Food Microbiology 32 (2012) 243–253

Contents lists available at SciVerse ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Yeast–yeast interactions revealed by aromatic profile analysis of Sauvignon Blanc wine fermented by single or co-culture of non-*Saccharomyces* and *Saccharomyces* yeasts

Mohand Sadoudi [a], Raphaëlle Tourdot-Maréchal [a], Sandrine Rousseaux [a], Damien Steyer [d], Joan-Josep Gallardo-Chacón [a], Jordi Ballester [c], Stefania Vichi [e], Rémi Guérin-Schneider [b], Josep Caixach [e], Hervé Alexandre [a,*]

[a] UMRA 02 102 PAM laboratoire VALMIS, Institut Universitaire de la Vigne et du Vin Jules Guyot, Université de Bourgogne, 21078 Dijon cedex, France
[b] Institut Français de la Vigne et du Vin UMT QUALINNOV, Pôle Rhône Méditerranée, 2 Place Pierre Viala Bâtiment 28, 34060 Montpellier, France
[c] Centre des Sciences du Goût et de l'Alimentation, UMR6265 CNRS, UMR1324 INRA, Université de Bourgogne, Agrosup Dijon, F-21000 Dijon, 15, rue Hugues Picardet, 21000 Dijon, France
[d] TWISTAROMA, 28 rue de Herrlisheim, 68021 Colmar, France
[e] Laboratory of Mass Spectrometry/Organic Pollutants, IDAEA-CSIC, Jordi Girona, 18-26 08034 Barcelona, Spain

ARTICLE INFO

Article history:
Received 20 March 2012
Received in revised form
27 June 2012
Accepted 27 June 2012
Available online 13 July 2012

Keywords:
Non-*Saccharomyces*
Saccharomyces
Yeast interactions
Wine
Volatile thiols
Torulaspora delbrueckii
Metschnikowia pulcherrima
Candida zemplinina
Wine aroma

ABSTRACT

There has been increasing interest in the use of selected non-*Saccharomyces* yeasts in co-culture with *Saccharomyces cerevisiae*. The main reason is that the multistarter fermentation process is thought to simulate indigenous fermentation, thus increasing wine aroma complexity while avoiding the risks linked to natural fermentation. However, multistarter fermentation is characterised by complex and largely unknown interactions between yeasts. Consequently the resulting wine quality is rather unpredictable. In order to better understand the interactions that take place between non-*Saccharomyces* and *Saccharomyces* yeasts during alcoholic fermentation, we analysed the volatile profiles of several mono-culture and co-cultures. *Candida zemplinina*, *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* were used to conduct fermentations either in mono-culture or in co-culture with *S. cerevisiae*. Up to 48 volatile compounds belonging to different chemical families were quantified. For the first time, we show that *C. zemplinina* is a strong producer of terpenes and lactones. We demonstrate by means of multivariate analysis that different interactions exist between the co-cultures studied. We observed a synergistic effect on aromatic compound production when *M. pulcherrima* was in co-culture with *S. cerevisiae*. However a negative interaction was observed between *C. zemplinina* and *S. cerevisiae*, which resulted in a decrease in terpene and lactone content. These interactions are independent of biomass production. The aromatic profiles of *T. delbrueckii* and *S. cerevisiae* in mono-culture and in co-culture are very close, and are biomass-dependent, reflecting a neutral interaction. This study reveals that a whole family of compounds could be altered by such interactions. These results suggest that the entire metabolic pathway is affected by these interactions.

1. Introduction

Wine production is based on spontaneous fermentations or inoculated must fermentations; in both cases, *Saccharomyces* plays a major role. Spontaneous grape juice fermentations sometimes become stuck or sluggish. This lack of reproducibility and predictability has favoured the use of active dry yeast, generally strains of *Saccharomyces cerevisiae*. However, other non-*Saccharomyces* species are also responsible for alcoholic fermentation and can contribute to the sensory characteristics of the final product. Non-*Saccharomyces* yeasts have been shown to have a positive impact on wine organoleptic characteristics (Ciani et al., 2010). Nevertheless, selected *S. cerevisiae* strains predominate in starter cultures to ensure rapid and reliable grape juice fermentation and, thus, consistent and predictable wine quality. However, there has been increasing recognition that wines made with *Saccharomyces* starter cultures are less complex, producing standardised wines (Rainieri and Pretorius, 2000; Mannazzu et al., 2002).

* Corresponding author. Tel.: +33 80 39 63 93; fax: +33 80 39 62 65.
E-mail address: rvalex@u-bourgogne.fr (H. Alexandre).

0740-0020/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fm.2012.06.006

In the last few years, there has been increased interest in the role of non-*Saccharomyces* yeast during the alcoholic fermentation of grape juice. These studies have shown that non-*Saccharomyces* species could have an impact on the chemical composition of the wine and that the contribution of these yeasts to wine character is significant (Rodríguez et al., 2010; Comitini et al., 2011). Unfortunately, fermentation conducted with natural flora brings diminished predictability of the process and inconsistencies in wine quality. For these reasons an alternative process has gained interest, in particular the use of non-*Saccharomyces* starter cultures together with *Saccharomyces* starters.

Multistarters containing non-*Saccharomyces* and *Saccharomyces* strains have been shown to mimic natural fermentation, avoiding the risk of stuck fermentations (Bisson and Kunkee, 1993; Heard, 1999; Rojas et al., 2003; Romano, 2003; Ciani et al., 2006; Jolly et al., 2006). For example, it has been shown that *Torulaspora delbrueckii*/*S. cerevisiae* co-cultures produce less volatile acidity and acetaldehyde in high sugar fermentation (Bely et al., 2008).

When fermentations are conducted with different yeasts, complex interactions between organisms occur (Fleet, 2003; Alexandre et al., 2004). Great differences have been shown in the metabolism of *S. cerevisiae* in single and in co-culture with *Kloeckera apiculata* or *T. delbrueckii*. Moreira et al. (2005) reported an increase in the quantity of desirable compounds, such as higher alcohols and esters, when *S. cerevisiae* co-fermented with *Hanseniaspora uvarum*. However, all these studies investigated a rather low number of compounds belonging basically to higher alcohol and ester families (Zohre and Erten, 2002; Clemente-Jimenez et al., 2005; Viana et al., 2009) and give no information about yeast interactions. Howell et al. (2006) compared the metabolic profile of several *S. cerevisiae* strains in mono-culture and in co-culture. The principal component analysis showed that the profiles of compounds present in wines made by co-culture fermentation were different from those where yeasts were grown in mono-culture fermentation. Interestingly, the authors demonstrated that mixed cultures of *Saccharomyces* wine yeasts gave a combination of volatile aroma substances distinctly different from those in wines made by blending together mono-culture wines made with the same component yeast strains. These results indicate metabolic interaction between component strain and species. The final flavour of the wine is determined in part by the composite of volatile aroma compounds produced by the co-culture reaction (Lambrechts and Pretorius, 2000; Fleet, 2003). More information is needed to understand the growth profiles of particular species and strains of wine yeasts in co-culture and their impact on the production of aroma volatiles.

In this study, we compared the metabolic profiles of *Saccharomyces* and three non-*Saccharomyces* yeasts in single and co-cultures in order to elucidate the nature of the interactions between yeasts during the alcoholic fermentation of Sauvignon Blanc must.

2. Materials and methods

2.1. Yeast strains

The yeast strains used in this study are listed in Table 1. *S. cerevisiae* neutral killer commercial strain PB2023 (purchased as active dry yeast by SPINDAL-AEBgroup, France) was used as the fermentation control strain during culture experiments. The non-*Saccharomyces* strains were previously isolated from samples of grape juice from Burgundy wineries, working in spontaneous fermentation. The yeast species were identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of internal transcribed spacers (ITS), as described in Esteve-Zarzoso et al. (1999).

All the strains were conserved in a YPD medium (20 g l^{-1} glucose, 5 g l^{-1} yeast extract, 10 g l^{-1} peptone, 0.2 g l^{-1} chloramphenicol, 20 g l^{-1} Agar), containing 50% glycerol at 40% (v/v), kept frozen at −80 °C.

2.2. DNA sequencing and sequence analysis

To confirm the identification of isolated strains, sequencing was performed on 5.8S-ITS DNA fragments generated by PCR. The primers ITS1/ITS4 were used. Beckman Coulter Genomics (England) sequenced the PCR products in both orientations. The sequences obtained were compared with sequences available in the EMBL database using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). These sequences were aligned with ClustalW and restriction fragments with the http://biotools.umassmed.edu/tacg4/ program.

2.3. Fermentations

Fermentations were carried out for the four strains and their mixed cultures with *S. cerevisiae* PB2023.

2.3.1. Pure cultures

Pure cultures were carried out in 1 l sterile Erlenmeyer flasks, closed with dense cotton plugs, containing 400 ml of pasteurised (10 min at 100 °C) Sauvignon Blanc grape must (110 g l^{-1} glucose, 109 g l^{-1} fructose, 2.77 g l^{-1} L-malic acid, 588 mg l^{-1} total nitrogen, pH 3.45) supplemented with sulphur dioxide (30 mg l^{-1}) (vintage 2010). Each experiment was performed in triplicate. Yeasts were pre-cultured in a YPD liquid medium at 30 °C for 48 h. Then pasteurised Sauvignon Blanc grape must was inoculated with 10^6 cells ml^{-1} and incubated at 20 °C, without agitation (semi-anaerobic conditions). Fermentation progress and yeast growth were monitored throughout the fermentation process, by assessing sugar concentration and by viable cell counts. Cell viability was determined by flow cytometry using fluorescein diacetate (FDA) as a marker of viability (Malacrinò et al., 2001).

2.3.2. Mixed cultures

Mixed cultures were grown in 1 l Erlenmeyer flasks containing 400 ml pasteurised Sauvignon Blanc grape must supplemented with sulphur dioxide (30 mg l^{-1}) (vintage 2010) as described previously. Each experiment was performed in triplicate. The pasteurised Sauvignon Blanc grape must was inoculated with 48-h pre-cultures grown in a YPD liquid medium at 30 °C, as follows: the non-*Saccharomyces* strain (initial population 10^7 cells ml^{-1}) and *S. cerevisiae* PB2023 strain (initial population 10^6 cells ml^{-1}) (ratio 10:1). The must was incubated at 20 °C, without agitation (semi-

Table 1
Yeast strains used in this study.

Strains	Species	PCR amplified product	Restriction fragments			Accession number	Origin
			*Cfo*I	*Hae*III	*Hin*fI		
MCR-24	*Metchnikowia pulcherrima*	400	205 + 100+95	280 + 100	200 + 190	JX234570	Pinot noir juice (grape harvest 2010)
MCR-9	*Candida zemplinina*	475	215 + 110+80 + 60	475	235 + 235	JX234569	Pinot noir juice (grape harvest 2010)
BB-MV:3FA5	*Torulaspora delbrueckii*	800	330 + 220+150 + 100	800	410 + 380	JX234568	Chardonnay juice (grape harvest 2010)
PB2023	*Saccharomyces cerevisiae*	–	–	–	–	–	SPINDAL-AEBgroup, France

anaerobic conditions). *S. cerevisiae* PB2023 was inoculated 24-h after *T. delbrueckii* and *Candida zemplinina* and 48-h after *Metschnikowia pulcherrima* strains. In the case of the co-culture *S. cerevisiae*/*M. Pulcherrima, S. cerevisiae* was inoculated after 48 h instead of 24 h, otherwise a dramatic decrease in *M. pulcherrima* viability was observed. Samples were taken from each flask throughout the fermentation process to perform viable cell counts. One hundred µl aliquots of serial dilutions of each sample were plated onto both YPD medium (total yeast population) and Lysine Agar medium (non-*Saccharomyces* yeast population). Lysine Agar medium (Oxoid LTD, Hampshire, England) is a selective medium which does not support the growth of *S. cerevisiae* (Lin, 1975), and was therefore used for the viable count of the non-*Saccharomyces* yeasts cultured in mixed fermentation.

