LWT - Food Science and Technology 109 (2019) 241–249

Contents lists available at ScienceDirect

LWT - Food Science and Technology

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

Impact of co-inoculation of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum* and *Oenococcus oeni* autochthonous strains in controlled multi starter grape must fermentations

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ARTICLE INFO

Keywords:
Wine
Mixed-starter
Hanseniaspora uvarum
Oenococcus oeni
Autochthonous yeast

ABSTRACT

The use of multi-species starter cultures is an approach of increasing significance for winemakers in order to improve the general quality and safety of the final product. As first step of the present study, we isolated and characterize two *Saccharomyces cerevisiae* yeast starter strains, denoted as ITEM 167292 and ITEM 17293, from natural must fermentations of "Negroamaro" grapes. As second step, we studied the interactions during grape must fermentation between these two strains, the *Hanseniaspora uvarum* strain ITEM 8785 and five autochthonous *Oenococcus oeni* strains, by microbial counts and by quantifying L-malic acid and ethanol concentrations. The best performing *O. oeni* strain, namely OT4, was used to create, with the *H. uvarum* strain ITEM 8785, two mixed starter formulations with the strains ITEM 167292 and ITEM 17293. The three microbial species showed to be compatible and to complete the fermentative processes producing wines denoted by reduced acetic acid concentrations. The performance of the mixed starter formulations were then validated by carrying pilot-scale vinifications. At the best of our knowledge, this report is the first study regarding the utilization of selected *H. uvarum/S. cerevisiae/O. oeni* autochthonous strains in a simultaneous multi-starter inoculation for the industrial production of regional typical wines.

1. Introduction

Traditionally, the vinification process of red wines includes two essential stages, i.e. the alcoholic fermentation (AF) and the malolactic fermentation (MLF). During the AF, the sugars of the must are transformed into ethanol and this process is carried out by the yeasts, principally by *Saccharomyces cerevisiae* (Garofalo, Tristezza, Grieco, Spano, & Capozzi, 2016). However, non-*Saccharomyces* species have a role in the AF and they contribute to enhance the organoleptic properties of wine (Liu, Lu, Duan &Yan, 2016; Petruzzi et al., 2017). Several non-*Saccharomyces* species have been studied in mixed fermentations with the scope of adding peculiar features to the wine (Ciani, Beco, & Comitini, 2006; Ciani, Comitini, Mannazzu, & Domizio, 2009; Comitini et al., 2011; Suzzi et al., 2012; Tristezza et al., 2016b). These mixed cultures have an additional interest when they are formed by autochthonous selected yeasts, since they are adapted to the conditions of a specific wine-production area (Capozzi, Garofalo, Chiriatti, Grieco, & Spano, 2015; Lopes, Rodriguez, Sangorrin, Querol, & Caballero, 2007; Tofalo et al., 2016) and may ensure the maintenance of the typical oenological and sensory characteristics of wine (Rodríguez et al., 2010).

The development of efficient malolactic starter cultures is crucial for the oenological industry (Berbegal et al., 2016, Brizuela et al., 2018). Several are the strain-specific features requested for a malolactic starter culture, such as the capacity to stand low pH, high ethanol and SO_2 concentrations, the absence of biogenic amines production, the compatibility with yeast selected strains (Berbegal et al., 2016; Capozzi et al., 2010). Besides, a critical step in the employment of MLF starters is the time of inoculation. Lactic acid bacteria (LAB) starters can be co-inoculated with yeast at the beginning of AF, or sequentially inoculated after the AF (Bartowsky, Costello, & Chambers, 2015). Several recent investigations have indicated that when bacteria are directly inoculated into the must they performed better than they when added after the end

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https://doi.org/10.1016/j.lwt.2019.04.045
Received 1 March 2019; Received in revised form 11 April 2019; Accepted 12 April 2019
Available online 15 April 2019
0023-6438/

to the AF (Abrahamse & Bartowsky, 2011; Trani, Verrastro, Punzi, Faccia, & Gambacorta, 2016; Tristezza et al., 2016a). The *H. uvarum* ITEM 8795 was selected because of its contribution in increasing the wine organoleptic quality and reducing the volatile acidity (De Benedictis, Bleve, Grieco, Tristezza, & Tufariello, 2011). The oenological potential of this strain in co-inoculation and in a sequential inoculation with *S. cerevisiae* was also assessed by industrial wine production (Tristezza et al., 2016b).

