermentation was examined by many authors. Usually, the non-conventional yeasts were associated with *S. cerevisiae* in order to prevent sluggish or stuck fermentations (see the reviews of Ciani et al. 2010; Jolly et al. 2014; Padilla et al. 2016). An alternative way could be to take advantage of the natural non-*Saccharomyces* population naturally present on grapes in the vineyard (Pretorius 2000). Moreover, the cellar equipment could act as an inoculum source of a non-*Saccharomyces* population that may persist in the winery environment from one year to another (Ciani et al. 2004; Grangeteau et al. 2015). However, very few studies described the impact of the natural non-*Saccharomyces* community during the prefermentation stage on the wine composition and quality. Indubitably, *S. cerevisiae* growth is a key point for controlling the development of the non-*Saccharomyces* population in the early stages of winemaking. Indeed, the rapid development of *S. cerevisiae* (either indigenous or inoculated) risks to outcompete the non-*Saccharomyces* flora by nutrient depletion (Andorrà et al. 2012). In order to promote the impact on the non-*Saccharomyces* community, one strategy consists of delaying *S. cerevisiae* starter development by applying a sequential inoculation or by lowering its inoculation level (e.g., 10^3 cells/mL). An alternative way is the use of a *S. cerevisiae* strain inoculated at a normal rate (4–5 × 10^6 cells/mL) but showing a long lag phase duration. Indeed, lag phase is a quantitative trait that varies between few hours and few days among *S. cerevisiae* strains in enological conditions (Camarasa et al. 2011; Marullo et al. 2006). The genetic control of this parameter has been partially identified by a Quantitative Trait Loci (QTL) mapping approach (Marullo et al. 2007). A subsequent study reveals that two distinct chromosomal translocation events involving the gene encoding the sulfite pump *SSU1* mainly control this trait (Zimmer et al. 2014). The identification of these translocations paves the way for controlling the lag phase duration using marker-assisted breeding programs as done previously for other enological traits (Dufour et al. 2013; Marullo et al. 2007).

For some scientists and enologists, the growth of non-*Saccharomyces* yeast during the early stage of winemaking is still considered as an uncontrollable risk, whereas for the

others, their development is one way to increase the wine quality. In this project, our initial hypothesis was that the non-*Saccharomyces* component of the microbial community during the prefermentation stage could impact wine composition. We aimed to evaluate the organoleptic benefit associated with the development of a complex yeast community during the prefermentation stage and to quantify its impact on the fruity notes and wine complexity. For that purpose, we developed and used near isogenic *S. cerevisiae* strains with short or long lag phase in order to modulate the duration of the prefermentation phase and the non-*Saccharomyces* impact.

Material and methods

Media and culture conditions

The *S. cerevisiae* strains were grown at 30 °C on complete YPD-2 medium containing 1% yeast extract (Difco Laboratories, Detroit, Michigan), 1% peptone (Difco), and 2% dextrose solidified with 2% agar when necessary. Sporulation was induced on acetate medium (1% potassium acetate, 2% agar) for 3 days at 24 °C. Auxotrophies were detected on SD medium (0.67% YNB, 2% dextrose, 2% agar) supplemented with lysine (30 mg/L) or uracil (10 mg/L). The non-*Saccharomyces* strains were grown at 25 °C in YPD-6 differing for YPD-2 by its dextrose concentration (6%).

Molecular genotyping of chromosome XVI forms and *ho* allele

The homo or heterothallic (*HO* or *ho*) status of spore clones was checked by PCR using the following primers: p301, AGTCACATCAAGATCGTTTATGG; p302, GCACGGAA TATGGGACTACTTCG; and P303, ACTCCACTTCAAGT AAGAGTTTG (Huxley et al. 1990). The different allelic forms of chromosome XVI were detected by using the PCR tests developed by Zimmer et al. (2014). According to the primer set, the presence/absence of the allelic forms XVI-wt (p788, TCTTTTTGGGCTGGTAGGAT; p789, ATATTTGT AGTGCCTGCACA), XV-t-XVI (P758, AAAGAAGT TGCATGCGCCTA; p765, GACACCCATGACCATCAC), and VIII-t-XVI (p764, TCGAACATCGAGCATGCA; p765, GACACCCATGACCATCAC) was detected. The wild-type copies of chromosomes VIII, XV, and XVI were detected using the primers p786, CGCATCCAGTACAAAGAAATG; p787, CTGAGTGATTTGTTTCCCGA; p758, AAAGAAGT TGCATGCGCCTA; p761, GAGTTTTTTGCGCCTGCATT; p788, TCTTTTTGGGCTGGTAGGAT; and p789, ATATTTGTAGTGCCTGCACA, respectively. The parental strain YPM64 (CRBO L1317) is derived from a monosporic clone of the commercial strain Zymaflore VL3 (Laffort, Bordeaux, France) and carries the translocation XV-t-XVI that reduces the lag phase length in sulfited grape juice (Zimmer et al. 2014). The parental strain YPM22 (CRBO L1303) is derived from the Σ1278b lab strain and carries the XVI-wt form. None of the strains used in this work have the other translocated form VIII-t-XVI previously described (Perez-Ortin et al. 2002). All PCR fragments were analyzed using multi-NA apparatus using the DNA-1000 bp kit (Shimatzu, Noisiel, France). The translocation PCR was also used as control to confirm the correct implantation of the strains at the end of the alcoholic fermentation.

Construction of near isogenic strains with a short and a long lag phase

Backcross experiments were carried out using auxotrophic markers as previously described (Dufour et al. 2013). Haploid strains and homothallic spore clones were crossed by mixing 10^6 haploid cells with 10 spores/mL treated by cytohelicase (2 mg/L) (Sigma, L'Isle d'Abeau Chesnes, France) for an hour. Zygote formation was observed in liquid YPD-2 after 6 h. Then, 100 μL of the mixture was plated on SD medium for selecting prototrophic hybrids. The hybrid nature of growing colonies was confirmed by the Mendelian segregation of lysine and uracil auxotrophies by tetrad dissection using a Singer micromanipulator MSM 200 (Singer Instruments, Roadwater, Somerset, UK). The use of the parental strains YPM22 (*ho*, *ura3*, XVI-wt) and YPM64 (*HO/HO*, *lys2/lys2*, XV-t-XVI/XV-t-XVI) allowed the construction of the hybrid VL3-BC4-LAG. At each backcross step, one meiotic segregant was selected for the following genotype: *ho*, *LYS2*, *ura3*, *XVI-wt*, and backcrossed with the strain YPM64. Auxotrophies were selected on appropriate media, while *ho* and XVI-wt genotypes were selected by molecular typing. After four backcrosses, the hybrid VL3-BC4-LAG was obtained. Six diploid progeny clones with the following phenotypes *HO/HO*, *LYS2/LYS2*, and *URA3/URA3* were finally selected. Three of them carried the XVI-wt form, while the others three carried the XV-t-XVI form. Table 1 summarizes the relevant genotypes of *S. cerevisiae* strains used in the backcross experiment.

Non-*S. cerevisiae* strains

Five non-conventional yeast strains were used (Table 1), either from Centre de Ressources Biologiques Oenologique, Bordeaux, France (CRBO) or Centre de Recherche Pernod-Ricard, Créteil, France (CRPR) collections: *H. uvarum* CRBO L0551, *T. delbrueckii* CRBO L0705 (commercialized as Zymaflore Alpha by Laffort, Bordeaux, France), *C. zemplinina* CRBO L0471, *Pichia kluyveri* CRPR NZ318, and *Metschnikowia* spp. CRBO L0563, close to *Metschnikowia andauensis* (Chasseriaud et al. 2015).

Table 1 Yeast strains used in this study

Strain	Species	Genotype	Origin
CRBO L0471 (Cz)	*Candida zemplinina*		CRBO L0471
CRBO L0705 (Td)	*Torulaspora delbrueckii*		CRBO L0705
CRBO L0551 (Hu)	*Hanseniaspora uvarum*		CRBO L0551
CRPR NZ318 (Pk)	*Pichia kluyveri*		CRPR NZ318
CRBO L0563 (Mp)	*Metschnikowia* spp.		CRBO L0563
YPM22	*Saccharomyces cerevisiae*	*ho*, *ura3*, *XVI-wtho*, *ura3*, *XVI-wt*	CRBO L1303
YPM64	*Saccharomyces cerevisiae*	*HO/HO*, *lys2/lys2*, *XV-t-XVI/XV-t-XVIHO/HO*, *lys2/lys2*, *XV-t-XVI/XV-t-XVI*	CRBO L1317
VL3-BC1	*Saccharomyces cerevisiae*	*HO/ho*, *LYS2/lys2*, *URA3/ura3*, *XV-t-XV/XVIHO/ho*, *LYS2/lys2*, *URA3/ura3*, *XV-t-XV/XVI*	This study
VL3-BC1-2B	*Saccharomyces cerevisiae*	*ho*, *mat* ***a***, *ura3*, *LYS2*, *XVI-wtho*, *mat* ***a***, *ura3*, *LYS2*, *XVI-wt*	This study
VL3-BC2	*Saccharomyces cerevisiae*	*HO/ho*, *LYS2/lys2*, *URA3/ura3*, *XV-t-XV/XVIHO/ho*, *LYS2/lys2*, *URA3/ura3*, *XV-t-XV/XVI*	This study
VL3-BC2-4A	*Saccharomyces cerevisiae*	*ho*, *mat* ***a***, *ura3*, *LYS2*, *XVI-wtho*, *mat* ***a***, *ura3*, *LYS2*, *XVI-wt*	This study
VL3-BC3	*Saccharomyces cerevisiae*	*HO/ho*, *LYS2/lys2*, *URA3/ura3*, *XV-t-XV/XVIHO/ho*, *LYS2/lys2*, *URA3/ura3*, *XV-t-XV/XVI*	This study
VL3-BC3-2C	*Saccharomyces cerevisiae*	*ho*, *mat* ***a***, *ura3*, *LYS2*, *XVI-wtho*, *mat* ***a***, *ura3*, *LYS2*, *XVI-wt*	This study
VL3-BC4	*Saccharomyces cerevisiae*	*HO/ho*, *LYS2/lys2*, *URA3/ura3*, *XV-t-XV/XVI HO/ho*, *LYS2/lys2*, *URA3/ura3*, *XV-t-XV/XVI*	This study
VL3-BC4-msp1	*Saccharomyces cerevisiae*	*HO/HO*, *mat* ***a***/*mat* α, *URA3/URA3 LYS2/LYS2*, *XV-t-XVI/XV-t-XVIHO/HO*, *mat* ***a***/*mat* α, *URA3/URA3 LYS2/LYS2*, *XV-t-XVI/XV-t-XVI*	This study
VL3-BC4-msp2	*Saccharomyces cerevisiae*	*HO/HO*, *mat* ***a***/*mat* α, *URA3/URA3 LYS2/LYS2*, *XV-t-XVI/XV-t-XVI*	This study
VL3-BC4-msp3 (Sh-LP)	*Saccharomyces cerevisiae*	*HO/HO*, *mat* ***a***/*mat* α, *URA3/URA3 LYS2/LYS2*, *XV-t-XVI/XV-t-XVI*	This study
VL3-BC4-msp4 (Lg-LP)	*Saccharomyces cerevisiae*	*HO/HO*, *mat* ***a***/*mat* α, *URA3/URA3 LYS2/LYS2*, *XVI-wt/XVI-wt*	This study
VL3-BC4-msp5	*Saccharomyces cerevisiae*	*HO/HO*, *mat* ***a***/*mat* α, *URA3/URA3 LYS2/LYS2*, *XVI-wt/XVI-wt*	This study
VL3-BC4-msp6	*Saccharomyces cerevisiae*	*HO/HO*, *mat* ***a***/*mat* α, *URA3/URA3 LYS2/LYS2*, *XVI-wt/XVI-wt*	This study

Fermentation assays

Fermentation assays in a model synthetic medium were performed as previously described (Marullo et al. 2006). The synthetic medium was filtered through a 0.45-mm nitrate-cellulose membrane (Millipore, Molsheim, France) before use. The SO_2 amount was adjusted at concentrations of 0 or 40 mg/L (only for the fermentation assays, not for the precultures).