After fermentation, in both pure and mixed cultures, the fermented musts were centrifuged (10 min at 7000 rpm) to remove yeast cells. 50 mg l^{-1} of SO_2 was added to fermented musts, which were kept at 4 °C before analysis.

2.4. Analytical determinations

Glucose, fructose, acetic acid and ethanol were determined by enzymatic kits following the manufacturer's instructions (Bio-SenTec, France). Total acidity was determined by potentiometric method on decarbonated wine and then titrated by NaOH 0.1 N solution to pH 7. The result is expressed in g l^{-1} H_2SO_4.

Volatile aroma compounds were analysed with the Stir Bar Sorptive Extraction Liquid Desorption method (SBSE-LD) (Coelho et al., 2009) adapted to our laboratory conditions. Each wine sample (20 ml) was spiked with 2 mg l^{-1} of 3-octanol and ethyl heptanoate (Fluka) as internal standard. The analyses were performed with an Agilent 6890N gas chromatograph equipped with an Agilent 7683 automatic liquid sampler coupled to an Agilent 5975B inert MSD (Agilent Technologies), 1 µl of solvent was injected. The gas chromatograph was fitted with a DB-Wax capillary column (60 m × 0.32 mm i.d. × 0.50 µm film thickness, J&W Scientific) and helium was used as carrier gas (1 ml min^{-1} constant flow). The GC oven temperature was programmed without initial hold time at a rate of 2.7 °C min^{-1} from 70 °C to 235 °C (hold 10 min). The injector was set to 250 °C and used in pulsed splitless mode (25 psi for 0.50 min). The temperatures of the interface, MS ion source and quadrupole were 270 °C, 230 °C and 150 °C, respectively. The Mass spectrometer was operated in electron impact ionisation mode (EI, 70 eV). The MS analyses were performed in full scan mode (TIC mode) with a scan range of 29–300 amu. Agilent MSD ChemStation software (G1701DA, Rev D.03.00) was used for instrument control and data processing. Identification of the compounds was performed using the NIST 05 mass spectral database, retention indices (RIs) reported in the literature (Tao and Zhang, 2010) and authentic standards (Fluka). For semi-quantification purposes, the relative peak areas of the 44 analytes were divided by the relative peak area of the 3-octanol (for alcohol, aldehyde and terpenol compounds) and by the relative peak area of the ethyl heptanoate for the other compounds, then multiplied by 2000 (data are given in µg l^{-1} relative to internal standard).

The thiol analysis was performed using the method reported by Rodríguez-Bencomo et al. (2009). Deuterated internal standards (3 MH d2, 500 ng l^{-1}; 3MHA d5, 50 ng l^{-1}; 4MMP d10, 20 ng l^{-1}) were added to each wine sample (100 ml). Then EDTA (250 mg), cystein (158 mg) and o-methylhydroxylamine (100 mg) were added. The flask was purged with nitrogen and heated at 50 °C for 45 min. After cooling, pH was adjusted to 7 with an NaOH solution, and the thiols were extracted using a C18 cartridge (500 mg Bond Elut-ENV, Varian), previously conditioned with 5 ml methylene chloride, 5 ml methanol and 5 ml water. After the sample elution, various solution were passed through the cartridge: 20 ml phosphate buffer (0.2 M in 40% methanol solution), 5 ml distilled water, 5 ml 1,8-Diazabicyclo-[5,4,0]-undec-7-ene (DBU, 6.7% in water), 1 ml 2,3,4,5,6-Pentaflurobenzylbromide (PFBBr, 200 mg l^{-1} in hexane) wich was allowed to react for 20 min, 1 ml mercaptoglycerol solution (400 mg l^{-1} in DBU solution (6.7%), 20 min) and 5 ml water. The derivatised thiols were finally eluted using a mixture of hexane/diethyl ether (4 mL, 1/3 v/v). The extract was washed with a NaCl solution (5 × 1 ml, 200 g l^{-1}), dried over anhydrous sodium sulphate, and concentrated to dryness under nitrogen flow. The analysis was performed by SPME using a Divinylbenzene/carboxen/PDMS fibre (2 cm, Supelco). The analytes were trapped on the fibre at 110 °C for 30 min and then thermically desorbed into the GC–MS injector at 270 °C for 10 min (splitless mode). The GC was equipped with an Optima-Wax capillary column (30 m × 0.25 mm × 0, 25 µm, Macherey–Nagel), programmed as follows : isotherm at 80 °C for 10 min, increase to 220 °C at 5 °C min^{-1}, increase to 245 °C at 15 °C min^{-1} and isotherm at 245 °C for 15 min. Detection was performed by negative chemical ionisation using methane and Single Ion Monitoring.

2.5. Statistical analysis

For all three replicates, outliers were removed from the raw data using the Q test (Rorabacher, 1991). Metabolite concentrations were then submitted to one-way Analysis of Variance (ANOVA) in order to test for significant differences between the wines, whether from mono-culture or co-culture. When significance was reached, a Tukey (HSD) post-hoc test (confidence interval: 95%) was performed. Principal component analysis (PCA) was also carried out on the concentration of volatile compounds (raw and normalised to the initial population) in order to visualise relationships between variables and between samples and variables. A second PCA was conducted on metabolite concentrations after data normalisation to the final concentration of cells (calculated in µg 10^6 $cells^{-1}$) for all samples. For *M. pulcherrima*/*S. cerevisiae* co-culture data, the final cell concentration was calculated with the final *S. cerevisiae* population plus the initial population of *M. pulcherrima*. The reason for this exception is that, unlike the other co-cultures, the *M. pulcherrima* population decreased dramatically by the fourth day when cultured with *S. cerevisiae* (see Results). Hierarchical Cluster Analysis (HCA) with the Ward criteria was finally applied to the factorial coordinates of the wines in the spaces defined by both PCA in order to determine which groups of samples are different according to their chemical composition. Statistical analyses were performed with XLSTAT version 7.5.2 software.

3. Results and discussion

3.1. Biomass evolution and sugar consumption in single and co-cultures

Yeast growth and fermentation kinetics were monitored for each single culture and co-culture (Fig. 1). The single culture of *S. cerevisiae* PB2023 completed alcoholic fermentation in 7 days with a maximal viable population of 1.46 10^8 cells ml^{-1} (Fig. 1A). The fermentation kinetic of the pure culture of *C. zemplinina* MCR-9 indicated a maximal population (2 × 10^8 cells ml^{-1}) obtained after 6 days with almost total fructose consumption (Fig. 1B). The fructophilic character of *C. zemplinina* has already been shown in previous works (Soden et al., 2000; Mills et al., 2002; Magyar and Tóth, 2011; Tofalo et al., 2012). *C. zemplinina* fermented glucose at a significantly lower rate (22 days) (Table 2). The maximal population obtained with the single culture of *M. pulcherrima* MCR-24

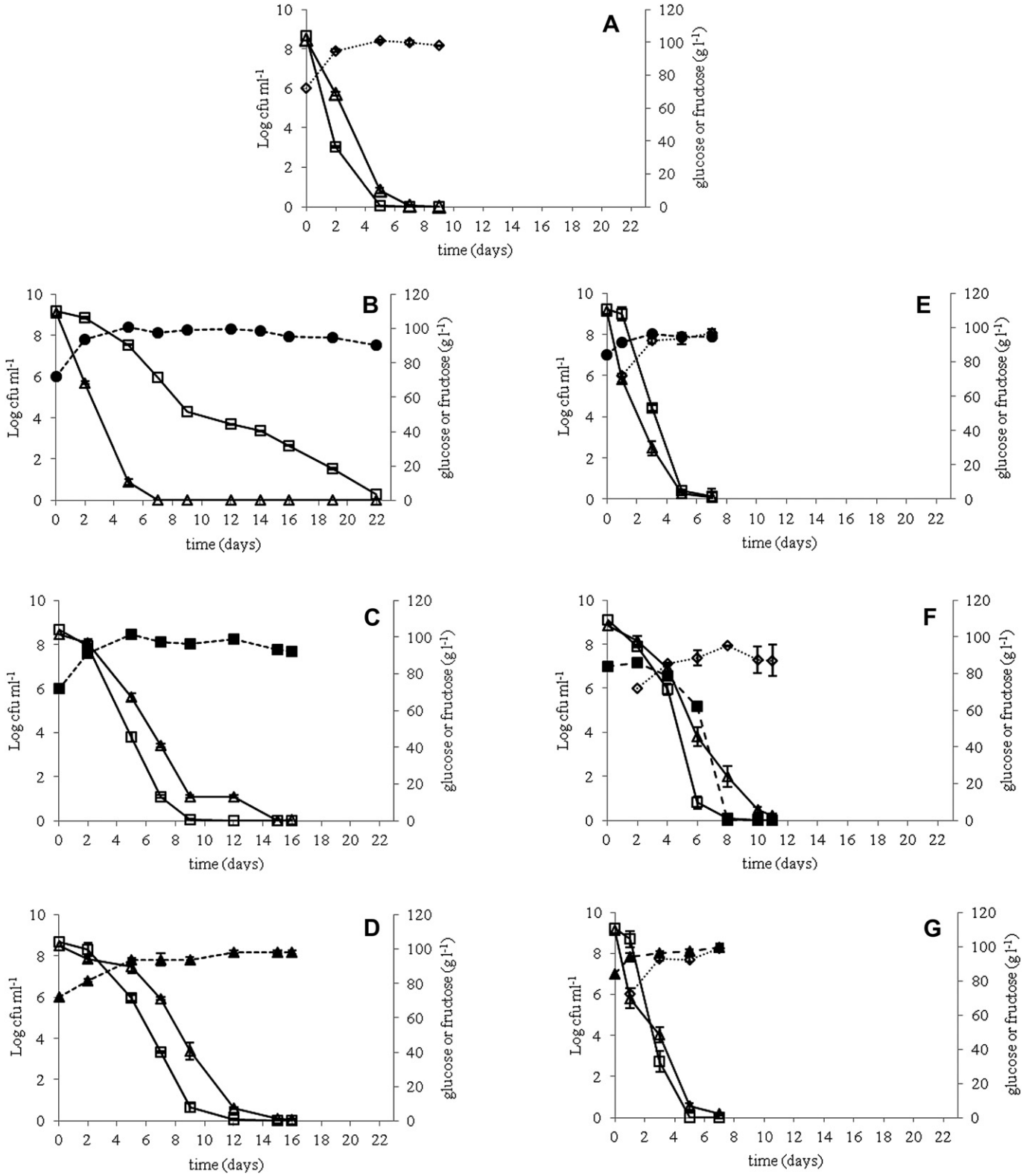

Fig. 1. Growth kinetics and sugar consumption of yeasts during mono-cultures and co-cultures: A) *S. cerevisiae* PB2023 (◇), B) *C. zemplinina* MCR-9 (●), C) *M. pulcherrima* MCR-24 (■), D) *T. delbruekii* BB-MV:3FA5, E) *S. cerevisiae* PB2023 (◇) and *C. zemplinina* MCR-9 (●), F) *S. cerevisiae* PB2023 (◇) and *M. pulcherrima* MCR-24 (■), G) *S. cerevisiae* PB2023 (◇) and *T. delbrueckii* BB-MV:3FA5 (▲). Glucose (□) and fructose (△). Data are representative of tree independent experiments.

was 1.7 10^8 cells ml^{-1} after 6 days of culture (Fig. 1C). Sugars were completely consumed, however the fermentation was longer than with *S. cerevisiae* (15 days), the difference being linked to a lower rate of glucose and fructose consumption. In the case of *T. delbrueckii* BB-MV:3FA5, single fermentation went to completion after 15 days (Fig. 1D), with a maximal population of 1.4×10^8 cells ml^{-1}. As for *M. pulcherrima*, fructose was consumed more slowly over the course of fermentation.