In the present investigation, we report the selection of Apulian autochthonous *S. cerevisiae* and *O. oeni* strains to design of a mixed starter culture with *H. uvarum* ITEM 8795 to simultaneously perform the AF and MLF. Furthermore, we evaluated the compatibility between the different microorganisms employed in the autochthonous mixed starter culture and the best inoculation time of *O. oeni* strains. At the best of our knowledge, this study described, for the first time the fermentative performance of a non-*Saccharomyces/Saccharomyces/O. oeni* mixed starter formulation.

2. Material and methods

2.1. Microorganisms

Yeast strains used in the present study are deposited in Agro- Food Microbial Culture Collection of ISPA (http://www.ispacnr.it/collezioni-microbiche/). All yeast strains were cultured in YPD (Sigma-Aldrich, USA) and incubated at 28 °C 24–48 h. *O. oeni* strains were previously isolated from Nero di Troia wine (Capozzi et al., 2014) and they are deposited in the collection of the Industrial Microbiology Laboratory (University of Foggia). LAB strains were cultured in MRS broth (Sigma-Aldrich, USA) and incubated at 28 °C for 4–7 days.

2.2. Yeast isolation and S. cerevisiae strains identification

The enological selection was carried out according to Tufariello et al. (2019) from spontaneous fermentations of Negroamaro grapes collected in the "Brindisi" PDO/DOC area. Briefly, yeast isolates were firstly screened for their ability to produce hydrogen sulphide on Biggy agar (Sigma, USA). H_2S-low producer isolates (i.e. white or light brown colonies) were selected for genetic characterization. The isolates were identified at species-level by PCR analysis or the ribosomal RNA region (Tufariello et al., 2019) and at strain-level by interdelta typing (Tristezza, Gerardi, Logrieco, & Grieco, 2009). The amplified DNA products were visualized and analyzed by agarose gel electrophoresis (Hay et al., 1994).

2.3. Lab-scale vinification

The identified *S. cerevisiae* strains were tested by micro-fermentation assays conducted in Negroamaro grape must (21.5° Babo; 7.2 g/L total acidity; pH 3.4) added with 100 mg/L potassium metabisulfite. One liter of treated must was inoculated with 10^6 CFU/mL of yeast culture. The vinifications were carried out in triplicate at 25 °C and daily monitored by measuring the reducing sugars concentration. Wines were then filtered, separately bottled and stored at 18 °C for the sensorial analysis (Tufariello et al., 2019).

2.4. Co-inoculation tests

For the co-inoculation trials, yeast and bacteria starter cultures were prepared by growing strains in YPD or MRS medium as described above and then inoculating in triplicate the strains into 200 mL of Negroamaro grape must from (21° Babo; 7.2 g/L total acidity; 2.57 g/L malic acid; pH 3.78). Using the 2 selected *S. cerevisiae*, 1 *H. uvarum* and 5 *O. oeni* strains, a total of 10 different starter culture combinations were carried out. In the mixed starter cultures, the *H. uvarum* strain was simultaneously inoculated with *S. cerevisiae* in a 1:100 inoculum ratio (respectively 10^4 CFU/mL and 10^6 CFU/mL). *O. oeni* strains were co-inoculated (ethanol content 0%) or sequentially inoculated during AF, when ethanol content was 2%, 4%, 6%, 8%, 10% or 12% (v/v) with a final concentration of 1×10^6 CFU/mL. The kinetics of the fermentations was monitored for 7 days. After AF, L-malic acid was determined by enzymatic kits (Biogamma, Italy).

Table 1
Main oenological and technological properties determined in 15 autochthonous *S. cerevisiae* strains.