For fermentation assays in natural grape must, white grape must was obtained from Sauvignon grapes, harvested in vineyards in the Bordeaux area (2011 vintage or 2014 vintage). Tartaric acid was stabilized (precipitation) before long storage at −20 °C. The initial assimilable nitrogen 152 and 157 mg N/L respectively for 2011 and 2014 vintages was adjusted to 200 mg N/L using Thiazote (Laffort, Bordeaux, France) in order to prevent nitrogen deficiencies (Bely et al. 1990a). The sugar concentration was 203 and 194 g L^{-1}, respectively, for 2011 and 2014. The free and total SO_2 were 25 and 75 mg/L for 2011 and 4 and 18 mg/L for 2014. The grape must was filtered through a 0.45-μm nitrate-cellulose membrane before inoculation for precultures only. The initial indigenous yeast population in grape must, estimated by YPD-plate counting before filtration, was low, i.e., around 20 colony-forming unit (CFU) per mL.

Non-*Saccharomyces* yeasts were individually precultured in filtered and half-diluted medium/must during 24 h at 24 °C and were then used to inoculate the medium/must at 1×10^6 viable cells/mL for *H. uvarum* and *C. zemplinina* or at 1×10^4 viable cells/mL for *P. kluyveri*, *T. delbrueckii*, and *Metschnikowia* spp. For *S. cerevisiae*, precultures were performed in filtered half-diluted medium/must at 24 °C during 24 h (short lag phase, Sh-LP) or 72 h (long lag phase, Lg-LP). *S. cerevisiae* strains were then inoculated at 1×10^6 viable cells/mL, except for the "low inoculum" modality where Sh-LP was inoculated at 1×10^3 viable cells/mL. The cell number and viability was determined by flow cytometry (see below). All fermentations assays were repeated three to four times.

Fermentations were run in closed 1.2-L glass reactors, locked to maintain anaerobiosis, with permanent stirring (≈ 150 rpm) (Bely et al. 1990b). CO_2 was released through a sterile air outlet condenser, and the precise amount of CO_2 released was determined by measurement of glass reactor weight loss. The temperature was set at 13 °C in order to mimic a prefermentative stage at cold temperature. As soon as the fermentation begun (CO_2 release > 2.5 g/L), the temperature was set at 18 °C and was finally adjusted at 20 °C when 75% of the reaction was reached (CO_2 release > 72 g/L) in order to mimic the temperature management at the

industrial scale. At 75% of the alcoholic fermentation, *S. cerevisiae* strain implantation (Sh-LP/short lag phase or Lg-LP/long lag phase) was controlled using the translocation PCR described in the present study.

At the end of alcoholic fermentation (AF) (no more CO_2 release), stirring was stopped and lees settled as sediment during 48 h at 13 °C. After racking, SO_2 was added (50 mg/L) and the wines were bottled before further chemical and/or sensory analysis.

For the so-called binary cultures, fermentations were conducted in 750-mL bottles (four replicates per modality), locked to maintain anaerobiosis without stirring in order to mimic winery conditions. The amount of CO_2 released was determined by measurement of glass reactor weight loss. A prefermentative stage was conducted (13 °C), then the temperature was set at 18 °C when the fermentation started (CO_2 release > 2.5 g/L). The long lag-phased *S. cerevisiae* Lg-LP was inoculated at 1×10^6 viable cells/mL and mixed with only one NS species inoculated at 1×10^6 viable cells/mL for *H. uvarum* and *C. zemplinina* and 1×10^4 viable cells/mL for *P. kluyveri*, *T. delbrueckii*, and *Metschnikowia* spp.

Fermentation kinetic monitoring

The amount of CO_2 released was determined by measurement of glass reactor weight loss. Different kinetic parameters were calculated: the *lag phase* (h) was the time between inoculation and the beginning of CO_2 release (CO_2 release higher than 2.5 g/L). The end of the fermentation was determined when the CO_2 release reached 97.5% of the total amount of expected CO_2. This point allowed us to estimate the *a*lcoholic *f*-ermentation time (*AF time*, h) which was the time necessary to ferment the sugars in the medium, excluding the lag phase. The *t30*, *t35*, *t50*, and *t80* were the time needed to reach 30, 35, 50, and 80% of the total amount of expected CO_2, respectively. All modalities (except Cz) were able to achieve the fermentation (i.e., to consume over 98.5% of initial sugar). Finally, the *CO_2tot* was the total amount of CO_2 released at the end of the fermentation (g/L).

Dosage of entering and sorting AF metabolites

The standard chemical parameters of wines were measured by the analytical laboratory SARCO (Bordeaux, France): ethanol concentration (% (*v*/*v*)) was determined by infrared reflectance (Infra-Analyzer 450, Technicon, Plaisir, France), acetic acid production (g/L) was measured by colorimetry (*A*460) in continuous flux (Sanimat, Montauban, France), and both residual D-glucose and D-fructose (g/L) were quantified using an enzymatic method (Boehringer Kit D glucose/D fructose, R-Biopharm, Darmstadt, Germany) on the supernatants. All assays showed complete fermentations (i.e., residual sugars at the end of AF lower than 1 g/L). External glycerol (g/L) was assayed by enzymatic method (Boehringer kits 10 148 270 035, R-Biopharm, Darmstadt, Germany). The total SO_2 and free SO_2 (mg/L) were measured by pararosaniline titration (Pate et al. 1962).

Alternatively, when only small volumes were available (i.e., for binary cultures), small samples were harvested (800 μl), stored at −20 °C, and analyzed thanks to the metabolomic platform of Bordeaux by semi-automatized enzymatic assays (http://metabolome.cgfb.u-bordeaux.fr/). The concentrations of the following organic metabolites were measured: acetic acid, glycerol, malic acid, pyruvate, acetaldehyde, and total SO_2 using the respective enzymatic kits, K-ACETGK, K-GCROLGK, K-LMAL-116A, K-PYRUV, K-ACHYD, and K-TSULPH (Megazyme, Bray, Ireland), following the instructions of the manufacturer. Glucose and fructose were assayed as described by Stitt et al. (1989).

Global population growth monitoring

The population growth was monitored by regular samplings using a flow cytometer (Quanta SC MPL, Beckman Coulter, Villepinte, France), equipped with a 488-nm laser (22 mW) and a 670-nm long-pass filter. Cell samples were diluted in McIlvaine buffer pH 4 (0.1 M citric acid, 0.2 M sodium phosphate dibasic) supplemented with propidium iodide (0.3% *v*/*v*) in order to stain dead cells (red fluorescence measure in FL3 channel). The experimental points were used to estimate three population growth parameters: the time of growth start *tgrowth* (h) was defined as the time between inoculation and effective doubling of the initial inoculated population, the maximum population size *K* (cells mL^{-1}), and the *Klag* (cells mL^{-1}) which is the population reached at the *tgrowth*.

Thiol and ester quantification

A HS-SPME-GC/MS method developed and validated by Antalick et al. (2010) was used to quantify 32 esters which included ethyl fatty acid esters, acetates of higher alcohol, ethyl branched acid esters, isoamyl esters, methyl esters, ethyl cinnamates, and some others. For volatile thiols, a specific extraction was performed according to Tominaga et al. (1998).

Sensory analysis

For sensory analyses, triplicates of the same modality were blended (equimolar ratio). Fifty milliliters of the five resulting wines were poured into black wine glasses, labeled with random three-digit codes, and covered with half of a plastic Petri dish. Evaluations were performed in a dedicated room, at room temperature (around 20 °C). All the 24 panelists were staff of the research laboratory at ISVV, Bordeaux University, or from the Laffort Company and had previous experience with the sensory evaluation of wines. The evaluation of the

overall complexity was performed on a continuous scale illustrated by a series of six pictures that facilitate the comprehension of the complexity (Meillon et al. 2010). Analyses were carried out by orthonasal evaluations. The samples were presented simultaneously to the panel who estimated the intensity of the fruitiness and complexity of the wines on a discontinuous scale from 1 to 7. The resulting data were normalized for each panelist, in order to take into account individual variation before variance analysis (ANOVA) followed by Duncan test (alpha = 0.05) by means of R and *agricolae* package (R Development Core Team 2010).

Statistical analyses

All data were analyzed either through non-parametric statistics (Wilcoxon test) or variance analysis (ANOVA) followed by Duncan test (alpha = 0.05) by means of R (R Development Core Team 2010). A principal component analysis (PCA) was performed using R and *ade4* package (Chessel et al. 2004).

Results

Construction of nearly isogenic strains with short and long lag phase

In a previous work, a major genetic determinism of the lag phase in oenology has been deciphered by describing the role of two translocation forms targeting the promoter region of *SSU1* gene on chromosome XVI (Zimmer et al. 2014). These two types of gross chromosomal rearrangements enhance the transcription of the *SSU1* gene that encodes a sulfite pump playing a major role in sulfite resistance during the first hour of alcoholic fermentation (Divol et al. 2012). Both translocated forms are frequently found among wine starter cultures ensuring their rapid growth in grape must contain free SO_2 (Zimmer et al. 2014). Therefore, the rare non-translocated form XVI-wt was used to construct a wine yeast strain showing a long lag phase. By a marker-assisted backcross program, we produced nearly isogenic strains showing important differences in their lag phase.

The VL3-BC4-LAG hybrid was obtained by introgressing in the YPM64 (VL3-like background) the XVI-wt form of the strain YPM22 (Table 1). As the strain YPM64 possesses the XV-t-XVI translocated form, the genotype for the VL3-BC4-LAG hybrid is heterozygous (XV-t-XVI/XV-wt) for this locus. Previous works demonstrated that four rounds of backcrosses are mostly sufficient to obtained nearly isogenic lines sharing more than 93% of genome identity (Marullo et al. 2007, 2009). To avoid manual spore to spore pairing, the backcross program was carried out using the auxotrophic markers *ura3* and *lys2* as previously described (Dufour et al. 2013). At the final step, homothallic spore clones with the following genotype (*URA3*/*URA3*, *LYS2*/*LYS2*, *HO*/*HO*) were selected from the VL3-BC4-LAG hybrid in order to get prototrophic, diploid monosporic clones expected to be fully homozygous. The XVI-wt and XV-t-XVI alleles segregated in a Mendelian way (five full tetrads analyzed). The kinetic properties were measured for six progeny clones on a Sauvignon Blanc grape juice (2011 vintage) in duplicate (Online Resource 1). As expected, the lag phase was strongly affected by the inheritance of the chromosome XVI forms, with more than 90 h of difference (Wilcoxon test, alpha = 0.05) in fermentation start. In contrast, the other kinetic parameters were statistically similar between the two groups suggesting that the progenies tested have broadly the same technological properties under fermentative conditions except for the lag phase. According to their short and long lag phase, two of these clones carrying the XV-t-XVI and XVI-wt forms were named Sh-LP and Lg-LP, respectively. Sh-LP and Lg-LP strains were subsequently compared more thoroughly in a synthetic grape juice, with or without sulfite (Fig. 1), and several parameters were measured (Table 2): five kinetic parameters (*lag phase*, *AF time*, time to complete 30, 35, 50, or 80% of AF *t30*, *t35*, *t50*, *t80*) and nine entering or sorting AF metabolites (acetaldehyde, pyruvate, glucose, fructose, glycerol, malic acid, acetic acid, total SO_2, CO_2max). Our results indicated that these two nearly isogenic strains displayed identical fermentation kinetics, except for *lag phase* that increased from 25 to 28 h (Sh-LP with or without sulfite and Lg-LP without sulfite) to 193 h for Lg-LP in the presence of sulfite. Regarding metabolites, a significant "sulfite" effect was identified for pyruvate: both Lg-LP and Sh-LP fermentations displayed higher amounts of pyruvate in the presence of sulfite (0.012–0.014 g/L compared to 0–0.002 g/L). Finally, the concentration of total SO_2 was significantly higher in synthetic medium with sulfite as expected (30–40 versus 1–8 mg/L). Moreover, the amount of total SO_2 was also higher for Sh-LP compared to Lg-LP (around 7 mg/L more SO_2), with or without sulfite, in accordance with previous experiments showing that the translocation harbored by Sh-LP confers an increased expression of the sulfite pump (Zimmer et al. 2014). Altogether, those results indicated that Sh-LP and Lg-LP displayed identical phenotypes under fermentative conditions, except, as expected, for lag phase and sulfite pumping.