Table 2
Main enological characteristics of wines produced from pure cultures by different species: *C. zemplinina*, *M. pulcherrima*, *T. delbrueckii* and the control *S. cerevisiae* and from mixed cultures by different yeast couples: *C. zemplinina/S. cerevisiae*, *M. pulcherrima/S. cerevisiae*, *T. delbrueckii/S. cerevisiae*. Initial sugar content of the juice was 219 g l^{-1}. Data are average of three replicates ± standard deviations.

Species	Strains	Residual sugar (g l^{-1})		Ethanol (v/v %)	Sugars used for 1% ethanol production (g)	pH	Total acidity (g l^{-1} sulphuric acid)	Acetic acid (g l^{-1})***	Time of alcoholic fermentation (days)
		Glucose	Fructose						
S. cerevisiae	PB2023	nd	0.04 ± 0.00	11.02 ± 0.2	19.87	3.43 ± 0.03	3.63 ± 0.06	0.41 ± 0.04^{c}	7
C. zemplinina	MCR-9	3.51 ± 0.01	Nd	9.98 ± 0.29	21.59	3.65 ± 0.01	3.82 ± 0.05	0.86 ± 0.03^{a}	22
C. zemplinina/S. cerevisiae	MCR-9 / PB2023	1.76 ± 0.08	1.22 ± 0.07	10.69 ± 0.35	20.21	3.47 ± 0.02	3.99 ± 0.1	0.51 ± 0.04^{c}	6
M. pulcherrima	MCR-24	nd	0.31 ± 0.01	10.89 ± 0.32	20.08	3.55 ± 0.02	3.51 ± 0.08	0.16 ± 0.07^{d}	15
M. pulcherrima/S. cerevisiae	MCR-24/ PB2023	0.1 ± 0.05	2.69 ± 0.08	10.22 ± 0.41	21.17	3.54 ± 0.03	3.25 ± 0.14	0.23 ± 0.02^{d}	11
T. delbruekii	BB-MV:3FA5	nd	0.95 ± 0.01	10.66 ± 0.13	20.45	3.48 ± 0.02	4.30 ± 0.03	0.65 ± 0.05^{b}	14
T. delbruekii/S. cerevisiae	BB-MV:3FA5 /PB2023	nd	2.01 ± 0.06	11 ± 0.25	19.73	3.44 ± 0.02	3.95 ± 0.03	0.24 ± 0.04^{d}	7

Values with the same letters are not significantly different according to the Tukey test (95%).
Significance level of the one way ANOVA ($p < 0.001$), nd: not detected.

When alcoholic fermentations were conducted with co-cultures of *S. cerevisiae* with either *C. zemplinina*, *M. pulcherrima* or *T. delbrueckii* at an inoculation ratio 1:10, all fermentations progressed to completion in 6–11 days. The maximum population reached 2×10^8 cells ml^{-1} for *T. delbrueckii*, 1.4×10^8 cells ml^{-1} for *S. cerevisiae*, 9.3×10^7 cells ml^{-1} for *C. zemplinina*, and 3.9×10^7 cells ml^{-1} for *M. pulcherrima* (Fig. 1). Each strain tested shows different growth kinetics. As expected, *S. cerevisiae* (Fig. 1A) has a rapid exponential phase and reached stationary phase after two days, compared to 6 days for the other strains. The viability of the cells remained high throughout the fermentation process and started to decline at the end of alcoholic fermentation (Fig. 1A,B,C,D).

Three different behaviours could be observed, depending on the couple tested. When *C. zemplinina* MCR-9 and *S. cerevisiae* PB2023 were co-inoculated, both strains grew in the must and alcoholic fermentation was completed in 7 days (Fig. 1E). However, *C. zemplinina* showed a marked effect on *S. cerevisiae* growth. The maximum population reached 7.45×10^7 cells ml^{-1}, which is 49% lower than *S. cerevisiae* monoculture (Fig. 1A). These observations support previous findings (Ciani et al., 2006; Mendoza et al., 2007; Comitini et al., 2011). It is worth noting that fructose consumption in co-culture was faster than in *S. cerevisiae* mono-culture, which is probably due to the fructophilic character of *C. zemplinina*. Co-culture of *M. pulcherrima* MCR-24 and *S. cerevisiae* PB2023 showed a different profile (Fig. 1F). Although alcoholic fermentation was completed after 8 days and despite the rather high ethanol tolerance of our strain (production of 10.8% v/v ethanol in single culture), the *M. pulcherrima* population dropped quickly and no viable cells were detected after 8 days. Such an antagonistic effect has been reported previously (Jolly et al., 2003; Rodríguez et al., 2010; Comitini et al., 2011). According to Nguyen and Panon (1998), this antagonistic effect was attributed to a killer type interaction. However the *S. cerevisiae* strain used in our study was neutral for killer character. Nissen and Arneborg (2003) postulated that early cell death would be mediated by a cell–cell contact mechanism. Competition for oxygen could also explain the rapid death of *M. pulcherrima* cells. Oxygen is a limiting factor for yeast growth. While *S. cerevisiae* is capable of rapid growth under strictly anaerobic conditions, other yeasts, including the wine-related genera *Hanseniaspora* (*Kloeckera*), grow poorly under such conditions (Díaz-Montaño and Córdova, 2009).

For the couple *S. cerevisiae* PB2023/*T. delbrueckii* (BB-MV:3FA) (Fig. 1G), both yeast species reached a high level of cells, more than 1.8×10^8 cells ml^{-1}. Viability remained high until the end of the alcoholic fermentation (Fig. 1G).

3.2. Metabolic profiles of wines

3.2.1. Oenological characteristics of mono- and co-cultures

The chemical compositions of the wines, whether from mono- or co-cultures, reveal significant differences (Table 2). As stated above, a mono-culture of *C. zemplinina* MCR-9 was characterised by the presence of residual sugar (3.5 g l^{-1} glucose). However, this level of sugar is very low. Recently, Tofalo et al. (2012) have shown that most *C. zemplinina* strains are not able to consume glucose completely. However, 3 isolates out of 36 consume almost all the glucose during alcoholic fermentation. *C. zemplinina* is also characterised by a lower ethanol yield and greater production of acetic acid, which are well-known traits of this yeast species (Soden et al., 2000; Magyar and Tóth, 2011). The results obtained in this study confirm the high production of acetic acid (0.86 g l^{-1}) but do not show a poor ethanol yield (approximately 10% ethanol). A mono-culture of *T. delbrueckii* BB-MV:$_3$FA$_5$ also induced high acetic acid production (0.65 g l^{-1}). This result is contradictory with previous findings, which showed that *T. delbrueckii* species are characterised by low volatile acidity production (Ciani and Maccarelli, 1998; Renault et al., 2009). This seems to be a general trait, especially for high sugar content must (Renault et al., 2009). According to these authors, major variations in volatile acidity production are observed among *T. delbrueckii* species. Some *T. delbrueckii* strains produced similar or even higher amounts of volatile acidity than *S. cerevisiae*, as observed in our study. *M. pulcherrima* in mono-culture is rather a low producer of volatile acidity which confirms previous findings (Comitini et al., 2011). It is worth noting that our strain of *M. Pulcherrima* produces unusual amount of alcohol compared to what is reported in the literature (Comitini et al., 2011). Another interesting finding is the lower acetic production of the co-culture *M. pulcherrima/S. cerevisiae* when compared to *S. cerevisiae* mono-culture. This decrease could be either due to yeast–yeast interaction or to acetic acid co-metabolism as described by Dos Santos et al. (2003).

One point of interest in co-culture was the considerable decline in acetic acid content in wines inoculated with the couple *S. cerevisiae*/*C. zemplinina* (Table 2) which confirmed previous results (Soden et al., 2000).

3.2.2. Influence of interactions on aromatic compounds produced and released by yeast

The main issue in our study was to assess metabolic changes in aromatic compounds. Because of good sensitivity and reproducibility, the high-throughput SBSE-LD-GC-MS was applied here for

aromatic compound analysis. A total of 44 aromatic compounds were quantified. Results from the univariate analysis of the aroma concentration dataset are presented in Table 3. Samples showed significant differences for all the aroma compounds analysed. A PCA was conducted to gain insight into the nature of the multivariate data and to evaluate biological interaction as well as to check fermentation reproducibility. The resulting PCA accounted for 61.06% of the total variance for the first two principal components (Fig. 2). Replicates clustered quite well, which shows high reproducibility of the experimental procedure. Based on classical ascendant hierarchy, four experimental groups of mono- and co-culture fermentation could be distinguished (Fig. 2) by their

Table 3
Concentrations and univariate statistical analysis of the quantified volatile compounds (μg l^{-1}) in the wines produced by the pure cultures of *S. cerevisiae* PB2023 (Sc), *M. pulcherrima* MCR-24 (Mp), *T. delbrueckii* BB-MV:3FA5 (Td), and *C. zemplinina* MCR-9 (Cz) and by mixed cultures of *T. delbrueckii/S. cerevisiae* (Td−Sc), *C. zemplinina/S. cerevisiae* (Cz−Sc) and *M. pulcherrima/S. cerevisiae* (Mp−Sc). Data are average of three replicates ± standard deviations.