Isolate	FP	AYC	AC	H_2S	Foam
P1	0.04	0.62	12.6	+ +	-
P2	0.03	0.64	13.7	-	-
P5	0.03	0.64	14.0	-	-
P6	0.03	0.63	13.6	+	-
P9	0.04	0.59	12.5	+	-
P13	0.03	0.63	13.6	-	-
P14	0.03	0.63	13.6	+	+ +
P20	0.03	0.64	13.9	-	-
P25	0.04	0.64	13.8	-	-
P26	0.03	0.65	14.1	-	-
P28	0.03	0.65	14.1	-	-
P32	0.05	0.64	14.0	-	+
P33	0.03	0.65	14.0	-	-
P34	0.03	0.64	13.8	-	-
P35	0.03	0.65	14.1	-	-
Control	0.04	0.63	13.3		

Data, measured at the end of fermentation, represent the average of three replicates. FP fermentation purity [volatile acidity (g/L)/ethanol (% v/v)], AYC alcohol yield coeficient [alcohol (% v/v/initial sugars (%) -Final sugars (%)], AC alcohol content (% v/v). H_2S and foam production: absent (-); low (+), high (+ +), very high (+ + +).

2.5. Pilot-scale vinification

The vinification was carried out in an experimental cellar using sterile stainless steel 100-L vessels by inoculating 90 L of Negroamaro must (18.9° Babo; pH 3.22; nitrogen 176.4 g/L), as single or mixed inoculum with 10^6 CFU/mL of *H. uvarum* ITEM 8795, 10^4 CFU/mL of *S. cerevisiae* ITEM 17292 or ITEM 17293 and 10^6 CFU/mL of *O. oeni* OT4. The dynamics of the alcoholic fermentation process was daily monitored and samples of wines were stored at −20 °C for further analyses.

2.6. Analytical determinations

The main product components (ethanol, residual sugars, pH, glucose, fructose, malic acid, lactic acid, tartaric acid, citric acid, volatile acidity, total acidity, glycerol brix, density, SO_2, total polyphenols, antocyans, CO_2, absorbance at 420, 520 and 620 nm) of wine and must under fermentation were evaluated by Fourier Transform Infrared Spectroscopy the (FTIR) by employing the WineScan Flex (FOSS Analytical, DK). Samples were centrifuged at 8000 rpm for 10 min and then analyzed following the supplier's instructions. The major volatile constituents [acetaldehyde, ethyl acetate, 2-methyl-1-propanol, 1-propanol, higher alcohols, acetoin] were determined by gas-chromatography according to Di Toro et al. (2015). The internal standard solution used was 4-methyl-2-pentanol. Identification and quantification of the volatile compounds by GC–MS were carried out using an internal standard as already described (Tufariello et al., 2014). Volatile compounds were extracted in triplicate by solid phase extraction (SPE) technique (Garofalo et al., 2018). The samples were injected into a DB-WAX capillary column (60 m × 0.25 mm I.D., 0.25 μm film thickness; Agilent, USA) and then analyzed with a 6890N series gas chromatograph (Agilent, USA) equipped with an Agilent 5973 mass spectrometer selective detector (MSD). The analysis was performed as previously reported (Tufariello et al., 2014). Technological parameters were obtained as previously described (Tufariello et al., 2019).

Table 2
Concentration of major chemical compounds in wines obtained with 15 autochthonous strain of *S. cerevisiae*.