Fermentation kinetics of short and long lag-phased *S. cerevisiae* with or without a non-*Saccharomyces* reconstructed consortium

In order to estimate the impact of both lag phase duration and complex yeast consortium including *S. cerevisiae* and non-*Saccharomyces* yeasts, we conducted prefermentation maceration and AF in a Sauvignon Blanc grape must (2011 vintage). Five different modalities were tested (in triplicates), including either *S. cerevisiae* Sh-LP or Lg-LP inoculated at 1×10^6 viable cells/mL. The fermentations were run as either pure or complex culture. In this latter case, a non-*Saccharomyces* community (NS

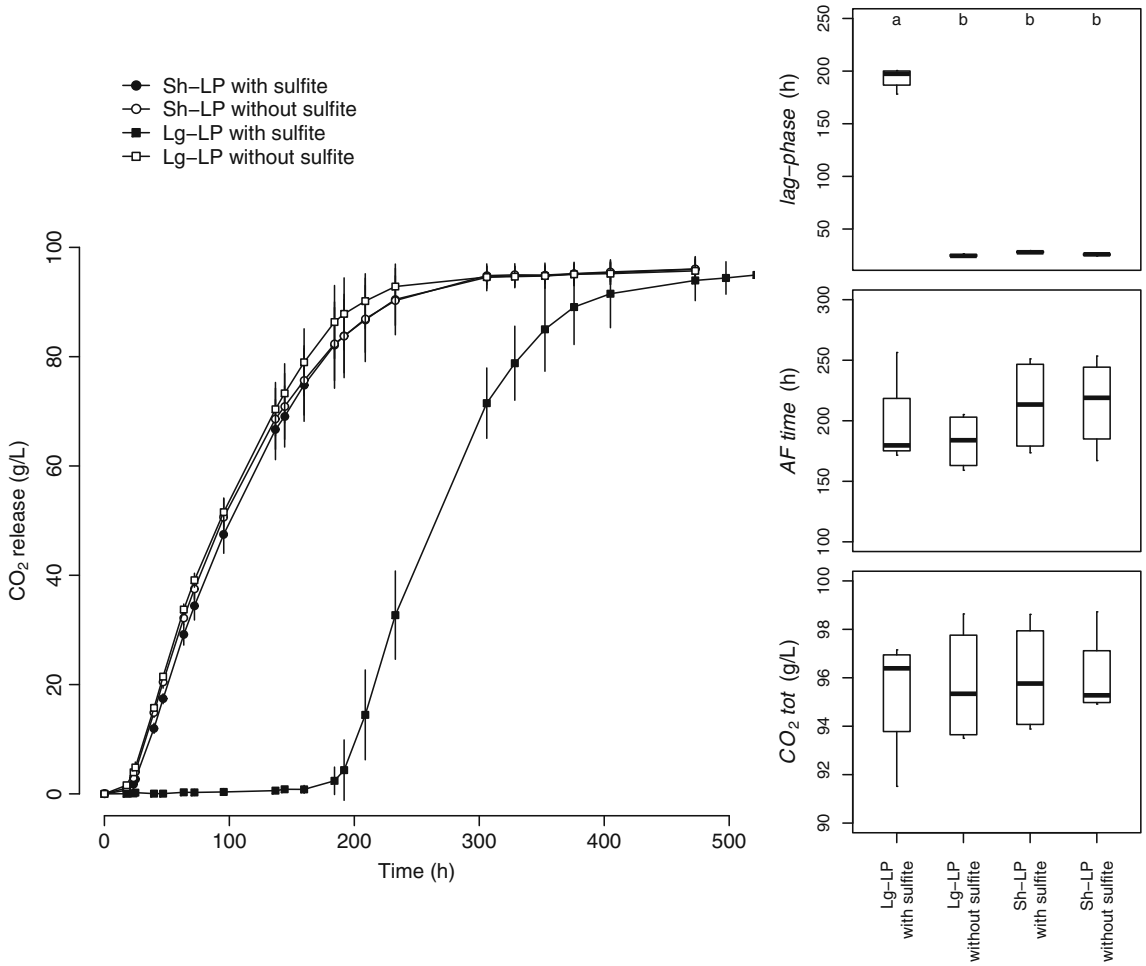

Fig. 1 Impact of sulfite on the fermentation kinetics of short and long lag phase strains in a synthetic grape must. Sh-LP and Lg-LP were tested in synthetic medium with or without sulfite (40 mg/L). For fermentation kinetics, error bars represent standard deviation of the triplicates. The boxplots represent some parameters of interest (*lag phase*, *AF Time*, *CO2tot*). Boxplots with different letter are significantly different (Duncan test following ANOVA, alpha = 0.05, only lag phase parameter was significant)

consortium) was mimicked using a mix of five strains belonging to five different species. The highly abundant *H. uvarum* and *C. zemplinina* were inoculated at 1×10^6 viable cells/mL while the less frequent *P. kluyveri*, *T. delbrueckii*, and *Metschnikowia* spp. were inoculated at 1×10^4 viable cells/mL. Finally, a "low inoculum" modality was also tested (Sh-LP inoculated at 1×10^3 viable cells/mL added with NS consortium) in order to assess the impact of a long lag phase performed by a short lag phase strain. A prefermentation stage was simulated by setting the temperature at 13 °C until the actual beginning of CO_2 release. The three quarters of the AF were then performed at 18 °C and the last quarter at 20 °C to mimic a winery-like temperature management at the end of fermentation. The corresponding fermentation kinetics are shown in Fig. 2, and fermentation parameters are summarized in Table 3. The fermentation kinetic profiles were very similar: no significant differences were recorded for the total amount of CO_2 released (*CO_2tot*) or for *AF time* (calculated without lag phase). Indeed, the main difference between the different modalities was due as expected to the lag phase duration: the modalities with the short lag phase *S. cerevisiae* strain (Sh-LP) presented identical lag phases, and no difference was associated with or without non-*Saccharomyces* (69.3 and 68.1 h, respectively). The "low inoculum" modality with the short lag phase *S. cerevisiae* strain (Sh-LP (low inoculum) + NS) displayed, as expected, a significantly greater lag phase of 128.6 h. Finally, the modalities with the long lag phase *S. cerevisiae* strain alone (Lg-LP) presented a higher lag phase of 242.1 h (without NS consortium). Interestingly, the same strain associated with NS showed a shorter lag phase (201.3 h).

Population growth

The total population growth was evaluated by flow cytometry. As the different species could not be differentiated, the overall yeast population was globally evaluated (Fig. 2 and Table 3). For all modalities, the start of the population growth (*tgrowth*) preceded slightly (a few hours) the actual beginning of CO_2 release referred

Table 2 Comparison of short lag phase and long lag phase strains in a synthetic grape must with or without sulfite

Modalities	Acetaldehyde (g/L)	Pyruvate (g/L)	Acetic acid (g/L)	Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Malic acid (g/L)	Total SO_2 (g/L)
Lg-LP with sulfite	0.089 ± 0.035 ns	0.014 ± 0.003 a	0.42 ± 0.09 ns	0.064 ± 0.127 ns	0.064 ± 0.127 ns	1.52 ± 1.63 ns	0.071 ± 0.056 ns	33.11 ± 3.41 b
Lg-LP without sulfite	0.078 ± 0.037 ns	0 ± 0 b	0.38 ± 0.14 ns	0 ± 0 ns	0 ± 0 ns	4.73 ± 0.81 ns	0.095 ± 0.031 ns	1.52 ± 1.34 d
Sh-LP with sulfite	0.055 ± 0.016 ns	0.012 ± 0.004 a	0.5 ± 0.15 ns	0 ± 0 ns	0 ± 0 ns	1.95 ± 2.27 ns	0.076 ± 0.038 ns	40.57 ± 2.64 a
Sh-LP without sulfite	0.081 ± 0.033 ns	0.002 ± 0.002 b	0.46 ± 0.06 ns	0 ± 0 ns	0 ± 0 ns	4.05 ± 2.46 ns	0.085 ± 0.019 ns	7.46 ± 1.79 c

Modalities	Pyruvate (g/L)	*CO_2max* (g/L)	*t50* (h)	*t80* (h)
Lg-LP with sulfite	0.014 ± 0.003 a	95.4 ± 2.6 ns	67.2 ± 3.7 ns	130.3 ± 11.9 ns
Lg-LP without sulfite	0 ± 0 b	95.7 ± 2.5 ns	63.9 ± 2.8 ns	129.7 ± 12.4 ns
Sh-LP with sulfite	0.012 ± 0.004 a	96 ± 2.3 ns	69.1 ± 4.4 ns	141.1 ± 16.4 ns
Sh-LP without sulfite	0.002 ± 0.002 b	96 ± 1.8 ns	65.5 ± 4.6 ns	140.2 ± 16.4 ns

Data presented are the means of four fermentations, ±standard deviation. A Kruskal-Wallis test was used to assess significant differences between modalities: modalities sharing the same letters are not significantly different

ns not significant (alpha = 0.05)

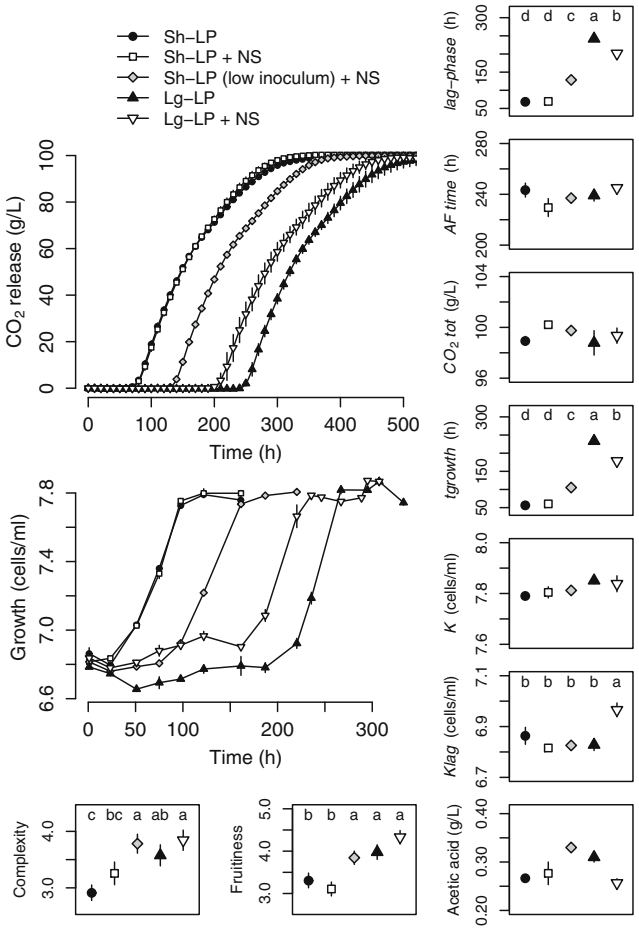

Fig. 2 Effect of the *Saccharomyces cerevisiae* lag phase and the non-*Saccharomyces* consortium. Five modalities were tested in triplicate, including short or long lag-phased *S. cerevisiae* strain (Sh-LP and Lg-LP, respectively) inoculated at classical concentration (1×10^6 viable cells/mL) or with low inoculum (1×10^3 viable cells/mL), with or without non-*Saccharomyces* (NS) ecosystem. Error bars represent standard deviation of the triplicates. The boxplots represent some parameters of interest (*lag phase*, *AF time*, *CO2tot*, *tgrowth*, *K*, *Klag*, acetic acid). Boxplots with different letter are significantly different (Duncan test following ANOVA, alpha = 0.05). Growth and growth parameters (*K*, *Klag*) are shown in log(cells/mL). For sensory analyses, triplicates of the same modality were blended (equimolar ratio). Orthonasal evaluations were conducted by 24 panelists to estimate the intensity of the fruitiness and complexity of the five resulting wines

herein as the *lag phase*. Consequently, significant differences within the modalities were detected for this parameter. In contrast, the maximal yeast population (*K*) was similar (7.79 to 7.85 log10 cells/mL). We estimated the maximal population during the lag phase, before the actual beginning of CO_2 release (*Klag*). Interestingly, for the long lag phase "Lg-LP + NS" modality, a significant increase in yeast population during the lag phase was recorded (*Klag* of 6.97 log10 cells/mL compared to 6.81–6.86 log10 cells/mL in other modalities), possibly as a consequence of NS growth.