Compounds	Sc	Mp	Td	Cz	Cz−Sc	Mp−Sc	Td−Sc
Alcohols							
Isoamyl alcohol***	7454 ± 334^{a,b}	6130 ± 667^{b,c}	5970 ± 313^{c}	1932 ± 110^{d}	5879 ± 622^{c}	8031 ± 587^{a}	8852 ± 504^{a}
1-Pentanol, 3-methyl***	nd^{c}	9.28 ± 0.62^{a,b}	8.47 ± 1.16^{b}	nd^{c}	7.27 ± 0.13^{b}	8.09 ± 0 .08^{b}	11.16 ± 1.06^{a}
1-Propanol, 3-ethoxy***	nd^{c}	155 ± 8^{a}	9.47 ± 0.01^{c}	nd^{c}	nd^{b}	nd^{c}	102.74 ± 7.99^{b}
2-(2-Butoxyethoxy)-Ethanol***	46.78 ± 2.93^{c}	51.55 ± 4.15^{b,c}	45.78 ± 5.52^{c}	17.63 ± 3.67^{d}	48.40 ± 1.26^{b,c}	57.06 ± 2.54^{a,b}	63.09 ± 0.86^{a}
Isobutanol***	2505 ± 320^{b}	1093 ± 45^{d}	3510 ± 99^{a}	3219 ± 174^{a}	1926 ± 110^{c}	2368 ± 256^{b,c}	1125 ± 136^{d}
Butanol, 3-methyl***	46744 ± 2903^{a}	32342 ± 1102^{b}	33511 ± 1165^{b}	11660 ± 825^{c}	35694 ± 1471^{b}	48095 ± 3194^{a}	36620 ± 1638^{b}
2-Phenylethyl alcohol***	32784 ± 2653^{b,c,d}	39908 ± 2216^{a,b}	28504 ± 2345^{c,d}	24628 ± 2730^{d}	37035 ± 2053^{b,c}	48751 ± 4970^{a}	47126 ± 5309^{a}
Hexanol***	965.5 ± 130.6^{b,c}	1345 ± 198^{a}	768.6 ± 76.6^{c}	795.1 ± 30.3^{c}	1208 ± 54^{a,b}	690.1 ± 75.3^{c}	1373 ± 24^{a}
trans-3-Hexen-1-ol*	12.15 ± 1.19^{c}	19.16 ± 0.17^{a}	15.88 ± 0.09^{b}	19.32 ± 0.88^{c}	15.19 ± 0.29^{b}	14.79 ± 0.41^{b}	19.32 ± 0.88^{a}
cis-3-Hexene-1-ol***	77.19 ± 0.98^{c,d}	99.29 ± 0.86^{b}	78.27 ± 5.47^{c}	66.14 ± 3.57^{e}	90.98 ± 3.91^{b}	76.95 ± 3.46$^{c,\ d}$	111.6 ± 3.1^{a}
∑ Alcohols***	91831 ± 775^{b,c}	83559 ± 2639^{c,d}	74131 ± 917^{d}	38006 ± 5150^{e}	83961 ± 1590^{c,d}	110264 ± 6064^{a}	98626 ± 4202^{b}
Aldehydes							
2-Hexenal***	15.92 ± 1.71^{a}	nd^{b}	nd^{b}	nd^{b}	nd^{b}	nd^{b}	nd^{b}
Benzaldehyde, 4-methyl***	107.7 ± 14.4^{a}	102.0 ± 8.8^{a,b}	96.10 ± 6.53^{a,b}	27.47 ± 1.78^{c}	77.55 ± 5.32^{b}	46.64 ± 2.45^{c}	115.9 ± 6.2^{a}
∑ Aldehydes***	123.6 ± 12.9^{a}	102.0 ± 8 .8^{a,b,c}	96.1 ± 6.5^{b,c}	27.5 ± 1.8^{d}	77.6 ± 5.3^{c}	46.6 ± 2.5^{d}	116 ± 6.2^{a,b}
Terpenols							
Linalool***	33.98 ± 2.73^{d}	30,82 ± 2.05^{d,e}	27.38 ± 1.44^{e}	68.88 ± 1.57^{a}	41.46 ± 1.66^{c}	51.42 ± 0.50^{b}	44.48 ± 0.97^{c}
Hotrienol***	nd^{d}	nd^{d}	nd^{d}	22.56 ± 2.33^{b}	nd^{d}	51.90 ± 1.89^{a}	9.56 ± 0.42^{c}
4-Terpineol***	nd^{b}	nd^{b}	nd^{b}	9.66 ± 0.51^{a}	nd^{b}	10.14 ± 0.38^{a}	nd^{b}
Citronellol***	34.75 ± 1.53^{c}	nd^{g}	29.91 ± 1.51^{d}	16.16 ± 1.49^{f}	60.45 ± 1.05^{b}	21.13 ± 0.03^{e}	77.72 ± 5.22^{a}
Geraniol***	27.77 ± 2.20^{e}	66.87 ± 6.00^{c}	40.99 ± 1.60^{d}	159.8 ± 3.7^{a}	99.23 ± 2.19^{b}	30.97 ± 1.43^{d,e}	74.84 ± 2.11^{c}
Nerolidol***	nd^{c}	23.96 ± 0.80^{a,b}	17.53 ± 1.90^{b}	37.75 ± 10.77^{a}	14.95 ± 0.77^{b}	nd^{c}	35.36 ± 4.38^{a}
Farnesol***	nd^{d}	103.0 ± 10.7^{b}	67.24 ± 2.50^{c}	244.7 ± 32.7^{a}	86.90 ± 5.02^{b,c}	63.58 ± 2.76^{c}	86.53 ± 5.20^{b,c}
∑ Terpenols***	96.5 ± 5.9^{d}	216.6 ± 11.5^{b,c,d}	150.7 ± 61.0^{c,d}	395.9 ± 77.5^{a}	264.9 ± 68.4^{a,b,c}	222.1 ± 10.2^{b,c,d}	283.2 ± 31.5^{a,b}
Norisoprenoids							
β-Damascenone***	36.44 ± 5.47^{b}	27.21 ± 1.79^{c}	29.66 ± 2.66^{b,c}	64.55 ± 2.39^{a}	30.25 ± 1.38^{b,c}	65.82 ± 2.61^{a}	31.99 ± 1.72^{b,c}
Lactones							
γ−Nonalactone***	25.57 ± 6.68^{c,d}	33.52 ± 3.39^{b,c}	36.20 ± 2.13^{a,b}	nd^{e}	21.17 ± 0.93^{d}	23.70 ± 0.78^{d}	42.76 ± 1.35^{a}
γ−Decalactone***	nd^{b}	nd^{b}	nd^{b}	17.25 ± 1.72^{a}	nd^{b}	nd^{b}	nd^{b}
δ-Dodecalactone***	nd^{c}	nd^{c}	73.96 ± 1.95^{b}	83.13 ± 5.11^{a}	nd^{c}	nd^{c}	nd^{c}
∑ Lactones***	25.6 ± 6.7^{d,e}	33.5 ± 3.4^{c,d}	111.4 ± 2.1^{a}	395.9 ± 77.5^{b}	21.2 ± 0.9^{e}	23.7 ± 0.8^{d,e}	42.8 ± 1.4^{c}
Acetates							
n-Amyl acetate***	8.24 ± 0.55^{b}	nd^{c}	nd^{c}	nd^{c}	nd^{c}	989 ± 0.26^{a}	nd^{c}
Isoamyl acetate***	3669 ± 62^{b}	92.41 ± 0.43^{d}	496.6 ± 28.5^{c,d}	45.62 ± 5.68^{d}	671.7 ± 87.5^{c,d}	4736 ± 370^{a}	878.0 ± 84.5^{c}
cis-Hexenyl acetate***	100.9 ± 1.6^{b}	9.83 ± 0.39^{d}	10.51 ± 0.73^{d}	nd^{e}	10.91 ± 1.51^{d}	121.8 ± 6.0^{a}	22.48 ± 1.09^{c}
Hexyl acetate***	765.8 ± 143.4^{a}	14.41 ± 0.50^{b}	27.02 ± 2.84^{b}	5.51 ± 2.33^{b}	100.6 ± 14.5^{b}	860.4 ± 69.6^{a}	115.6 ± 18.1^{b}
Heptyl acetate***	8.03 ± 0.46^{b}	nd^{c}	nd^{c}	nd^{c}	nd^{c}	10.70 ± 0.51^{a}	nd^{c}
2-Phenylethyl acetate***	871.7 ± 58.5^{b}	170.2 ± 9.6^{d}	202.7 ± 31.7^{d}	21.00 ± 0.95^{d}	542.4 ± 13.3^{c}	2212 ± 189^{a}	574.3 ± 3.0^{c}
∑ Acetates***	5538 ± 71^{b}	251 ± 68^{d}	724 0.3 ± 75.2^{c,d}	71.5 ± 4.5^{d}	1326 ± 117^{c}	7947 ± 612^{a}	1590 ± 101^{c}
Esters							
Ethyl 2-butanoate***	6.48 ± 0.50^{a}	nd^{c}	nd^{c}	nd^{c}	5.52 ± 0.26^{a}	6.70 ± 0.72^{a}	3.28 ± 0.42^{b}
Ethyl 4-hydroxybutanoate***	16.50 ± 0.82^{c}	25.42 ± 1.50^{b}	41.38 ± 3.24^{a}	nd^{e}	17.90 ± 0.36^{c}	8.35 ± 1.13^{d}	27.00 ± 0.17^{b}
Ethyl 3-hexenoate***	5.33 ± 0.16^{d}	6.36 ± 0.49^{d}	18.70 ± 0.02^{a}	nd^{e}	6.23 ± 0.57^{d}	12.28 ± 0.19^{c}	14.21 ± 0.57^{b}
Ethyl hexanoate***	850.6 ± 54.3^{b}	412.5 ± 8.5^{d}	720.7 ± 39.2^{c}	44.34 ± 0.46^{e}	924.6 ± 24.5^{b}	1786 ± 55^{a}	857.2 ± 65.1^{b}
Ethyl octanoate***	622 ± 77^{b}	88.86 ± 6.85^{e}	459.6 ± 59^{c}	8.91 ± 2.88^{e}	487.1 ± 14.4^{b,c}	864.9 ± 30.8^{a}	242.8 ± 25.7^{d}
Ethyl decanoate***	41.14 ± 5.71^{c,d}	22.25 ± 2.50^{d}	43.30 ± 3.19^{c}	nd^{e}	83.62 ± 3.21^{b}	195.0 ± 11.6^{a}	51.29 ± 2.69^{d}
Ethyl 9-decenoate***	225.1 ± 15.9^{b}	50.48 ± 2.10^{d,e}	92.71 ± 33.76^{c,d}	nd^{e}	147.6 ± 19.7^{b,c}	306.0 ± 28.3^{a}	122.2 ± 31.1^{c,d}
Diethyl succinate***	10.82 ± 1.83^{b}	5.78 ± 0.01^{c,d}	24.25 ± 0.64^{a}	2.29 ± 0.49^{d}	10.82 ± 0.67^{b}	4.88 ± 0.08^{d}	9.18 ± 0.49^{b,c}
Diethyl glutarate***	nd^{b}	nd^{b}	nd^{b}	nd^{b}	6.79 ± 0.08^{a}	nd^{b}	nd^{b}
∑ Esters***	1759 ± 116^{b}	584.4 ± 24.4^{e}	1358 ± 157^{c,d}	54.2 ± 4.7^{f}	1686 ± 29^{b,c}	3072 ± 109^{a}	1185 ± 198^{d}
Fatty acids							
Octanoic acid***	586 ± 188^{b}	79.81 ± 11.51^{d,e}	369.3 ± 45.2^{b,c,d}	nd^{e}	470.4 ± 20.5^{b,c}	1305 ± 188^{a}	269.2 ± 4.0$^{c,\ d,\ e}$
Decanoic acid***	38.90 ± 4.16^{c}	3.15 ± 0.32^{c}	5.18 ± 0.81^{c}	26.71 ± 1.34^{c}	246.9 ± 33.6^{a}	286.6 ± 17.3^{a}	137.0 ± 17.6^{b}
∑ Fatty acids***	612 ± 198^{b}	83.0 ± 11.8^{c,d}	374.5 ± 44.4^{b,c,d}	26.7 ± 1.3^{d}	717.3 ± 13.6^{b}	1601 ± 186^{a}	441.9 ± 45.7^{b,c}
Various							
1-Ethyl-4-methylbenzene***	5.13 ± 0.59^{c}	5.26 ± 0.08^{c}	5.72 ± 0.12^{c}	nd^{d}	9.99 ± 0.32^{a}	9.81 ± 1.06^{a}	8.00 ± 0.19^{b}
Formamide***	20.01 ± 0.87^{b}	22.11 ± 2.52^{b}	22.13 ± 0.19^{b}	nd^{c}	20.01 ± 0.87^{b}	20.15 ± 0.20^{b}	27.04 ± 1.09^{a}
1-Methyl-2-Pyrrolidinone***	79.65 ± 3.69^{b}	88.49 ± 9.78^{b}	83.77 ± 4.20^{b}	17.70 ± 0.94^{c}	82.71 ± 3.89^{b}	96.01 ± 0.60^{b}	114.1 ± 2.3^{a}
2,4-bis (1,1-dimethylethyl)-phenol***	149 ± 19.56^{b,c}	174.6 ± 5.4^{b}	148.5 ± 7.1^{b,c}	77.95 ± 42.68^{c}	164.6 ± 7.4^{b,c}	2120 ± 67^{a}	205.8 ± 6.8^{b}

Values with the same letters are not significantly different according to the Tukey test (95%).
***Significance level of the one way ANOVA (* 0,05 > p > 0,01; ** 0,01 > p > 0.001; ***p < 0.001), nd: not detected.

significant metabolic differences. The relevance of different yeasts in the production of aromatic molecules is clearly demonstrated in the Principal Component plot shown in Fig. 2.