Strain	Ethanol	Sugars	TA	VA	pH	Malic	Lactic	Tartaric	Citric	Glycerol
P 1	13.2 ± 0.15	4.94 ± 0.95^{b}	6.26 ± 0.05	0.41 ± 0.11	3.39 ± 0.55	1.41 ± 0.16	0.25 ± 0.05	2.04 ± 0.44	0.47 ± 0.11	8.21 ± 0.67
P 2	13.68 ± 0.45	3.40 ± 0.66^{a}	5.78 ± 0.31	0.44 ± 0.16	3.39 ± 0.47	1.26 ± 0.13	0.14 ± 0.07	1.89 ± 0.28	0.47 ± 0.13	7.99 ± 1.11
P 5	14.05 ± 0.87	1.92 ± 0.24^{a}	5.99 ± 0.65	0.44 ± 0.07	3.41 ± 0.38	1.41 ± 0.24	0.07 ± 0.03	1.99 ± 0.65	0.47 ± 0.07	8.66 ± 0.94
P 6	13.74 ± 0.55	3.11 ± 0.43^{a}	6.19 ± 0.16	0.42 ± 0.16	3.37 ± 0.31	1.49 ± 0.33	0.12 ± 0.04	1.88 ± 0.48	0.48 ± 0.14	7.55 ± 0.55
P 9	13.07 ± 0.92	7.26 ± 2.35^{b}	6.21 ± 0.35	0.58 ± 0.21	3.37 ± 0.37	1.45 ± 0.27	0.05 ± 0.02	1.68 ± 0.33	0.43 ± 0.19	7.67 ± 0.07
P 13	13.70 ± 0.40	1.87 ± 0.34^{a}	6.57 ± 0.95	0.41 ± 0.15	3.39 ± 0.62	1.59 ± 0.34	0.12 ± 0.04	2.01 ± 0.07	0.48 ± 0.15	8.64 ± 0.27
P 14	13.60 ± 1.05	2.05 ± 0.07^{a}	6.51 ± 0.44	0.42 ± 0.11	3.39 ± 0.38	1.58 ± 0.37	0.14 ± 0.03	1.98 ± 0.27	0.51 ± 0.08	8.16 ± 0.18
P 20	13.92 ± 0.88	2.15 ± 0.12^{a}	5.81 ± 0.27	0.45 ± 0.22	3.39 ± 0.17	1.29 ± 0.28	0.08 ± 0.03	1.50 ± 0.37	0.45 ± 0.15	8.78 ± 0.05
P 25	14.08 ± 0.27	2.11 ± 0.44^{a}	6.35 ± 0.65	0.33 ± 0.08	3.45 ± 0.73	1.36 ± 0.54	0.31 ± 0.07	1.52 ± 0.27	0.47 ± 0.12	10.27 ± 0.77
P 26	14.12 ± 0.84	2.24 ± 0.23^{a}	6.84 ± 0.38	0.47 ± 0.23	3.38 ± 0.37	1.70 ± 0.17	0.23 ± 0.11	1.47 ± 0.65	0.50 ± 0.20	8.76 ± 0.93
P 28	14.31 ± 0.11	1.76 ± 0.28^{a}	6.98 ± 0.48	0.32 ± 0.08	3.39 ± 0.51	1.72 ± 0.52	0.26 ± 0.08	2.04 ± 0.12	0.51 ± 0.14	9.20 ± 3.10
P 32	14.01 ± 0.41	1.49 ± 0.33^{a}	7.50 ± 0.38	0.66 ± 0.12	3.38 ± 0.93	1.94 ± 0.17	0.25 ± 0.11	1.34 ± 0.26	0.44 ± 0.18	8.18 ± 0.66
P 33	14.02 ± 0.60	2.69 ± 0.76^{a}	5.99 ± 0.95	0.48 ± 0.07	3.39 ± 0.45	1.40 ± 0.66	0.09 ± 0.03	1.91 ± 0.54	0.47 ± 0.15	8.48 ± 0.10
P 34	13.87 ± 0.76	2.36 ± 0.27^{a}	6.06 ± 0.55	0.47 ± 0.08	3.42 ± 0.61	1.25 ± 0.27	0.13 ± 0.04	2.24 ± 0.38	0.43 ± 0.12	8.37 ± 0.65
P 35	14.26 ± 0.36	3.17 ± 0.94^{a}	6.33 ± 0.95	0.44 ± 0.11	3.41 ± 0.75	1.41 ± 0.52	0.09 ± 0.03	2.27 ± 0.25	0.46 ± 0.25	8.36 ± 0.05

TA, total acidity. VA, volatile acidity. Values are expressed in g/L. The ethanol concentration is expressed in g/100 mL. Results are the mean of three injections of each replicate (n = 9); the standard deviation values (±) are indicated. Different letters in the column denote significant differences between yeast strains, at $p < 0.05$.

Table 3
Concentration of selected volatile compounds determined by GC in wines obtained with the 15 autochthonous strain of *S. cerevisiae*.