Wine analyses

At the end of AF, several classical chemical parameters of the wine were measured (ethanol, acetic acid, glycerol, residual sugars, free and total SO_2; see Table 3). No significant differences among modalities were found, except for acetic acid that was slightly increased in the "low inoculum" modality (0.33 g/L) compared to Sh-LP (0.27 g/L) (Fig. 2). Volatile thiols (4MSP, 3SH, A3SH) and several esters were also quantified. No significant differences were recorded for volatile thiols among the different modalities, while five esters displayed abundance variation depending on the modalities (C2C3, C2iC4, C9C2, C2PhC2, dhcinnC2).

A principal component analysis was drawn based on ester abundances (Fig. 3). The first two axes explained 56.8% of the total variation of the 32 esters and showed a clear clustering based on the lag phase duration, with the modalities including long lag phase *S. cerevisiae* Lg-LP as well as the low inoculum modality with Sh-LP grouped together. Neither specific clustering according to the presence/absence of the NS consortium nor according to the *S. cerevisiae* background (Sh-LP versus Lg-LP) was evidenced.

Finally, a sensory analysis was conducted: the fruitiness and complexity of the wines resulting from the five different modalities were assessed by 24 panelists. Our results show that the modalities with actual long lag phase (i.e., modalities with either the long lag-phased *S. cerevisiae* Lg-LP or the short lag-phased Sh-LP at low inoculum) displayed significantly more intense complexity and fruitiness (Fig. 2). Long lag phases were not associated with acetic acid production, as acetate was lower for Lg-LP + NS and Sh-LP modalities.

Investigating lag phase duration through binary cultures

In order to determine whether the shortening of the long lag phase was due to a specific NS species or to the whole NS consortium, we performed binary cultures including the long lag-phased *S. cerevisiae* (Lg-LP) and either the whole NS consortium or individual NS species (Fig. 4). The fermentations were performed without stirring in order to mimic winery conditions. No significant differences among the modalities for the ethanol, residual sugars, and acetic acid were found (Table 4). Our results confirmed that lag phase varied depending on the modalities: 353 h for Lg-LP, against 179 and 181 h for Lg-LP + NS and Lg-LP + Cz, respectively. Three other NS species (*Metschnikowia* spp., *P. kluyveri*, and *T. delbrueckii*) also allowed a significant, but slighter, shortening of the lag phase (306–319 h) compared to Lg-LP pure culture. In addition, the fermentation duration varied significantly depending on the modalities: Lg-LP + NS modality displayed the shortest *AF time* (249 h), followed by Lg-LP + Cz (266 h), Lg-LP + Hu (376 h), Lg-LP + Mp (405 h) and Lg-LP, Lg-LP + Pk, and Lg-LP + Td (446, 447, and 506 h, respectively).

Table 3. Impact of the lag-phase and the non-*Saccharomyces* consortium in a Sauvignon Blanc grape must (2011 vintage)

Modalities	*CO2tot (g/L)*	*Lag-phase (h)*	*AF time (h)*	*K (log [cells/mL])*	*Klag (log [cells/mL])*	*tgrowth (h)*	Ethanol (%v/v)	Residual glucose & fructose (g/L)	Acetic acid (g/L)
Sh-LP	99 +/- 1 ns	68.1 +/- 1.4 d	243.3 +/- 9.5 ns	7.79 +/- 0.02 ns	6.86 +/- 0.06 b	56.4 +/- 3.1 d	12.59 +/- 0.04 ns	0.58 +/- 0.54 ns	0.27 +/- 0.01 bc
Sh-LP + NS	100 +/- 0 ns	69.3 +/- 2 d	229.6 +/- 12.2 ns	7.8 +/- 0.04 ns	6.82 +/- 0.02 b	60.3 +/- 2.4 d	12.63 +/- 0.04 ns	0.1 +/- 0.03 ns	0.28 +/- 0.04 bc
Sh-LP (low inoculum) + NS	100 +/- 0 ns	128.6 +/- 1.6 c	237 +/- 6.2 ns	7.81 +/- 0.02 ns	6.83 +/- 0.01 b	105.3 +/- 3.7 c	12.64 +/- 0.03 ns	0.09 +/- 0.02 ns	0.33 +/- 0.02 a
Lg-LP	99 +/- 2 ns	242.1 +/- 2.2 a	239.1 +/- 7.7 ns	7.85 +/- 0.02 ns	6.83 +/- 0.04 b	233.4 +/- 5.1 a	12.54 +/- 0.08 ns	1.3 +/- 1.61 ns	0.31 +/- 0.02 ab
Lg-LP + NS	99 +/- 1 ns	201.3 +/- 6.4 b	245 +/- 4.3 ns	7.84 +/- 0.05 ns	6.97 +/- 0.05 a	178.6 +/- 5.6 b	12.58 +/- 0.03 ns	0.18 +/- 0.17 ns	0.26 +/- 0.02 c

Modalities	Glycerol (g/L)	4MMP (ng/L)	3MH (ng/L)	A3MH (ng/L)	C3C2 (μg/L)	C4C2 (μg/L)	C6C2 (μg/L)	C8C2 (μg/L)	C10C2 (μg/L)
Sh-LP	5.52 +/- 0.16 ns	0.6 +/- 1 ns	834 +/- 285 ns	112 +/- 35 ns	68.8 +/- 3.1 ns	381 +/- 23 ns	704 +/- 169 ns	521 +/- 71 ns	81.3 +/- 10.6 ns
Sh-LP + NS	5.74 +/- 0.41 ns	0 +/- 0 ns	1005 +/- 917 ns	101 +/- 47 ns	71.7 +/- 4.5 ns	367 +/- 5 ns	628 +/- 48 ns	503 +/- 15 ns	76 +/- 11.2 ns
Sh-LP (low inoculum) + NS	5.51 +/- 0.02 ns	0 +/- 0 ns	459 +/- 14 ns	56 +/- 23 ns	69.5 +/- 4.6 ns	388 +/- 45 ns	778 +/- 122 ns	545 +/- 18 ns	79.6 +/- 6.2 ns
Lg-LP	5.57 +/- 0.16 ns	0.6 +/- 1 ns	524 +/- 126 ns	67 +/- 39 ns	62.8 +/- 1.2 ns	403 +/- 6 ns	814 +/- 119 ns	513 +/- 60 ns	77.9 +/- 5.5 ns
Lg-LP + NS	5.59 +/- 0.15 ns	0 +/- 0 ns	910 +/- 729 ns	95 +/- 62 ns	70.8 +/- 8.1 ns	398 +/- 21 ns	759 +/- 37 ns	533 +/- 33 ns	77.9 +/- 12 ns

Modalities	C12C2 (μg/L)	C2C3 (μg/L)	C2iC4 (μg/L)	C2C4 (μg/L)	C2iC5 (μg/L)	C2C6 (μg/L)	C2C8 (μg/L)	iC4C2 (μg/L)	mC4C2 (μg/L)
Sh-LP	7.87 +/- 4.41 ns	24.9 +/- 1.9 a	101 +/- 3 a	5.3 +/- 0.8 ns	6480 +/- 1779 ns	118 +/- 38 ns	0.015 +/- 0 ns	69 +/- 8 ns	3.4 +/- 0.6 ns
Sh-LP + NS	8.75 +/- 4.18 ns	23.3 +/- 0.6 ab	98 +/- 3 a	4.2 +/- 0.6 ns	6955 +/- 325 ns	95 +/- 3 ns	NaN +/- NA ns	75 +/- 4 ns	3.6 +/- 0.1 ns
Sh-LP (low inoculum) + NS	4.71 +/- 1.64 ns	19.5 +/- 2.2 c	78 +/- 15 b	4.6 +/- 0.7 ns	6236 +/- 1460 ns	107 +/- 31 ns	0.015 +/- 0 ns	79 +/- 9 ns	3.3 +/- 0.3 ns
Lg-LP	3.02 +/- 1.74 ns	21 +/- 1.2 bc	88 +/- 6 ab	4.7 +/- 0.4 ns	6585 +/- 87 ns	85 +/- 29 ns	0.015 +/- 0 ns	79 +/- 9 ns	3.1 +/- 0.1 ns
Lg-LP + NS	3.22 +/- 0.57 ns	20.2 +/- 1.3 c	86 +/- 2 ab	4.6 +/- 0.5 ns	6408 +/- 535 ns	104 +/- 8 ns	0.015 +/- 0 ns	87 +/- 6 ns	3.2 +/- 0.2 ns

Modalities	iC5C2 (μg/L)	C5C2 (μg/L)	C7C2 (μg/L)	C9C2 (μg/L)	C4C1 (μg/L)	C6C1 (μg/L)	C8C1 (μg/L)	C10C1 (μg/L)	C4iC5 (μg/L)
Sh-LP	7.5 +/- 0.8 ns	0.75 +/- 0.11 ns	0.18 +/- 0.01 ns	0.046 +/- 0.004 b	0.33 +/- 0 ns	1.08 +/- 0.16 ns	0.92 +/- 0.22 ns	0.063 +/- 0.015 ns	0.27 +/- 0.05 ns
Sh-LP + NS	7.6 +/- 0.1 ns	0.63 +/- 0.12 ns	0.23 +/- 0.02 ns	0.064 +/- 0.01 a	0.33 +/- 0 ns	1.01 +/- 0.03 ns	0.86 +/- 0.08 ns	0.061 +/- 0.009 ns	0.24 +/- 0.02 ns
Sh-LP (low inoculum) + NS	6.7 +/- 0.3 ns	0.91 +/- 0.19 ns	0.29 +/- 0.03 ns	0.045 +/- 0.004 b	0.33 +/- 0 ns	1.21 +/- 0.15 ns	1.04 +/- 0.14 ns	0.065 +/- 0.006 ns	0.29 +/- 0.07 ns
Lg-LP	6.6 +/- 0.6 ns	0.87 +/- 0.09 ns	0.37 +/- 0.2 ns	0.046 +/- 0.009 b	0.33 +/- 0 ns	1.37 +/- 0.27 ns	1.11 +/- 0.26 ns	0.059 +/- 0.004 ns	0.26 +/- 0.05 ns
Lg-LP + NS	6.4 +/- 0.9 ns	0.66 +/- 0.29 ns	0.4 +/- 0.12 ns	0.048 +/- 0.006 b	0.33 +/- 0 ns	1.19 +/- 0.04 ns	0.94 +/- 0.03 ns	0.06 +/- 0.006 ns	0.26 +/- 0.05 ns

Modalities	C6iC5 (μg/L)	C8iC5 (μg/L)	hexC2 (μg/L)	C6iC4 (μg/L)	C1 trans-ger (μg/L)	PhC2C2 (μg/L)	C2PhC2 (μg/L)	dhcinnC2 (μg/L)	cinnC2 (μg/L)
Sh-LP	1.29 +/- 0.2 ns	2.85 +/- 0.25 ns	0.378 +/- 0.089 ns	0.109 +/- 0.017 ns	0.09 +/- 0.059 ns	0.55 +/- 0.04 a	433 +/- 38 a	0.34 +/- 0 a	1.34 +/- 0.03 ns
Sh-LP + NS	1.28 +/- 0.15 ns	2.89 +/- 0.46 ns	0.338 +/- 0.02 ns	0.103 +/- 0.013 ns	0.099 +/- 0.013 ns	0.54 +/- 0.02 a	393 +/- 18 ab	0.33 +/- 0.01 a	1.33 +/- 0.01 ns
Sh-LP (low inoculum) + NS	1.22 +/- 0.27 ns	2.68 +/- 0.43 ns	0.452 +/- 0.061 ns	0.1 +/- 0.022 ns	0.043 +/- 0.004 ns	0.47 +/- 0.01 b	335 +/- 52 b	0.25 +/- 0.02 c	1.32 +/- 0.03 ns
Lg-LP	1.16 +/- 0.05 ns	2.78 +/- 0.17 ns	0.563 +/- 0.228 ns	0.113 +/- 0.035 ns	0.039 +/- 0.005 ns	0.48 +/- 0.01 b	372 +/- 4 b	0.27 +/- 0.02 c	1.32 +/- 0.05 ns
Lg-LP + NS	1.22 +/- 0.06 ns	2.84 +/- 0.07 ns	0.49 +/- 0.042 ns	0.101 +/- 0.008 ns	0.041 +/- 0.009 ns	0.52 +/- 0.05 ab	380 +/- 14 ab	0.3 +/- 0.02 b	1.32 +/- 0.04 ns