It appears that *C. zemplinina* MCR-9 is a low producer of higher alcohols. Approximately, 2.5 times lower amounts of higher alcohols were produced by this strain than by *S. cerevisiae* (Table 3). Furthermore, this strain was characterised by higher production of most lactones, norisoprenoids and terpenols (Table 3), with a great quantity of δ-dodecalactone, γ-decalactone, β-damascenone, geraniol and farnesol. To our knowledge, this is the first report highlighting greater production of terpenols, lactones and norisoprenoids by *C. zemplinina* in comparison with other yeast strains. The contribution of yeast to the formation of wine varietal aroma by action on grape glycosidic precursors is well documented in the literature. Terpenols and lactones are mostly released from their glycosidic precursors upon the action of glycosidases such as β-glucosidases, α-L-arabinofuranosidases, α-L-rhamnosidases and β-D-xylosidases (for a review, see Maicas and Mateo, 2005). Yeasts such as *S. cerevisiae* do not efficiently excrete monoterpenes (Carrau et al., 2005) but synthesise the phosphorylated form of geraniol, geranyl diphosphate (GDP), as an intermediate of farnesyl diphosphate (FDP) synthesis, a key molecule in the isoprenoid pathway that leads to the synthesis of dolicols, ubiquinones and sterols (Lees et al., 1999). It has also been shown that yeasts use terpenes as biosynthetic intermediates for sterol synthesis (Gamero et al., 2011). Thus, differences in the terpene profile of wines probably depend on β-D-glucosidase activity, terpene bioconversion rate and the percentage of terpenes accumulated by yeasts.

An interesting finding is that the PCA plot unravels the interaction that occurred during co-culture. While *C. zemplinina* alone is characterised by terpenol, lactone and norisoprenoid production, the aromatic profile changed completely in the presence of *S. cerevisiae* PB2023. The metabolic profile of this co-culture appears to be closer to that of *S. cerevisiae* alone. It is worth noting that, despite a greater population of *C. zemplinina* in the co-culture than in the mono-culture, the concentrations of terpenols

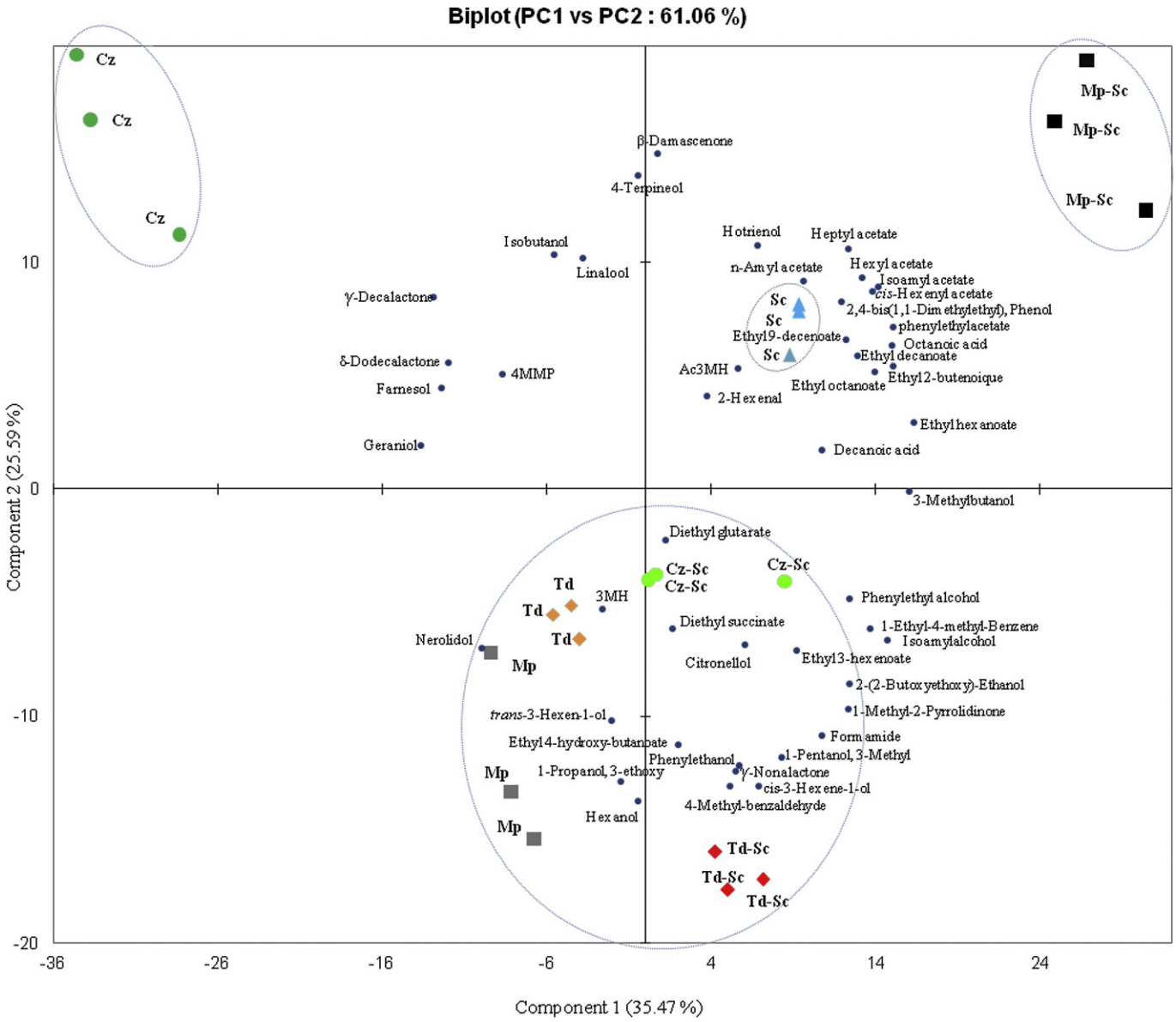

Fig. 2. Biplot of the principal components analysis (PC 1 vs. PC 2) of metabolite profiles from mono-culture fermentations : *S. cerevisiae* PB2023 (Sc:▲), *T. delbrueckii* BB-MV:3FA5 (Td:♦), *C. zemplinina* MCR-9 (Cz:●) and *M. pulcherrima* MCR-24 (Mp:■) and from co-culture fermentations: *C. zemplinina* and *S. cerevisiae* (Cz–Sc:●); *M. pulcherrima* and *S. cerevisiae* (Mp–Sc:■); *T. delbrueckii* and *S. cerevisiae* (♦). Ellipses represent clusters obtained from HCA.

and norisoprenoids were significantly lower in the co-culture than in the mono-culture (Table 3). The production of lactones was very close to the production observed after formation with *S. cerevisiae* in mono-culture. This difference could be linked to more rapid fermentation in the case of the co-culture. In this condition, *C. zemplinina* could not synthesise as many terpenols and lactones in 6 days as in 22 days, the time taken for fermentation with the mono-culture (Fig. 1B and E). In order to assess whether differences in biomass production rather than metabolism were responsible for the observed differences in volatile compound concentrations, we normalised compound concentrations to the biomass produced. Fig. 3 clearly shows that the level of two lactones out of three and three terpenols out of six (geraniol, farnesol and nerolidol) was not linked to a biomass effect whatever the type of culture. If this had been the case, mono- and co-cultures with *C. zemplinina* would have been clustered. Thus the decrease in production of these lactones and terpenols by *C. zemplinina* co-cultured with *S. cerevisiae* was probably due to a negative interaction.

The hypothesis of a biotransformation of terpenols by *S. cerevisiae* can be discarded. Studies related to these biotransformations have shown reduction of geraniol to citronellol, translocation of geraniol to linalool, isomerisation of nerol to geraniol and cyclisations of linalool to α-terpineol (Gramatica et al., 1982; King and Richard Dickinson, 2000; Zea et al., 1995; Zoecklein et al., 1997; Gamero et al., 2011). In fact, terpenols are transformed into other terpenes and never hydrolysed.

It should be underlined that terpenols, especially farnesol, have been shown to play a role in microbial interactions. The *Candida albicans* metabolite farnesol can modulate the expression of virulence genes in *Pseudomonas aeruginosa* (Cugini et al., 2007, 2010). Further investigations are needed to study the potential role of farnesol produced by *C. zemplinina* in yeast interactions.