Data presented are the means of triplicates, +/- standard deviation. A Kruskal-Wallis test was used to assess significant differences between modalities: modalities sharing the same letters are not significanty different; NS stands for not-significant (alpha=0.05). For esters name, see legend of Fig. 3

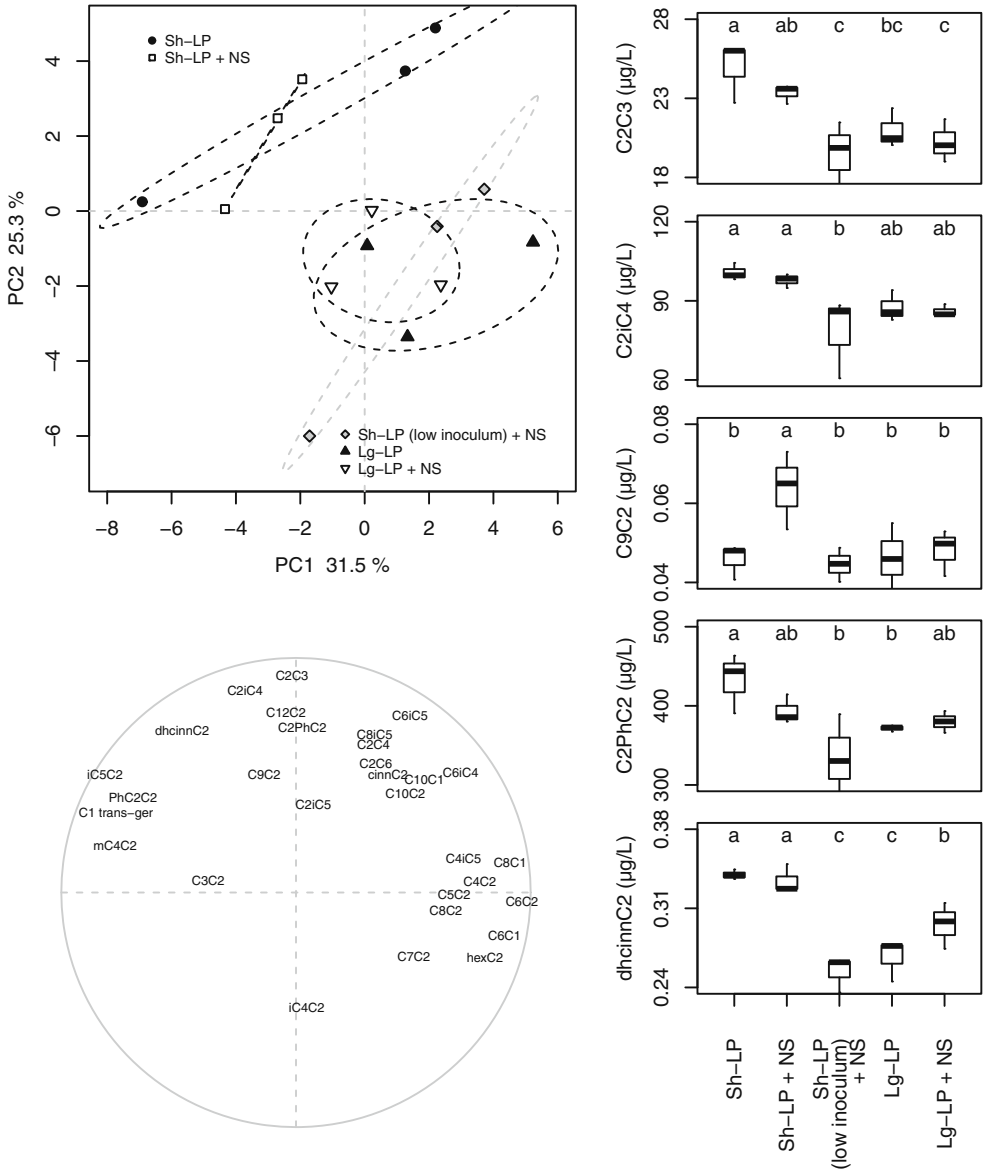

Fig. 3 Principal component analysis (PCA) of five wines on the basis of 32 ester abundances. Five modalities were tested in triplicate, including short or long lag-phased *S. cerevisiae* strain (Sh-LP and Lg-LP, respectively) inoculated at classical concentration (1×10^6 viable cells/mL) or with low inoculum (1×10^3 viable cells/mL), with or without non-*Saccharomyces* (NS) community. The first two principal components (PA) explained respectively 31.5 and 25.3% of the total variance. The boxplots represent the five esters found to be significantly different for at least one modality. Different letters indicate significantly different means (Duncan test following ANOVA, alpha = 0.05). Esters are noted as follows on the correlation circle: C2C4, butyl acetate; mC4C2, ethyl 2-methylbutyrate; C4C2, ethyl butyrate; cinnC2, ethyl cinnamate; C10C2, ethyl decanoate; dhcinnC2, ethyl dihydrocinnamate; C12C2, ethyl dodecanoate; C7C2, ethyl heptanoate; C6C2, ethyl hexanoate; iC4C2, ethyl isobutyrate; iC5C2, ethyl isovalerate, C9C2, Ethyl nonanoate; C8C2, Ethyl octanoate; PhC2C2, ethyl phenylacetate; C3C2, ethyl propanoate; hexC2, ethyl trans-2-hexenoate; C5C2, ethyl valerate; C2C6, hexyl acetate; C2iC5, isoamyl acetate; C4iC5, isoamyl butyrate; C6iC5, isoamyl hexanoate; C8iC5, isoamyl octanoate; C2iC4, isobutyl acetate; C6iC4, isobutyl hexanoate; C4C1, methyl butyrate; C10C1, methyl decanoate; C6C1, methyl hexanoate; C8C1, methyl octanoate; C1 trans-ger, methyl trans-geranate; C2C8, octyl acetate; C2PhC2, phenylethyl acetate; C2C3, propyl acetate

To assess whether acetaldehyde production was involved in the shortening of the lag phase in binary cultures with *C. zemplinina* species, additional fermentations were performed in a Sauvignon Blanc grape must (2014 vintage) with Lg-LP and *C. zemplinina* as pure species and both (Lg-LP + Cz). In the 2014 vintage, the shortening of the lag phase of Lg-LP was still significant (Table 5), with 44 h for the Lg-LP + Cz modality compared to 51 h for Lg-Lp alone. A sample was harvested at 40 h (Fig. 5), and several metabolites were measured. No significant differences were detected between Lg-LP and Lg-LP + Cz modalities for acetaldehyde, pyruvate, acetic acid, glycerol, malic acid, and total SO_2. The Cz modality was associated with decreased pyruvate and acetic acid

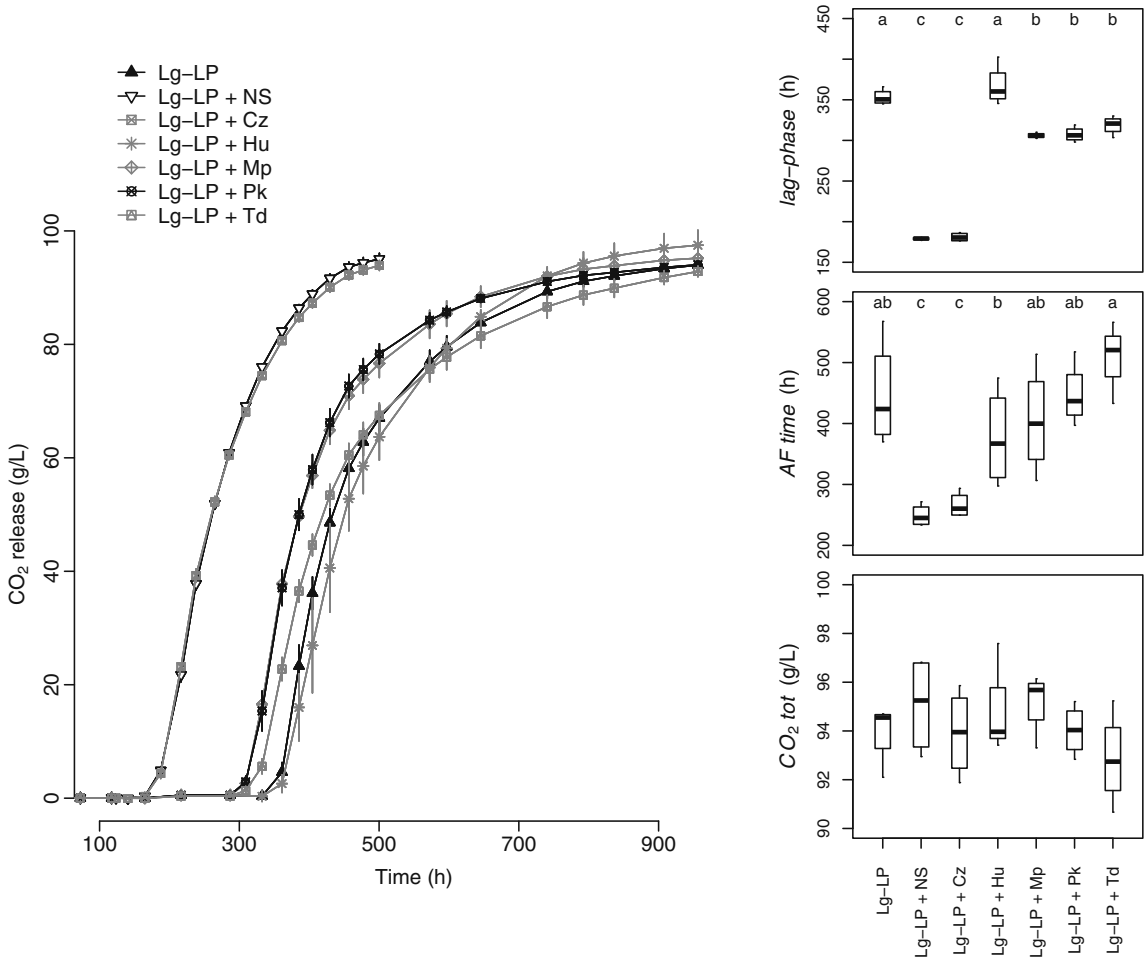

Fig. 4 Fermentation kinetics and parameters in a Sauvignon grape must for the long lag-phased *S. cerevisiae* Lg-LP, with or without NS species. Seven modalities were tested with the long lag-phased *S. cerevisiae* strain (Lg-LP), with or without non-*Saccharomyces* (NS) ecosystem, or with individual non-*Saccharomyces* noted as follows: Cz, *C. zemplinina*; Hu, *H. uvarum*; Mp, *Metschnikowia* spp.; Pk, *P. kluyveri*; Td, *T. delbrueckii*). For population kinetics, error bars represent standard deviation of the four replicates. The boxplots represent parameters of interest (*lag phase*, *AF time*, *CO2tot*). Boxplots with different letter are significantly different (Duncan test following ANOVA, alpha = 0.05)

content compared to Lg-LP + Cz. Finally, the same metabolites were measured at the end of AF, and no significant differences between Lg-LP and Lg-LP + Cz modalities were detected.

Table 4 Main characteristics of binary cultures including a long lag phase *S. cerevisiae*

Modalities	*Lag phase* (h)	*AF time* (h)	*CO_2tot* (g/L)	Ethanol (% *v/v*)	Residual glucose and fructose (g/L)	Acetic acid (g/L)
Lg-LP	353 ± 9 a	446 ± 88 a	94 ± 1 ns	12.23 ± 0.11 ns	0.91 ± 0.19 ns	0.2 ± 0.06 ns
Lg-LP + NS	179 ± 2 c	249 ± 18 c	95 ± 2 ns	12.12 ± 0.2 ns	1.07 ± 0.36 ns	0.3 ± 0.05 ns
Lg-LP + Cz	181 ± 5 c	266 ± 21 bc	94 ± 2 ns	12.37 ± 0.28 ns	0.65 ± 0.32 ns	0.24 ± 0.14 ns
Lg-LP + Hu	367 ± 25 a	376 ± 81 abc	95 ± 2 ns	12.4 ± 0.2 ns	0.72 ± 0.28 ns	0.29 ± 0.07 ns
Lg-LP + Mp	306 ± 3 b	405 ± 87 ab	95 ± 1 ns	12.37 ± 0.07 ns	0.77 ± 0.05 ns	0.23 ± 0.02 ns
Lg-LP + Pk	307 ± 9 b	447 ± 51 a	94 ± 1 ns	12.27 ± 0.24 ns	0.94 ± 0.35 ns	0.31 ± 0.05 ns
Lg-LP + Td	319 ± 11 b	506 ± 68 a	93 ± 2 ns	12.14 ± 0.14 ns	1.08 ± 0.27 ns	0.21 ± 0.1 ns

Data presented are the means of triplicated fermentations, ± standard deviation. A Kruskal-Wallis test was used to assess significant differences between modalities: modalities sharing the same letters are not significantly different

ns not significant (alpha = 0.05)

Table 5 Impact of *C. zemplinina* on the *S. cerevisiae* Lg-LP lag phase in a Sauvignon Blanc grape must (2014 vintage)

Modalities	40 h after inoculation						End of AF				
	Acetaldehyde (g/L)	Pyruvate (g/L)	Acetic acid (g/L)	Glycerol (g/L)	Malic acid (g/L)	Total SO_2 (g/L)	Acetaldehyde (g/L)	Pyruvate (g/L)	Acetic acid (g/L)	Fructose (g/L)	Glucose (g/L)
Cz	0.015 ± 0.002 ns	0.01 ± 0.008 b	0.085 ± 0.003 b	0.73 ± 0.35 ns	2 ± 0.21 ns	13.2 ± 9.1 ns	0.019 ± 0.006 ns	0.046 ± 0.012 a	0.767 ± 0.047 a	NR	NR
Lg-LP	0.014 ± 0.003 ns	0.017 ± 0.002 ab	0.129 ± 0.013 a	0.58 ± 0.49 ns	2.13 ± 0.28 ns	16.8 ± 2.3 ns	0.02 ± 0.005 ns	0 ± 0.001 b	0.134 ± 0.02 b	0 ± 0 ns	0 ± 0 ns
Lg-LP + Cz	0.016 ± 0.003 ns	0.024 ± 0.002 a	0.144 ± 0.008 a	0.12 ± 0.08 ns	1.97 ± 0.13 ns	19.6 ± 0.9 ns	0.019 ± 0.003 ns	0.001 ± 0.002 b	0.161 ± 0.016 b	0.127 ± 0.147 ns	0 ± 0 ns

Modalities	End of AF				Fermentation kinetic parameters					
	Glycerol (g/L)	Malic acid (g/L)	Total SO_2 (g/L)	Ethanol (% *v*/*v*)	*CO2max* (g/L)	*Lag phase* (h)	*AF time* (h)	*t30* (h)	*t50* (h)	*t80* (h)
Cz	6.08 ± 1.88 ns	1.11 ± 0.16 b	1.17 ± 1.55 ns	NR	67.8 ± 12.9 b	80.5 ± 4.4 a	258.4 ± 6.8 a	99.6 ± 7 a	131.5 ± 11.5 a	198.2 ± 10.5 a
Lg-LP	2.75 ± 2.07 ns	1.58 ± 0.08 a	5.13 ± 2.64 ns	11.5 ± 0.4 ns	91.1 ± 0.8 a	51.2 ± 1 b	205 ± 9.7 b	36.4 ± 0.8 b	57.4 ± 1.9 b	98.7 ± 5.2 b
Lg-LP + Cz	2.57 ± 2.97 ns	1.48 ± 0.09 a	6.06 ± 3.23 ns	11.6 ± 0.1 ns	91 ± 2.2 a	44.3 ± 1.2 c	210.3 ± 3.1 b	42.5 ± 1.7 b	63.1 ± 2.2 b	104.6 ± 3.7 b

Data presented are the means of four fermentations, ± standard deviation. A Kruskal-Wallis test was used to assess significant differences between modalities: modalities sharing the same letters are not significantly different

ns not significant (alpha = 0.05)

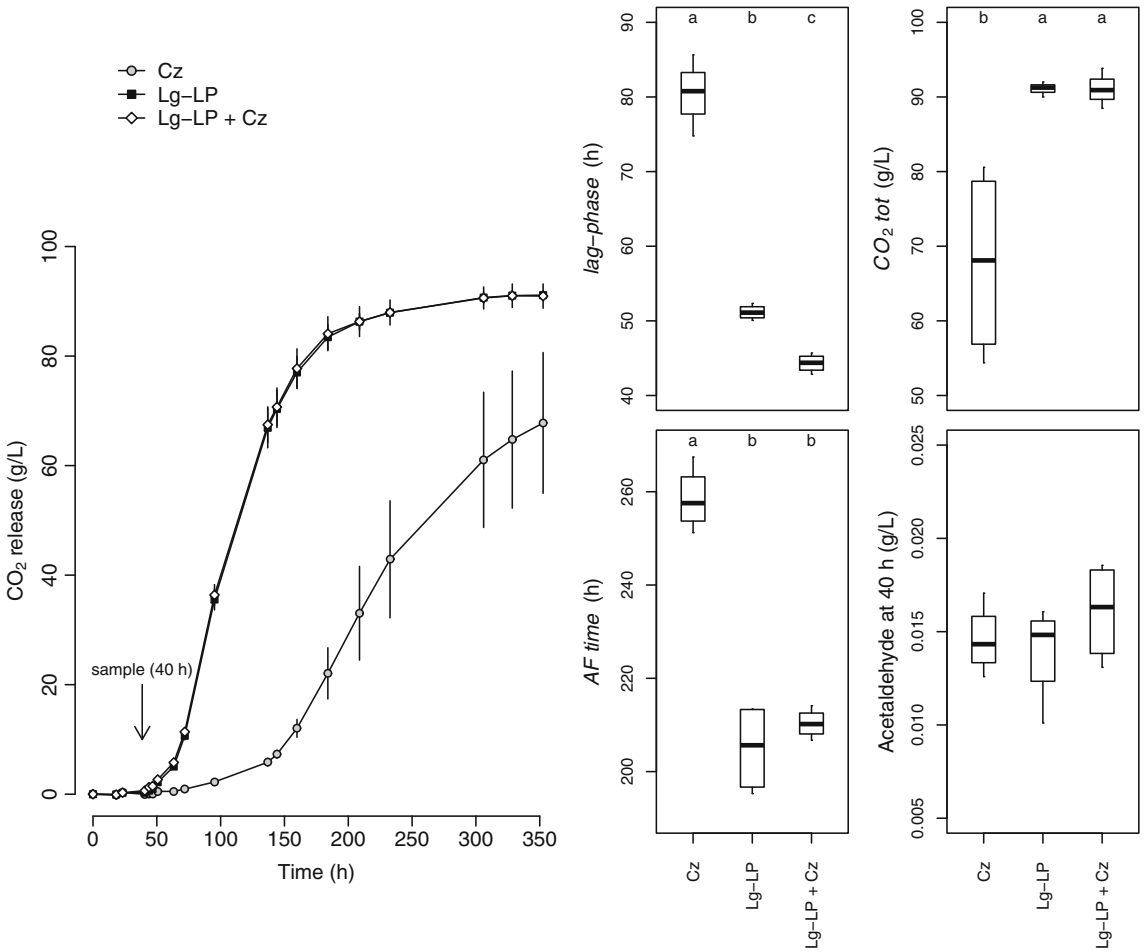

Fig. 5 Impact of *C. zemplinina* on the *fermentation kinetics* in a Sauvignon Blanc grape must (2014 vintage). Three modalities were tested: the long lag-phased *S. cerevisiae* strain (Lg-LP), the *C. zemplinina* strain (Cz), and mixed culture (Lg-LP + Cz). For population kinetics, error bars represent standard deviation of the four replicates. The boxplots represent parameters of interest (*lag phase*, *AF time*, *CO2tot*, acetaldehyde at 40 h after inoculation). Boxplots with different letter are significantly different (Duncan test following ANOVA, alpha = 0.05)

Discussion

The lag phase has a strong impact on wine composition

In this work, two nearly isogenic strains of *S. cerevisiae* showing major difference in their lag phase were successfully obtained through a breeding program based on four successive backcrosses. In our conditions, the modulation of the lag phase due to the differential inheritance of the chromosome XVI form was confirmed, with a difference in lag phase of up to 68 versus 242 h for the Sh-LP and Lg-LP, respectively, in the presence of sulfite. Both fermentation kinetics and population growth parameters were similar for the two nearly isogenic strains, as well as the acetic acid and glycerol contents. Thus, it was possible to modulate the lag phase without affecting the other fermentation properties of the strain. No significant differences concerning the volatile thiol content according to the nearly isogenic strain were shown. However, on the basis of abundance of 32 esters on the final wines, it was possible to differentiate the modalities according to the lag phase duration as revealed by the PCA analysis. Moreover, the wines fermented by the Lg-LP *S. cerevisiae* strain were judged to be more complex and fruity comparing with the wines fermented by the Sh-LP. Two different factors could explain such differences: (i) the lag phase duration itself impacts ester composition and sensory analysis and/or (ii) the genetic differences between the short and long lag phase strains (Sh-LP and Lg-LP are nearly isogenic with > 93% of identical genome) explain the differences of the ester composition and wine sensory analysis. The latter hypothesis seems unlikely, as the low inoculum modality that involved short lag phase strain (Sh-LP) displays the same pattern regarding ester composition and sensory analysis than Lg-LP, along with increased lag phase. In conclusion, it is the first time that a study suggests that the wine composition can be affected by the lag phase duration in a must that does not contain other species. The underlying mechanisms are still unclear and may range from physicochemical phenomena to enzymatic ones

(physicochemical or enzymatic reactions that may occur naturally in a grape must, provided that sufficient time is allowed) to biological processes (as a result of the specific metabolic activity of yeast strains associated with lag phase).

The availability of two nearly isogenic *S. cerevisiae* with different lag phase allowed us to modulate the duration of the prefermentation phase with the aim to promote the role of non-*Saccharomyces* before alcoholic fermentation by delaying the growth *of S. cerevisiae*. One modality based on a low inoculation rate of the Sh-LP was also added to our experimental design as an alternative strategy to postpone the growth of *S. cerevisiae*. According to previous studies on the non-*Saccharomyces* diversity and population level during the prefermentation stage, we formulated a combination of non-*Saccharomyces* based on two major species, *H. uvarum* and *C. zemplinina*, and three minor species, *P. kluyveri*, *T. delbrueckii*, and *Metschnikowia* spp., to mimic a possible natural non-*Saccharomyces* community during prefermentation phase. Thus, different prefermentation modalities associated *S. cerevisiae* (Sh-LP and Lg-LP) and non-*Saccharomyces* consortium were tested for their impact on wine composition and sensory analysis. No significant difference concerning the ethanol, glucose, and fructose was shown between *S. cerevisiae* monoculture and complex yeast consortium, except a significant albeit slight increase in acetic acid content for the Sh-LP low inoculum modality (0.27 to 0.33 g/L).

In our experimental conditions, a higher maximal population during the lag phase (*Klag*) was obtained for the "Lg-LP + NS" modality compared to the Sh-LP high and low inoculum + NS, probably due to a significant NS growth. The presence/absence of NS consortium did not impact the global ester composition of the wines. Still, some esters displayed content variation according to the lag phase duration and the presence of NS.

The wine sensorial analysis highlighted the positive impact of the long lag phase on the wine fruitiness and complexity perception, independently of the presence of NS. Our results seem to indicate that a long prefermentation stage, through either a long lag phase *S. cerevisiae* or a low inoculation rate, is associated with higher complexity and fruitiness for the resulting wines. This major effect seems slightly enhanced by the presence of NS; however, in this experiment, we could not definitively conclude concerning the positive impact of the non-*Saccharomyces* yeasts during the prefermentation stage on the wine fruitiness and complexity.