While levels of higher alcohols in mono-cultures of *M. pulcherrima* and *S. cerevisiae* are similar, a significant increase in the production of higher alcohols is observed in the *M. pulcherrima*/*S. cerevisiae* co-culture (Table 3). Although the aromatic profile for the *C. zemplinina*/*S. cerevisiae* co-culture is relatively close to that of *S. cerevisiae* in mono-culture, the *M. pulcherrima*/*S. cerevisiae* co-culture has a completely different aromatic profile (Fig. 2), characterised by high levels of fatty acids, ethyl esters, and acetates (especially phenylethyl acetate and isoamyl acetate), and by modification of the terpenol profile, with high levels of hotrienol, 4-

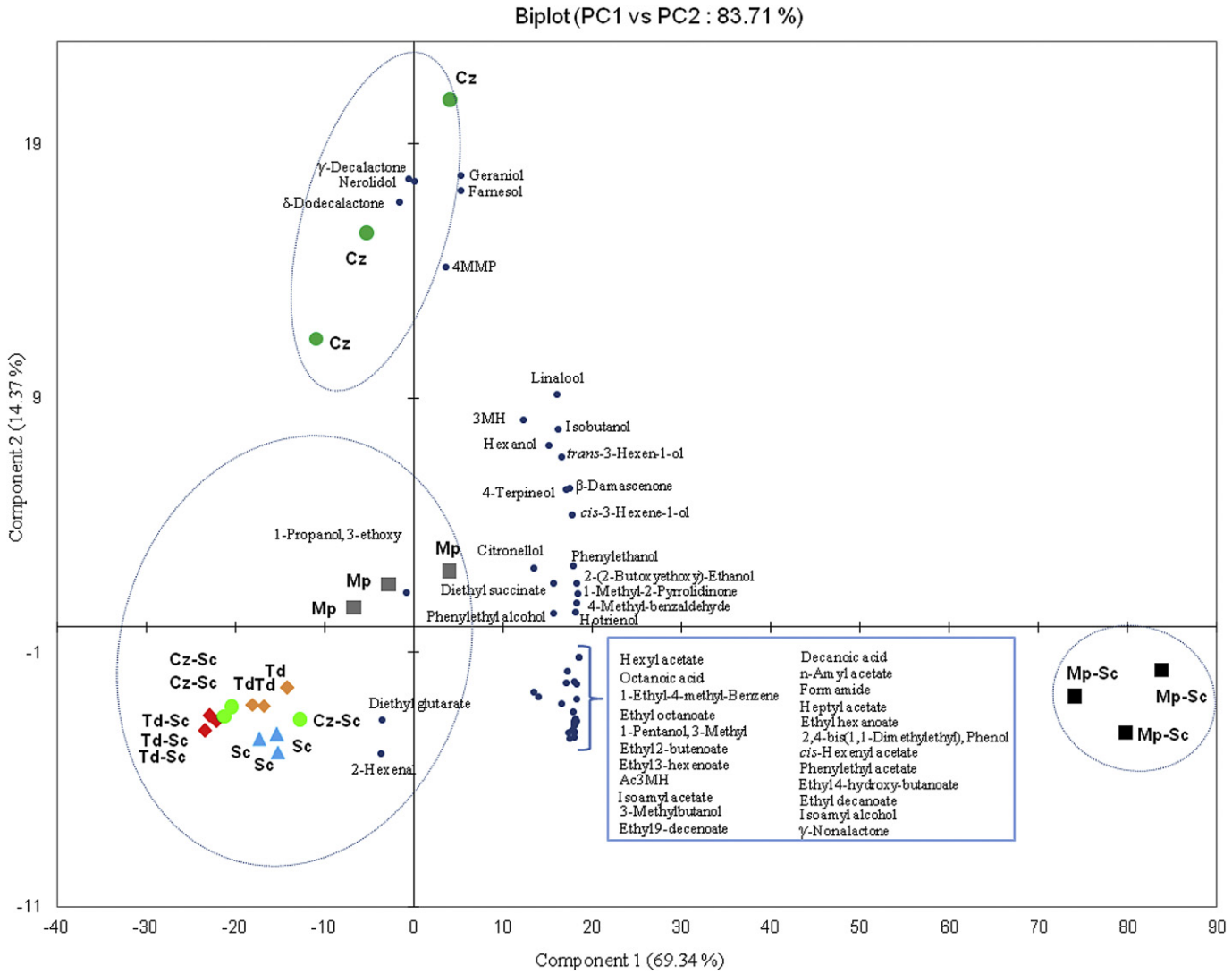

Fig. 3. Biplot of the principal components analysis (PC 1 vs. PC 2) of metabolite profiles normalised to the biomass from mono-culture fermentations: *S. cerevisiae* PB2023 (Sc:▲), *T. delbrueckii* BB-MV:3FA5 (Td:♦), *C. zemplinina* MCR-9 (Cz:●) and *M. pulcherrima* MCR-24 (Mp:■) and from co-culture fermentations: *C. zemplinina* and *S. cerevisiae* (Cz–Sc:●); *M. pulcherrima* and *S. cerevisiae* (Mp–Sc:■); *T. delbrueckii* and *S. cerevisiae* (♦). Ellipses represent clusters obtained from HCA.

terpineol and linalool. The concentration of β-damascenone is doubled, compared to the concentrations obtained in mono-cultures of *M. pulcherrima* (27 μg l^{-1}) or *S. cerevisiae* (36 μg l^{-1}) (Table 3). These data could reflect a synergistic effect (positive interaction) between the two yeasts for these compounds. This type of interaction has rarely been reported and only for one or two components. Toro and Vazquez (2002) have shown an enhancement of glycerol content with *S. cerevisiae/Candida cantarellii* in co-culture. Increased varietal thiol concentrations were also observed with *Pichia kluyveri/S. cerevisiae* in co-culture (Anfang et al., 2009). However our study clearly shows that the concentration levels of whole families of volatile compounds could be modified by co-culture interactions. Therefore, the volatile profile contribution of a yeast strain in co-culture cannot be predicted by the growth profile of that yeast alone.

Such behaviour is intriguing because, in co-culture, *M. pulcherrima* MCR-24 did not persist during alcoholic fermentation, with a rapid decline in cell viability (Fig. 1F). Furthermore, co-culture wines were clearly distinguishable from mono-culture wines, demonstrating that the numerically inferior yeast *M. pulcherrima* made a significant contribution to the metabolic profile in co-culture (Fig. 3).

As shown in Fig. 3, the higher level of acetate esters is not linked to a biomass effect in co-culture. The nature of the interaction is due to other factors. While acetate ester production depends on the concentration of Unsaturated Fatty Acids and oxygen in the fermentation medium and/or the carbon-to-nitrogen ratio (C/N), it is well recognised that the regulation of acetate ester synthesis is mainly due to the expression level of the known alcohol acetyltransferases ATF1 and ATF2 (Verstrepen et al., 2003; Saerens et al., 2008). This means that a higher production of acetate esters could be explained by a higher *ATF1* and/or *ATF2* gene expression in *S. cerevisiae* (Saerens et al., 2010). We can hypothesise that high production of acetate esters could be linked to oxygen depletion in the medium induced by the presence of both yeasts in the medium. In these conditions, the TCA cycle could not be fully used, which may lead to an accumulation of acetyl-CoA and a depletion of free CoA. Ester synthesis could be a way to regenerate free CoA without releasing a high concentration of free acetic acid. This hypothesis is sustained by the level of ethyl esters and medium-chain fatty acids (MCFAs), such as octanoic and decanoic acids, which are also involved in this mechanism. The levels of ethyl esters and MCFAs were greater in the co-culture of *M. pulcherrima/S. cerevisiae* than in mono-cultures. Accumulation of MCFAs generally occurs when fatty acid synthesis is inhibited, as is the case under anaerobic conditions (Dufour et al., 2003). These MCFAs are toxic for the yeast (Alexandre et al., 1996) and it has been proposed that MCFAs are converted to ethyl esters, which are less toxic and could diffuse more easily through the plasma membrane (Saerens et al., 2010).

The *T. delbrueckii* BB-FV:3FA5 aromatic profile differed from that obtained with *S. cerevisiae* (Fig. 2), more particularly because of high terpenols (farrnesol, nerolidol, and geraniol) and poor acetate productions (−87% compared to *S. cerevisiae*). The *T. delbrueckii/S. cerevisiae* co-culture profile is clearly different from the *S. cerevisiae* mono-culture profile (Fig. 2). A characteristic of the *T. Delbrueckii/S. cerevisiae* co-culture is an increase in C6 compounds, terpenols and 2-phenylethanol (Table 3). These results suggest that the metabolisms of both yeasts have a cumulative effect. This synergistic effect could be due either to an alteration of yeast metabolism or to a biomass effect. As shown in Fig. 3 in which concentrations are normalised versus biomass, both mono-culture and co-culture belong to the same cluster, which means that the higher production of these compounds is solely related to the biomass.

S. cerevisiae is the greatest producer of phenylethyl acetate when compared to other mono-cultures, but in co-culture conditions with *T. delbrueckii* or *C. zemplinina* phenylethyl acetate concentration showed a more than two-fold decrease, despite the same level of population (Table 3, Fig. 1E and G). These results are in agreement with the level of the precursor (2-phenylethanol), lower in *S. cerevisiae* mono-culture (32.8 mg l^{-1}) than in co-culture with *T. delbrueckii* (47.1 mg l^{-1}) or *C. zemplinina* (37.0 mg l^{-1}). It is interesting to note that an increase in phenylethyl acetate level was observed with the *M. pulcherrima/S. cerevisiae* co-culture, which is in agreement with the findings of Viana et al. (2009). These authors have shown that wines obtained with a co-culture (*Hanseniaspora osmophila/S. cerevisiae*) showed concentrations of 2-phenylethyl acetate approximately 3–9 times greater than that produced by a *S. cerevisiae* mono-culture. Rojas et al. (2003) reported that *H. guillermondii* was a stronger producer of 2-phenylethyl acetate than *S. cerevisiae*. However, the *S. cerevisiae/H. guillermondii* co-culture produced a lower level of phenylethyl acetate than the *H. guillermondii* mono-culture.

3.2.3. Influence of interactions on aromatic compounds released by yeast

Volatile thiols are a group of aroma compounds whose significance to wine aroma, particularly Sauvignon Blanc wines, has been widely studied (King et al., 2008). Thiols are not present in grape juice, but are released by yeast during fermentation from precursors that are present in juice. 4-mercapto-4-methylpentan-2-one (4MMP) and 3-mercaptohexan-1-ol (3MH) are produced from cysteinylated conjugates (cys-4MMP, cys-3MH) or glutathionylated conjugates by cleavage with β-lyase enzymes present in yeast (Peyrot des Gachons et al., 2002; Howell et al., 2006; Subileau et al., 2008; Fedrizzi et al., 2009). 3MHA is derived from 3MH acetylation by yeast during alcoholic fermentation. Volatile thiol production during alcoholic fermentation has been shown to depend on yeast strain, especially *Saccharomyces* (Dubourdieu et al., 2006; Swiegers et al., 2009). More recently, it has been shown that non-*Saccharomyces* yeasts are able to reveal 3MH, 4MMP and 3MHA (Anfang et al., 2009; Zott et al., 2011).

Table 4 shows the production of the three compounds analysed for each mono- or co-culture.

All the strains were able to release 3MH, but among the strains tested, *M. pulcherrima* MCR-24 and *S. cerevisiae* PB2023 were the greatest producers, followed by *C. zemplinina* MCR-9 and *T. delbrueckii* BB-MV:3FA5. These results are in agreement with those of Zott et al. (2011) regarding the ability of *M. pulcherrima* and *T. delbrueckii* to release 3 MH. The *C. zemplinina* strain tested in our study also appears to be a good 3 MH producer, suggesting that this production is strain-dependent. Indeed, *C. zemplinina* has already been shown to be a good producer of 3MH by Anfang et al. (2009). However, *C. zemplinina* does not produce this compound, according to Zott et al. (2011). *S. cerevisiae* produced significantly more 3MHA (513 ng l^{-1}) than all

Table 4
Concentrations and univariate statistical analysis of the quantified volatile thiols (ng l^{-1}) in the wines produced by the pure cultures of *S. cerevisiae* PB2023 (Sc), *C. zemplinina* MCR-9 (Cz), *M. pulcherrima* MCR-24 (Mp) and *T. delbrueckii* BB-MV:3FA5 (Td) and mixed cultures of *C. zemplinina/S. cerevisiae* (Cz–Sc), *M. pulcherrima/S. cerevisiae* (Mp–Sc) and *T. delbrueckii/S. cerevisiae* (Td–Sc). Data are average of three replicates ± standard deviations.