The non-*Saccharomyces* consortium impacts the lag phase of *S. cerevisiae*

Fermentation kinetic analysis revealed that the lag phase in the modality with the Lg-LP *S. cerevisiae* strain was shortened in the presence of non-*Saccharomyces* yeasts (201 h) compared to the pure *S. cerevisiae* modality (242 h, Table 3). In the modality "Lg-LP + NS," a slight growth of the NS population was noticed (37% increased of *Klag*). The presence of the NS consortium seemed to impact the growth start (*tgrowth*) of the Lg-LP strain, with 233 and 179 h for Lg-LP and Lg-LP + NS, respectively. To investigate whether this impact was due to the whole non-*Saccharomyces* community or to a given species, binary cultures were performed. Our results concerning the fermentation kinetics showed that the lag phase was shortened to a similar extent both with the non-*Saccharomyces* community and *C. zemplinina* alone and to a lesser extent with *Metschnikowia* spp., *H. uvarum*, and *P. kluyveri.* The shortening of the lag phase in the presence of *C. zemplinina* was furthermore confirmed in another grape must (2014 vintage), albeit in lesser proportion than for 2011 vintage, which is probably related to the lower amount of SO_2 in the 2014 vintage (total SO_2 of 75 and 18 mg/L for 2011 and 2014, respectively).

In winemaking conditions, different factors were reported to impact the lag phase: temperature (Marullo et al. 2009), initial sugar concentration, nutrients such as thiamin (Bataillon et al. 1996), nitrogen (Bely et al. 1990a), and SO_2 (Egli et al. 1998; Henick-Kling et al. 1998; Zimmer et al. 2014). Free SO_2 inhibits the growth of yeast by entering into the cells in its molecular form (Ingram 1948). As reviewed by Divol et al. (2012), yeast cells have different responses to the presence of SO_2 including the production of acetaldehyde and the active efflux of SO_2 by the sulfite pump Ssu1p. Both mechanisms play a major role in sulfite resistance during the first hour of alcoholic fermentation. Indeed, the expression of the *SSU1* gene was shown to be due to its genetic environment through different translocation events (Nardi et al. 2010; Perez-Ortin et al. 2002; Zimmer et al. 2014). In our study, the Lg-LP *S. cerevisiae* strain possesses the XVI-wt form which is supposed to express at a basal level the SSU1 gene. As a consequence, in the presence of SO_2, the physiology of the Lg-LP strain in the first hours after inoculation is affected as the cell has to trigger the expression regulation of *SSU1* and to pump out SO_2. This physiological adaptation delayed the initiation of growth and of fermentation. One can hypothesize that the non-*Saccharomyces* community could decrease the amount of available molecular SO_2, thus impacting the Lg-LP growth initiation and reducing the lag phase duration. The underlying mechanisms are unclear, but it has been shown that acetaldehyde production was involved in lag phase duration and yeast-yeast interactions (Cheraiti et al. 2005, 2010). However, in our case, the amount of acetaldehyde was not significantly altered in the presence of *C. zemplinina* during the lag phase, suggesting that other mechanisms could be involved. The molecular causes should be explored in a subsequent work and could be important for understanding the early yeast community dynamic in a complex must media colonized by non-*Saccharomyces* yeast and *S. cerevisiae*.

Author contributions WA, JC, VM, BCC, MBer, PM, and IMP conceived or designed the study. WA, AZ, CMS, MBel, and PM performed research, WA, JC, VM, BCC, MBel, PM, and IMP analyzed the data. WA, PM, and IMP wrote the paper.Funding InformationThis work was founded by Nouvelle Aquitaine Region, Biolaffort, and Pernod-Ricard companies.

Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest Warren Albertin, Cécile Miot-Sertier, Marina Bely, and Isabelle Masneuf-Pomarede declare that they have no conflict of interest.

Adrien Zimmer, Margaux Bernard, Joana Coulon, Virginie Moine, and Philippe Marullo are affiliated with Biolaffort company, and Benoit Colonna-Ceccaldi is affiliated with Pernod-Ricard company.

References

Albergaria H, Francisco D, Gori K, Arneborg N, Gírio F (2010) *Saccharomyces cerevisiae* CCMI 885 secretes peptides that inhibit the growth of some non-*Saccharomyces* wine-related strains. Appl Microbiol Biotechnol 86:965–972. https://doi.org/10.1007/s00253-009-2409-6

Andorra I, Landi S, Mas A, Esteve-Zarzoso B, Guillamon JM (2010) Effect of fermentation temperature on microbial population evolution using culture-independent and dependent techniques. Food Res Int 43:773–779. https://doi.org/10.1016/j.foodres.2009.11.014

Andorra I, Monteiro M, Esteve-Zarzoso B, Albergaria H, Mas A (2011) Analysis and direct quantification of *Saccharomyces cerevisiae* and *Hanseniaspora guilliermondii* populations during alcoholic fermentation by fluorescence in situ hybridization, flow cytometry and quantitative PCR. Food Microbiol 28:1483–1491. https://doi.org/10.1016/j.fm.2011.08.009

Andorrà I, Berradre M, Mas A, Esteve-Zarzoso B, Guillamón JM (2012) Effect of mixed culture fermentations on yeast populations and aroma profile. Food Sci Technol 49:8–13. https://doi.org/10.1016/j.lwt.2012.04.008

Anfang N, Brajkovich M, Goddard MR (2009) Co-fermentation with *Pichia kluyveri* increases varietal thiol concentrations in Sauvignon Blanc. Aust J Grape Wine Res 15:1–8. https://doi.org/10.1111/j.1755-0238.2008.00031.x

Antalick G, Perello M-C, de Revel G (2010) Development, validation and application of a specific method for the quantitative determination of wine esters by headspace-solid-phase microextraction-gas chromatography–mass spectrometry. Food Chem 121:1236–1245. https://doi.org/10.1016/j.foodchem.2010.01.011

Arneborg N, Siegumfeldt H, Andersen GH, Nissen P, Daria VR, Rodrigo PJ, Glückstad J (2005) Interactive optical trapping shows that confinement is a determinant of growth in a mixed yeast culture. FEMS Microbiol Lett 245:155–159. https://doi.org/10.1016/j.femsle.2005.03.008

Bataillon M, Rico A, Sablayrolles J-M, Salmon J-M, Barre P (1996) Early thiamin assimilation by yeasts under enological conditions: impact on alcoholic fermentation kinetics. J Ferment Bioeng 82:145–150. https://doi.org/10.1016/0922-338X(96)85037-9

Beckner Whitener ME, Carlin S, Jacobson D, Weighill D, Divol B, Conterno L, Du Toit M, Vrhovsek U (2015) Early fermentation volatile metabolite profile of non-*Saccharomyces* yeasts in red and white grape must: a targeted approach. Food Sci Technol 64:412–422. https://doi.org/10.1016/j.lwt.2015.05.018

Beltran G, Torija MJ, Novo M, Ferrer N, Poblet M, Guillamón JM, Rozès N, Mas A (2002) Analysis of yeast populations during alcoholic fermentation: a six year follow-up study. Syst Appl Microbiol 25:287–293. https://doi.org/10.1078/0723-2020-00097

Bely M, Sablayrolles JM, Barre P (1990a) Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in enological conditions. J Ferment Bioeng 70:246–252

Bely M, Sablayrolles JM, Barre P (1990b) Description of alcoholic fermentation kinetics—its variability and significance. Am J Enol Vitic 41:319–324

Branco P, Viana T, Albergaria H, Arneborg N (2015) Antimicrobial peptides (AMPs) produced by *Saccharomyces cerevisiae* induce alterations in the intracellular pH, membrane permeability and culturability of *Hanseniaspora guilliermondii* cells. Int J Food Microbiol 205:112–118. https://doi.org/10.1016/j.ijfoodmicro.2015.04.015

Camarasa C, Sanchez I, Brial P, Bigey F, Dequin S (2011) Phenotypic landscape of *Saccharomyces cerevisiae* during wine fermentation: evidence for origin-dependent metabolic traits. PLoS One 6:e25147. https://doi.org/10.1371/journal.pone.0025147

Chasseriaud L, Miot-Sertier C, Coulon J, Iturmendi N, Moine V, Albertin W, Bely M (2015) A new method for monitoring the extracellular proteolytic activity of wine yeasts during alcoholic fermentation of grape must. J Microbiol Methods 119:176–179. https://doi.org/10.1016/j.mimet.2015.10.025

Cheraiti N, Guezenec S, Salmon JM (2005) Redox interactions between *Saccharomyces cerevisiae* and *Saccharomyces uvarum* in mixed culture under enological conditions. Appl Environ Microbiol 71:255–260. https://doi.org/10.1128/AEM.71.1.255-260.2005

Cheraiti N, Guezenec S, Salmon JM (2010) Very early acetaldehyde production by industrial *Saccharomyces cerevisiae* strains: a new intrinsic character. Appl Microbiol Biotechnol 86:693–700. https://doi.org/10.1007/s00253-009-2337-5

Chessel D, Dufour AB, Thioulouse J (2004) The ade4 package-I: one-table methods. R News 4:5–10

Ciani M, Mannazzu I, Marinangeli P, Clementi F, Martini A (2004) Contribution of winery-resident *Saccharomyces cerevisiae* strains to spontaneous grape must fermentation. Antonie Van Leeuwenhoek 85:159–164. https://doi.org/10.1023/B:ANTO.0000020284.05802.d7

Ciani M, Beco L, Comitini F (2006) Fermentation behaviour and metabolic interactions of multistarter wine yeast fermentations. Int J Food Microbiol 108:239–245. https://doi.org/10.1016/j.ijfoodmicro.2005.11.012

Ciani M, Comitini F, Mannazzu I, Domizio P (2010) Controlled mixed culture fermentation: a new perspective on the use of non-*Saccharomyces* yeasts in winemaking. FEMS Yeast Res 10:123–133. https://doi.org/10.1111/j.1567-1364.2009.00579.x

Clemente-Jimenez J, Mingorance-Cazorla L, Martínez-rodríguez S, Heras-Vázquez FJL, Rodríguez-Vico F (2004) Molecular characterization and oenological properties of wine yeasts isolated during spontaneous fermentation of six varieties of grape must. Food Microbiol 21:149–155. https://doi.org/10.1016/S0740-0020(03)00063-7

Combina M, Elía A, Mercado L, Catania C, Ganga A, Martinez C (2005) Dynamics of indigenous yeast populations during spontaneous fermentation of wines from Mendoza, Argentina. Int J Food Microbiol 99:237–243. https://doi.org/10.1016/j.ijfoodmicro.2004.08.017

David V, Terrat S, Herzine K, Claisse O, Rousseaux S, Tourdot-Maréchal R, Masneuf-Pomarede I, Ranjard L, Alexandre H (2014) High-throughput sequencing of amplicons to monitor yeast biodiversity in must and wine. J Ind Microbiol Biotechnol 41:811–821

Divol B, Toit M, Duckitt E (2012) Surviving in the presence of sulphur dioxide: strategies developed by wine yeasts. Appl Microbiol Biotechnol 95:601–613. https://doi.org/10.1007/s00253-012-4186-x

Dufour M, Zimmer A, Thibon C, Marullo P (2013) Enhancement of volatile thiol release of *Saccharomyces cerevisiae* strains using molecular breeding. Appl Microbiol Biotechnol 97:5893–5905

Egli CM, Edinger WD, Mitrakul CM, Henick-Kling T (1998) Dynamics of indigenous and inoculated yeast populations and their effect on the sensory character of Riesling and chardonnay wines. J Appl Microbiol 85: 779–789. https://doi.org/10.1046/j.1365-2672.1998.00521.x

Fleet GH (2003) Yeast interactions and wine flavour. Int J Food Microbiol 86:11–22

Garcia A, Carcel C, Dulau L, Samson A, Aguera E, Agosin E, Günata Z (2002) Influence of a mixed culture with *Debaryomyces vanriji* and *Saccharomyces cerevisiae* on the volatiles of a Muscat wine. J Food Sci 67:1138–1143

Garofalo C, Tristezza M, Grieco F, Spano G, Capozzi V (2016) From grape berries to wine: population dynamics of cultivable yeasts associated to "Nero di Troia" autochthonous grape cultivar. World J Microbiol Biotechnol 32:59. https://doi.org/10.1007/s11274-016-2017-4

Goddard MR (2008) Quantifying the complexities of *Saccharomyces cerevisiae*'s ecosystem engineering via fermentation. Ecology 89: 2077–2082. https://doi.org/10.1890/07-2060.1

Grangeteau C, Gerhards D, Rousseaux S, von Wallbrunn C, Alexandre H, Guilloux-Benatier M (2015) Diversity of yeast strains of the genus *Hanseniaspora* in the winery environment: what is their involvement in grape must fermentation? Food Microbiol 50:70–77. https://doi.org/10.1016/j.fm.2015.03.009

Henick-Kling T, Edinger W, Daniel P, Monk P (1998) Selective effects of sulfur dioxide and yeast starter culture addition on indigenous yeast populations and sensory characteristics of wine. J Appl Microbiol 84:865–876

Hu K, Qin Y, Tao YS, Zhu XL, Peng CT, Ullah N (2016) Potential of glycosidase from non-*Saccharomyces* isolates for enhancement of wine aroma. J Food Sci 81:M935–M943. https://doi.org/10.1111/1750-3841.13253

Huxley C, Green ED, Dunham I (1990) Rapid assessment of *S. cerevisiae* mating type by PCR. Trends Genet 6:236

Ingram M (1948) The germicidal effects of free and combined sulphur dioxide. J Chem Technol Biotechnol 67:18–21. https://doi.org/10.1002/jctb.5000670107

Jolly NP, Augustyn OPH, Pretorius IS (2003) The occurrence of non-*Saccharomyces cerevisiae* yeast species over three vintages in four vineyards and grape musts from four production regions of the western cape. S Afr J Enol Vitic 24:35–42

Jolly NP, Varela C, Pretorius IS (2014) Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. FEMS Yeast Res 14:215–237. https://doi.org/10.1111/1567-1364.12111

Li S-S, Cheng C, Li Z, Chen J-Y, Yan B, Han B-Z, Reeves M (2010) Yeast species associated with wine grapes in China. Int J Food Microbiol 138: 85–90. https://doi.org/10.1016/j.ijfoodmicro.2010.01.009

Marullo P, Bely M, Masneuf-Pomarede I, Pons M, Aigle M, Dubourdieu D (2006) Breeding strategies for combining fermentative qualities and reducing off-flavor production in a wine yeast model. FEMS Yeast Res 6:268–279. https://doi.org/10.1111/j.1567-1364.2006.00034.x

Marullo P, Aigle M, Bely M, Masneuf-Pomarede I, Durrens P, Dubourdieu D, Yvert G (2007) Single QTL mapping and nucleotide-level resolution of a physiologic trait in wine *Saccharomyces cerevisiae* strains. FEMS Yeast Res 7:941–952. https://doi.org/10.1111/j.1567-1364.2007.00252.x

Marullo P, Mansour C, Dufour M, Albertin W, Sicard D, Bely M, Dubourdieu D (2009) Genetic improvement of thermo-tolerance in wine *Saccharomyces cerevisiae* strains by a backcross approach. FEMS Yeast Res 9:1148–1160

Masneuf-Pomarede I, Juquin E, Miot-Sertier C, Renault P, Laizet Y, Salin F, Alexandre H, Capozzi V, Cocolin L, Colonna-Ceccaldi B, Englezos V, Girard P, Gonzalez B, Lucas P, Mas A, Nisiotou A, Sipiczki M, Spano G, Tassou C, Bely M, Albertin W (2015) The yeast *Starmerella bacillaris* (synonym *Candida zemplinina*) shows high genetic diversity in winemaking environments. FEMS Yeast Res 15:fov045. https://doi.org/10.1093/femsyr/fov045

Mateo JJ, Jimenez M, Huerta T, Pastor A (1991) Contribution of different yeasts isolated from musts of monastrell grapes to the aroma of wine. Int J Food Microbiol 14:153–160. https://doi.org/10.1016/0168-1605(91)90102-U

Meillon S, Viala D, Medel M, Urbano C, Guillot G, Schlich P (2010) Impact of partial alcohol reduction in Syrah wine on perceived complexity and temporality of sensations and link with preference. Food Qual Prefer 21: 732–740. https://doi.org/10.1016/j.foodqual.2010.06.005

Nardi T, Corich V, Giacomini A, Blondin B (2010) A sulphite-inducible form of the sulphite efflux gene *SSU1* in a *Saccharomyces cerevisiae* wine yeast. Microbiology 156:1686–1696. https://doi.org/10.1099/mic.0.036723-0

Nisiotou AA, Nychas GJ (2007) Yeast populations residing on healthy or botrytis-infected grapes from a vineyard in Attica, Greece. Appl Environ Microbiol 73:2765–2768. https://doi.org/10.1128/AEM.01864-06

Nissen P, Arneborg N (2003) Characterization of early deaths of non-*Saccharomyces* yeasts in mixed cultures with *Saccharomyces cerevisiae*. Arch Microbiol 180:257–263. https://doi.org/10.1007/s00203-003-0585-9

Padilla B, Gil JV, Manzanares P (2016) Past and future of non-*Saccharomyces* yeasts: from spoilage microorganisms to biotechnological tools for improving wine aroma complexity. Front Microbiol 7:411. https://doi.org/10.3389/fmicb.2016.00411

Pate JB, Lodge JP, Wartburg AF (1962) Effect of pararosaniline in the trace determination of sulfur dioxide. Anal Chem 34:1660–1662. https://doi.org/10.1021/ac60192a001

Perez-Ortin JE, Querol A, Puig S, Barrio E (2002) Molecular characterization of a chromosomal rearrangement involved in the adaptive evolution of yeast strains. Genome Res 12:1533–1539. https://doi.org/10.1101/gr.436602

Pérez G, Fariña L, Barquet M, Boido E, Gaggero C, Dellacassa E, Carrau F (2011) A quick screening method to identify β-glucosidase activity in native wine yeast strains: application of Esculin Glycerol Agar (EGA) medium. World J Microbiol Biotechnol 27:47–55. https://doi.org/10.1007/s11274-010-0425-4

Pfliegler WP, Horvath E, Kallai Z, Sipiczki M (2014) Diversity of *Candida zemplinina* isolates inferred from RAPD, micro/minisatellite and physiological analysis. Microbiol Res 169:402–410. https://doi.org/10.1016/j.micres.2013.09.006

Pretorius IS (2000) Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. Yeast 16:675–729. https://doi.org/10.1002/1097-0061(20000615)16:8<675::AID-YEA585>3.0.CO;2-B

Development Core Team R (2010) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria

Renault PE, Albertin W, Bely M (2013) An innovative tool reveals interaction mechanisms among yeast populations under oenological conditions. Appl Microbiol Biotechnol 97:4105–4119. https://doi.org/10.1007/s00253-012-4660-5

Renault P, Coulon J, de Revel G, Barbe JC, Bely M (2015) Increase of fruity aroma during mixed *T. delbrueckii/S. cerevisiae* wine fermentation is linked to specific esters enhancement. Int J Food Microbiol 207:40–48. https://doi.org/10.1016/j.ijfoodmicro.2015.04.037

Rodriguez ME, Lopes CA, Barbagelata RJ, Barda NB, Caballero AC (2010) Influence of *Candida pulcherrima* Patagonian strain on alcoholic fermentation behaviour and wine aroma. Int J Food Microbiol 138:19–25. https://doi.org/10.1016/j.ijfoodmicro.2009.12.025

Rojas V, Gil JV, Pinaga F, Manzanares P (2001) Studies on acetate ester production by non-*Saccharomyces* wine yeasts. Int J Food Microbiol 70:283–289

Romano P, Suzzi G, Comi G, Zironi R, Maifreni M (1997) Glycerol and other fermentation products of apiculate wine yeasts. J Appl Microbiol 82:615–618

Rossouw D, Bauer FF (2016) Exploring the phenotypic space of non-*Saccharomyces* wine yeast biodiversity. Food Microbiol 55:32–46. https://doi.org/10.1016/j.fm.2015.11.017

Sadoudi M, Tourdot-Marechal R, Rousseaux S, Steyer D, Gallardo-Chacon JJ, Ballester J, Vichi S, Guerin-Schneider R, Caixach J, Alexandre H (2012) Yeast-yeast interactions revealed by aromatic profile analysis of Sauvignon Blanc wine fermented by single or co-culture of non-*Saccharomyces* and *Saccharomyces* yeasts. Food Microbiol 32:243–253. https://doi.org/10.1016/j.fm.2012.06.006

Salvadó Z, Arroyo-López FN, Barrio E, Querol A, Guillamón JM (2011) Quantifying the individual effects of ethanol and temperature on the fitness advantage of *Saccharomyces cerevisiae*. Food Microbiol 28: 1155–1161. https://doi.org/10.1016/j.fm.2011.03.008

Stitt M, Lilley RM, Gerhardt R, Heldt HW (1989) [32] Metabolite levels in specific cells and subcellular compartments of plant leaves. Meth Enzymol 174:518–552. https://doi.org/10.1016/0076-6879(89)74035-0

Swiegers JH, Pretorius IS (2005) Yeast modulation of wine flavor. Adv Appl Microbiol 57:131–175. https://doi.org/10.1016/S0065-2164(05)57005-9

Tofalo R, Schirone M, Torriani S, Rantsiou K, Cocolin L, Perpetuini G, Suzzi G (2012) Diversity of *Candida zemplinina* strains from grapes and Italian wines. Food Microbiol 29:18–26. https://doi.org/10.1016/j.fm.2011.08.014

Tominaga T, Furrer A, Henry R, Dubourdieu D (1998) Identification of new volatile thiols in the aroma of *Vitis vinifera* L. var. Sauvignon blanc wines. Flavour Fragr J 13:159–162. https://doi.org/10.1002/(sici)1099-1026(199805/06)13:3<159::aid-ffj709>3.0.co;2-7

Tristezza M, Vetrano C, Bleve G, Spano G, Capozzi V, Logrieco A, Mita G, Grieco F (2013) Biodiversity and safety aspects of yeast strains characterized from vineyards and spontaneous fermentations in the Apulia Region, Italy. Food Microbiol 36:335–342. https://doi.org/10.1016/j.fm.2013.07.001

Varela C, Siebert T, Cozzolino D, Rose L, MCLean H, Henschke PA (2009) Discovering a chemical basis for differentiating wines made by fermentation with 'wild' indigenous and inoculated yeasts: role of yeast volatile compounds. Aust J Grape Wine Res 15:238–248

Viana F, Gil JV, Genovés S, Vallés S, Manzanares P (2008) Rational selection of non-*Saccharomyces* wine yeasts for mixed starters based on ester formation and enological traits. Food Microbiol 25: 778–785. https://doi.org/10.1016/j.fm.2008.04.015

Wang C, Esteve-Zarzoso B, Mas A (2014) Monitoring of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum*, and *Starmerella bacillaris* (synonym *Candida zemplinina*) populations during alcoholic fermentation by fluorescence in situ hybridization. Int J Food Microbiol 191: 1–9. https://doi.org/10.1016/j.ijfoodmicro.2014.08.014

Wang C, Mas A, Esteve-Zarzoso B (2015) Interaction between *Hanseniaspora uvarum* and *Saccharomyces cerevisiae* during alcoholic fermentation. Int J Food Microbiol 206:67–74. https://doi.org/10.1016/j.ijfoodmicro.2015.04.022

Zimmer A, Durand C, Loira N, Durrens P, Sherman DJ, Marullo P (2014) QTL dissection of lag phase in wine fermentation reveals a new translocation responsible for *Saccharomyces cerevisiae* adaptation to sulfite. PLoS One 9:e86298. https://doi.org/10.1371/journal.pone.0086298

Zott K, Miot-Sertier C, Claisse O, Lonvaud-Funel A, Masneuf-Pomarede I (2008) Dynamics and diversity of non-*Saccharomyces* yeasts during the early stages in winemaking. Int J Food Microbiol 125:197–203. https://doi.org/10.1016/j.ijfoodmicro.2008.04.001

Zott K, Claisse O, Lucas P, Coulon J, Lonvaud-Funel A, Masneuf-Pomarede I (2010) Characterization of the yeast ecosystem in grape must and wine using real-time PCR. Food Microbiol 27:559–567. https://doi.org/10.1016/j.fm.2010.01.006

Zott K, Thibon C, Bely M, Lonvaud-Funel A, Dubourdieu D, Masneuf-Pomarede I (2011) The grape must non-*Saccharomyces* microbial community: impact on volatile thiol release. Int J Food Microbiol 151:210–215. https://doi.org/10.1016/j.ijfoodmicro.2011.08.026