Type of wine	3MHA***	3MH***	4MMP***
Sc	513.6 ± 27.6^{a}	3064.4 ± 139.3^{a}	30.2 ± 6.4^{a,b}
Cz	10.3 ± 4.4^{c}	2075.9 ± 37.3^{b,c}	44.0 ± 14.9^{a}
Mp	3.6 ± 3.2^{c}	3151.8 ± 71.8^{a}	27.8 ± 11.3^{a,b,c}
Td	13.0 ± 2.7^{c}	2388.3 ± 259.9^{b}	7.1 ± 0.3^{c}
Cz–Sc	31.6 ± 5.4^{c}	2121.4 ± 6.7^{b,c}	10.2 ± 0.9^{c}
Mp–Sc	125.4 ± 13.9^{b}	1648.2 ± 59.2^{c}	7.0 ± 1.1^{c}
Td–Sc	26.3 ± 1.8^{c}	2205.7 ± 79.6^{b}	11.4 ± 1.1^{b,c}

Values with the same letters are not significantly different according to the Tukey test (significance 95%) ***Significance level of the one way ANOVA (* $0{,}05 > p > 0{,}01$; ** $0{,}01 > p > 0.001$; $^{***}p < 0.001$).

the other strains. The production of 3MHA by the non-*Saccharomyces* strains is rather low, on average 10 times lower than *S. cerevisiae*. According to Swiegers et al. (2006) *S. cerevisiae* is able to convert 3MH to 3MHA, thus a positive correlation is usually observed between 3MH and 3MHA concentrations for the *S. cerevisiae* strains (Anfang et al., 2009). Our results suggest that the non-*Saccharomyces* strains under study have a limited capacity to convert 3MH to 3MHA. Finally, the *T. delbrueckii* strain is the lowest producer of 4-MMP compared to other mono-cultures. This confirms previous results in Zott et al. (2011), who reported the low ability of *T. delbrueckii* to release 4MMP. On the other hand, we show here that *M. pulcherrima* and *C. zemplinina* produce as much 4MMP as *S. cerevisiae*, which was not the case in the report by Zott et al. (2011).

Co-inoculations of non-*Saccharomyces* strains with *S. cerevisiae* at a ratio 10:1 affect the release of thiols. The 3MH level decreased by almost half with the *M. pulcherrima*/*S. cerevisiae* co-culture (Table 4). This decrease could be linked to the increase in 3MHA level obtained with the same co-culture, in comparison to the *M. pulcherrima* mono-culture. However, a decrease in 3MH and 3MHA was observed for all the strains tested in co-culture conditions, when compared to the *S. cerevisiae* mono-culture. In contrast, Zott et al. (2011) reported increased 3MH levels with alcoholic fermentation by *S. cerevisiae* and Non-*Saccharomyces* in co-culture, in comparison with the *S. cerevisiae* mono-culture. Anfang et al. (2009) also reported an increase in 3MHA in Sauvignon Blanc co-fermented with *P. kluyveri* and some commercial *S. cerevisiae* strains at a 9:1 ratio. In addition, using different *Saccharomyces* strains, they also reported that co-fermentation did not change 3MHA levels significantly or decrease them, in contrast with the results obtained in our study. The comparison of our results with those in the literature supports the idea that interactions are not species-dependent but rather strain-dependant, which renders studies of yeast interactions extremely complex. With regard to 4MMP, *S. cerevisiae* always produced lower amounts of this compound in co-culture than in mono-culture. The same trend is also observed for *C. zemplinina*. These results suggest a negative interaction for 4MMP production in co-culture. When normalised versus biomass (Fig. 3), our results confirmed that the decrease in 4MMP is due to negative interaction. The precise cause of this decrease is unknown. The metabolism related to 4MMP is complex and still not completely described. 4MMP is produced from cys-4MMP by cleavage with β-lyase. However, cysteinylated precursors account for only a small percentage of total varietal thiol production (Subileau et al., 2008). 4MMP could be produced from another precursor, glutathionylated 4MMP (Roland et al., 2010). Schneider et al. (2006) have suggested that there may be another pathway for formation of 4MMP. Before being cleaved, precursors need to be transported inside the cell. Transporters and β-cysteinelyase are regulated by the nitrogen level in the medium (Thibon et al., 2008; Subileau et al., 2008). Thus, a decrease in thiol compounds during co-culture could be linked to competition for nitrogen sources between yeasts, with consequences on the expression of genes involved both in transport of precursors and in their hydrolysis. Some of the genes involved in thiol formation have recently been identified (Thibon et al., 2008; Subileau et al., 2008; Roncorini et al., 2011). Monitoring the expression of these genes in *S. cerevisiae* during co-culture could provide interesting information about interactions.

4. Conclusion

The nature of the interactions between *S. cerevisiae* and non-*Saccharomyces* yeasts that occur during alcoholic fermentation has rarely been the main focus, and most studies have analysed a relatively low number of aromatic compounds (Rodríguez et al., 2010; Comitini et al., 2011). It is difficult to distinguish between passive and active yeast interactions. Our study, based on the analysis of 48 volatile compounds and on the comparison of 3 non-*Saccharomyces*/*S. cerevisiae* co-cultures, demonstrates the existence of several types of interactions independent of biomass production. The synergistic effect (positive interaction) observed between *M. pulcherrima* and *S. cerevisiae* resulted in a higher level of aromatic compounds than the sum of the aromatic compounds present in each mono-culture, independent of biomass. Passive interaction was characterised by the *T. delbrueckii*/*S. cerevisiae* co-culture, where the aromatic profile is close to the mono-culture profile. The lower concentration of aromatic compounds in *C. zemplinina*/*S. cerevisiae* co-culture compared to *C. zemplinina* mono-culture, suggests a possible negative interaction between these two yeasts.

The comparative analysis of changes induced by co-cultures in the profiles of several compounds belonging to the same aromatic family may provide very interesting investigative leads on the co-culture effect in the entire metabolic pathway. A dynamic metabolic approach will be useful to unravel the nature of these interactions.

Acknowledgements

The authors would like to thank SPINDAL-AEBgroup and Regional Council of Burgundy for financial support. We thank Carmela CHATEAU for proofreading the manuscript.

References

Alexandre, H., Mathieu, B., Charpentier, C., 1996. Alteration in membrane fluidity and lipid composition, and modulation of H+-ATPase activity in *Saccharomyces cerevisiae* caused by decanoic acid. Microbiology 142 (3), 469–475.

Alexandre, H., Costello, P.J., Remize, F., Guzzo, J., Guilloux-Benatier, M., 2004. *Saccharomyces cerevisiae–Oenococcus oeni* interactions in wine: current knowledge and perspectives. International Journal of Food Microbiology 93 (2), 141–154.

Anfang, N., Brajkovich, M., Goddard, M.R., 2009. Co-fermentation with *Pichia kluyveri* increases varietal thiol concentrations in Sauvignon Blanc. Australian Journal of Grape and Wine Research 15 (1), 1–8.

Bely, M., Stoeckle, P., Masneuf-Pomarède, I., Dubourdieu, D., 2008. Impact of mixed *Torulaspora delbrueckii–Saccharomyces cerevisiae* culture on high-sugar fermentation. International Journal of Food Microbiology 122 (3), 312–320.

Bisson, L.F., Kunkee, R.E., 1993. Microbial interactions during wine production. In: Zeikus, J.G., Johnson, E.A. (Eds.), Mixed Cultures in Biotechnology. McGraw-Hill, New York, pp. 37–68.

Carrau, F.M., Medina, K., Boido, E., Farina, L., Gaggero, C., Dellacassa, E., Versini, G., Henschke, P.A., 2005. De novo synthesis of monoterpenes by *Saccharomyces cerevisiae* wine yeasts. FEMS Microbiology Letters 243, 107–115.

Ciani, M., Maccarelli, F., 1998. Oenological properties of non-*Saccharomyces* yeasts associated with wine-making. World Journal of Microbiology and Biotechnology 14 (2), 199–203.

Ciani, M., Beco, L., Comitini, F., 2006. Fermentation behaviour and metabolic interactions of multistarter wine yeast fermentations. International Journal of Food Microbiology 108 (2), 239–245.

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 Research 10 (2), 123–133.

Clemente-Jimenez, J.M., Mingorance-Cazorla, L., Martínez-Rodríguez, S., Las Heras-Vázquez, F.J., Rodríguez-Vico, F., 2005. Influence of sequential yeast mixtures on wine fermentation. International Journal of Food Microbiology 98 (3), 301–308.

Coelho, E., Coimbra, M.A., Nogueira, J.M.F., Rocha, S.M., 2009. Quantification approach for assessment of sparkling wine volatiles from different soils, ripening stages, and varieties by stir bar sorptive extraction with liquid desorption. Analytica Chimica Acta 635 (2), 214–221.

Comitini, F., Gobbi, M., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., Ciani, M., 2011. Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*. Food Microbiology 28 (5), 873–882.

Cugini, C., Calfee, M.W., Farrow III, J.M., Morales, D.K., Pesci, E.C., Hogan, D.A., 2007. Farnesol, a common sesquiterpene, inhibits PQS production in *Pseudomonas aeruginosa*. Molecular Microbiology 65 (4), 896–906.

Cugini, C., Morales, D.K., Hogan, D.A., 2010. *Candida albicans*-produced farnesol stimulates *Pseudomonas* quinolone signal production in LasR-defective *Pseudomonas aeruginosa* strains. Microbiology 156 (10), 3096–3107.

Dos Santos, M.M., Gombert, A.K., Christensen, B., Olsson, L., Nielsen, J., 2003. Identification of in vivo enzyme activities in the cometabolism of glucose and

acetate by *Saccharomyces cerevisiae* by using 13C-labeled substrates. Eukaryotic Cell 2 (3), 599–608.
Dubourdieu, D., Tominaga, T., Masneuf, I., Peyrot des Gachons, C., Murat, M.L., 2006. The role of yeasts in grape flavor development during fermentation: the example of sauvignon blanc. American Journal of Enology and Viticulture 57 (1), 81–88.
Dufour, J.P., Malcorps, P., Silcock, P., 2003. Control of ester synthesis during brewery fermentation. In: Smart, K. (Ed.), 2003. Brewing Yeast Fermentation Performance, vol. 2. Blackwell Publishing, Oxford, pp. 213–233.
Díaz-Montaño, D.M., Córdova, J., 2009. The fermentative and aromatic ability of *Kloeckera* and *Hanseniaspora* yeasts. In: Satyanarayana, T., Kunze, G. (Eds.), Yeast Biotechnology: Diversity and Applications. Springer Netherlands, Dordrecht, pp. 281–305.
Esteve-Zarzoso, B., Belloch, C., Uruburu, F., Querol, A., 1999. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. International Journal of Systematic Bacteriology 49 (1), 329–337.
Fedrizzi, B., Pardon, K.H., Sefton, M.A., Elsey, G.M., Jeffery, D.W., 2009. First identification of 4-S-glutathionyl-4-methylpentan-2-one, a potential precursor of 4-mercapto-4-methylpentan-2-one, in Sauvignon Blanc juice. Journal of Agricultural and Food Chemistry 57 (3), 991–995.
Fleet, G., 2003. Yeast interactions and wine flavour. International Journal of Food Microbiology 86 (1–2), 11–22.
Gamero, A., Hernández-Orte, P., Querol, A., Ferreira, V., 2011. Effect of aromatic precursor addition to wine fermentations carried out with different *Saccharomyces* species and their hybrids. International Journal of Food Microbiology 147 (1), 33–44.
Gramatica, P., Manitto, P., Ranzi, B.M., Delbianco, A., Francavilla, M., 1982. Stereospecific reduction of geraniol to (R)-(+)-citronellol by *Saccharomyces cerevisiae*. Experientia 38 (7), 775–776.
Heard, G., 1999. Novel yeasts in winemaking: looking to the future. Food Australia 51 (8), 347–352.
Howell, K.S., Cozzolino, D., Bartowsky, E.J., Fleet, G.H., Henschke, P.A., 2006. Metabolic profiling as a tool for revealing *Saccharomyces* interactions during wine fermentation. FEMS Yeast Research 6 (1), 91–101.
Jolly, N.P., Pretorius, I.S., Augustyn, O.P.H., 2003. The use of *Candida pulcherrima* in combination with *Saccharomyces cerevisiae* for the production of Chenin Blanc wine. South African Journal of Enology and Viticulture 24 (2), 63–69.
Jolly, N.P., Augustyn, O.P.H., Pretorius, I.S., 2006. The role and use of non-*Saccharomyces* yeasts in wine production. Vine 27 (1), 15–39.
King, A., Richard Dickinson, J., 2000. Biotransformation of monoterpene alcohols by *Saccharomyces cerevisiae*, *Torulaspora delbrueckii* and *Kluyveromyces lactis*. Yeast 16 (6), 499–506.
King, E.S., Swiegers, J.H., Travis, B., Francis, I.L., Bastian, S.E.P., Pretorius, I.S., 2008. Coinoculated fermentations using *Saccharomyces* yeasts affect the volatile composition and sensory properties of *Vitis vinifera* L. cv. Sauvignon blanc wines. Journal of Agricultural and Food Chemistry. 56 (22), 10829–10837.
Lambrechts, M.G., Pretorius, I.S., 2000. Yeast and its Importance to wine aroma – a review. South African Journal for Enology and Viticulture 21 (Special Issue), 97–129.
Lees, N.D., Bard, M., Kirsch, D.R., 1999. Biochemistry and molecular biology of sterol synthesis in *Saccharomyces cerevisiae*. Critical Review in Biochemistry and Molecular Biology 34, 33–37.
Lin, Y., 1975. Detection of wild yeasts in the brewery. Efficiency of differential media. Journal of the Institute of Brewing 81 (5), 410–417.
Magyar, I., Tóth, T., 2011. Comparative evaluation of some oenological properties in wine strains of *Candida stellata, Candida zemplinina, Saccharomyces uvarum* and *Saccharomyces cerevisiae*. Food Microbiology 28 (1), 94–100.
Maicas, S., Mateo, J.J., 2005. Hydrolysis of terpenyl glycosides in grape juice and other fruit juices: a review. Applied Microbiology and Biotechnology 67 (3), 322–335.
Malacrinò, P., Zapparoli, G., Torriani, S., Dellaglio, F., 2001. Rapid detection of viable yeasts and bacteria in wine by flow cytometry. Journal of Microbiological Methods 45 (2), 127–134.
Mannazzu, I., Clementi, F., Ciani, M., 2002. Strategies and criteria for the isolation and selection of autochthonous starters. In: Ciani, M. (Ed.), Biodiversity and Biotechnology of Wine Yeasts. Research Signpost, India, Kerala, pp. 19–33.
Mendoza, L.M., Nadra, M.C.M., Farías, M.E., 2007. Kinetics and metabolic behavior of a composite culture of *Kloeckera apiculata* and *Saccharomyces cerevisiae* wine related strains. Biotechnology Letters 29 (7), 1057–1063.
Mills, D.A., Johannsen, E.A., Cocolin, L., 2002. Yeast diversity and persistence in Botrytis-affected wine fermentations. Applied and Environmental Microbiology 68 (10), 4884–4893.
Moreira, N., Mendes, F., Hogg, T., Vasconcelos, I., 2005. Alcohols, esters and heavy sulphur compounds production by pure and mixed cultures of apiculate wine yeasts. International Journal of Food Microbiology 103 (3), 285–294.
Nguyen, H.-V., Panon, G., 1998. The yeast *Metschnikowia pulcherrima* has an inhibitory effect against various yeast species. Sciences Des Aliments 18 (5), 515–526.
Nissen, P., Arneborg, N., 2003. Characterization of early deaths of non-*Saccharomyces* yeasts in mixed cultures with *Saccharomyces cerevisiae*. Archives of Microbiology 180 (4), 257–263.
Peyrot des Gachons, C., Tominaga, T., Dubourdieu, D., 2002. Localization of S-Cysteine conjugates in the berry: effect of skin contact on aromatic potential of *Vitis vinifera* L. cv. Sauvignon Blanc must. American Journal of Enology and Viticulture 53 (2), 144–146.
Rodríguez, M.E., Lopes, C.A., Barbagelata, R.J., Barda, N.B., Caballero, A.C., 2010. Influence of *Candida pulcherrima* Patagonian strain on alcoholic fermentation behaviour and wine aroma. International Journal of Food Microbiology 138 (1–2), 19–25.
Rainieri, S., Pretorius, I.S., 2000. Selection and improvement of wine yeasts. Annals of Microbiology 50 (1), 15–31.
Renault, P., Miot-Sertier, C., Marullo, P., Hernández-Orte, P., Lagarrigue, L., Lonvaud-Funel, A., Bely, M., 2009. Genetic characterization and phenotypic variability in *Torulaspora delbrueckii* species: potential applications in the wine industry. International Journal of Food Microbiology 134 (3), 201–210.
Rodríguez-Bencomo, J.J., Schneider, R., Lepoutre, J.P., Rigou, P., 2009. Improved method to quantitatively determine powerful odorant volatile thiols in wine by headspace solid-phase microextraction after derivatization. Journal of Chromatography 1216 (30), 5640–5646.
Rojas, V., Gil, J.V., Piñaga, F., Manzanares, P., 2003. Acetate ester formation in wine by mixed cultures in laboratory fermentations. International Journal of Food Microbiology 86 (1–2), 181–188.
Roland, A., Schneider, R., Guernevé, C.L., Razungles, A., Cavelier, F., 2010. Identification and quantification by LC-MS/MS of a new precursor of 3-mercaptohexan-1-ol (3MH) using stable isotope dilution assay: elements for understanding the 3MH production in wine. Food Chemistry 121, 847–855.
Romano, P., 2003. Function of yeast species and strains in wine flavour. International Journal of Food Microbiology 86 (1–2), 169–180.
Roncorini, M., Santiago, M., Hooks, D.O., Moroney, S., Harsch, M.J., Lee, S.A., Richards, K.D., Nicolau, L., Gardner, S.C., 2011. The yeast IRC7 gene encodes a b-lyase responsible for production of the varietal thiol 4-mercapto-4-methylpentan-2-one in wine. Food Microbiology 28 (5), 926–930.
Rorabacher, D., 1991. Statistical treatment for rejection of deviant values: critical values of Dixon Q parameter and related subrange ratios at the 95 percent confidence level. Analytical Chemistry 63 (2), 139–146.
Saerens, S.M.G., Delvaux, F., Verstrepen, K.J., Van Dijck, P., Thevelein, J.M., Delvaux, F.R., 2008. Parameters affecting ethyl ester production by *Saccharomyces cerevisiae* during fermentation. Applied and Environmental Microbiology 74 (2), 454–461.
Saerens, Sofie M.G., Delvaux, F.R., Verstrepen, K.J., Thevelein, J.M., 2010. Production and biological function of volatile esters in *Saccharomyces cerevisiae*. Microbial Biotechnology 3 (2), 165–177.
Schneider, R., Charrier, F., Razungles, A., Baumes, R., 2006. Evidence for an alternative biogenetic pathway leading to 3-mercaptohexanol and 4-mercapto-4-methylpentan-2-one in wines. Analytica Chimica Acta 563 (1–2), 58–64.
Soden, A., Francis, I.L., Oakey, H., Henschke, P.A., 2000. Effects of co-fermentation with *Candida stellata* and *Saccharomyces cerevisiae* on the aroma and composition of Chardonnay wine. Australian Journal of Grape and Wine Research 6 (1), 21–30.
Subileau, M., Schneider, R., Salmon, J.-M., Degryse, E., 2008. New insights on 3-mercaptohexanol (3MH) biogenesis in Sauvignon Blanc wines: cys-3MH and (E)-hexen-2-al are not the major precursors. Journal of Agricultural and Food Chemistry 56 (19), 9230–9235.
Swiegers, J.H., Willmott, R., Hill-Ling, A., Capone, D.L., Pardon, K.H., Elsey, G.M., Howell, K.S., et al., 2006. Modulation of volatile thiol and ester aromas by modified wine yeast. Flavour Science Recent Advances and Trends 43, 113–116.
Swiegers, J.H., Kievit, R.L., Siebert, T., Lattey, K.A., Bramley, B.R., Francis, I.L., King, E.S., 2009. The influence of yeast on the aroma of Sauvignon Blanc wine. Food Microbiology 26 (2), 204–211.
Tao, Y., Zhang, L., 2010. Intensity prediction of typical aroma characters of cabernet Sauvignon wine in Changli County (China). LWT - Food Science and Technology 43 (10), 1550–1556.
Thibon, C., Marullo, P., Claisse, O., Cullin, C., Dubourdieu, D., Tominaga, T., 2008. Nitrogen catabolic repression controls the release of volatile thiols by *Saccharomyces cerevisiae* during wine fermentation. FEMS Yeast Research 8 (7), 1076–1086.
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 Microbiology 29 (1), 18–26.
Toro, M.E., Vazquez, F., 2002. Fermentation behaviour of controlled mixed and sequential cultures of *Candida cantarellii* and *Saccharomyces cerevisiae* wine yeasts. World Journal of Microbiology and Biotechnology 18 (4), 347–354.
Verstrepen, Kevin J., Derdelinckx, G., Dufour, J.-P., Winderickx, J., Thevelein, J.M., Pretorius, I.S., Delvaux, F.R., 2003. Flavor-active esters: adding fruitiness to beer. Journal of Bioscience and Bioengineering 96 (2), 110–118.
Viana, F., Gil, J.V., Vallés, S., Manzanares, P., 2009. Increasing the levels of 2-phenylethyl acetate in wine through the use of a mixed culture of *Hanseniaspora osmophila* and *Saccharomyces cerevisiae*. International Journal of Food Microbiology 135 (1), 68–74.
Zoecklein, B.W., Marcy, J.E., Williams, J.M., Jasinski, Y., 1997. Effect of native yeasts and selected strains of *Saccharomyces cerevisiae* on glycosyl glucose, potential volatile terpenes, and selected aglycones of white riesling (*Vitis vinifera* L.) wines. Journal of Food Composition and Analysis 10 (1), 55–65.
Zea, L., Moreno, J., Ortega, J.M., Medina, M., 1995. Content of free terpenic compounds in cells and musts during vinification with three *Saccharomyces cerevisiae* races. Journal of Agricultural and Food Chemistry 43 (4), 1110–1114.
Zohre, D., Erten, H., 2002. The influence of *Kloeckera apiculata* and *Candida pulcherrima* yeasts on wine fermentation. Process Biochemistry 38 (3), 319–324.
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. International Journal of Food Microbiology 151 (2), 210–215.