Strain	acetaldehyde	ethyl acetate	1-propanol	2-metil-1-propanol	higher alcohols	acetoin
P2	17.58 ± 0.55^{a}	15.27 ± .057^{b}	10.33 ± 0.79^{d}	4.38 ± 0.37^{a}	58.11 ± 0.48^{b}	2.59 ± 0.09^{a}
P5	14.91 ± 0.61^{a}	21.33 ± 0.69^{d}	12.54 ± 0.53^{e}	4.19 ± 0.62^{a}	55.80 ± 1.41^{a}	2.10 ± 0.17^{a}
P13	16.25 ± 1.50^{a}	12.54 ± 0.45^{b}	5.91 ± 0.15^{b}	4.52 ± 0.41^{a}	56.09 ± 0.75^{a}	3.60 ± 0.50^{b}
P20	16.89 ± 0.12^{a}	22.79 ± 0.25^{d}	12.91 ± 0.25^{e}	3.70 ± 0.24^{a}	61.20 ± 1.07^{b}	1.92 ± 0.16^{a}
P25	21.89 ± 1.02^{b}	16.49 ± 0.08^{b}	8.86 ± 0.10^{c}	9.25 ± 0.19^{b}	56.19 ± 0.43^{a}	1.88 ± 0.12^{a}
P26	15.72 ± 0.53^{a}	22.03 ± 0.91^{d}	11.21 ± 0.33^{d}	3.91 ± 0.25^{a}	59.66 ± 0.12^{b}	1.62 ± 0.15^{a}
P28	22.79 ± 0.30^{b}	18.30 ± 0.35^{c}	7.97 ± 0.52^{c}	3.02 ± 0.48^{a}	58.15 ± 1.66^{a}	2.74 ± 0.19^{a}
P33	15.09 ± 0.83^{a}	3.28 ± 0.67^{a}	5.36 ± 0.41^{b}	4.61 ± 0.32^{a}	55.83 ± 0.54^{a}	4.17 ± 0.12^{b}
P34	21.49 ± 1.13^{b}	17.43 ± 0.83^{c}	8.52 ± 0.11^{c}	4.55 ± 0.10^{a}	51.79 ± 1.47^{a}	4.96 ± 0.17^{b}
P35	24.97 ± 0.53^{c}	19.49 ± 0.64^{c}	3.55 ± 0.06^{a}	2.44 ± 0.29^{a}	58.56 ± 0.44^{b}	2.39 ± 0.24^{a}

Compound concentration (mg/L). Values are the mean of three injections of each replicate (n = 9); the standard deviation values (±) are indicated. Different letters in the row denote significant differences between yeast strains, at $p < 0.05$.

2.7. Determination of microbial population

The enumeration of viable yeast cells during the fermentations was carried out on WL agar medium (Sigma-Aldrich, USA), that allowed to discriminate *S. cerevisiae* (large white colonies) and *H. uvarum* (green colonies) after 48 h incubation at 28 °C for. The counting of LAB viable cells was made in MRS supplemented with 10 mg/L cycloheximide (Sigma-Aldrich, USA) to avoid yeast growth, after 7 days incubation at 28 °C.

2.8. Sensory analysis

The sensory analysis was performed by a panel composed of 5 professional experts, chosen among oenologists and producers involved in Negroamaro wine production. The judges were asked to assign a score for different parameters of the wines, such as gustatory-intensity, balance, acidity, body and gustatory-persistence, using a sensory analysis-tasting sheet with a scale ranging from 0 (absence of perception) to 3 (maximum perception). The mean scores of attributes were submitted to Quantitative Descriptive Analysis (QDA) according to Trani, Verrastro, Punzi, Faccia, and Gambacorta (2016).

2.9. Statistical analysis

Chemical data were subjected to One-Way factor analysis of variance (ANOVA). Significant differences were separated using the Duncan test. The level of significance was set at $P < 0.05$. The comparison of volatile classes of compounds during fermentation was achieved by principal component analysis (PCA). All statistical analyses were carried out using the STATISTICA7.0 software (StatSoft software package, USA).

3. Results

3.1. Yeast isolation and identification

The oenological selection of autochthonous yeasts associated with natural fermentations of Negroamaro grapes, collected in the “Brindisi” PDO/DOC area, started with the isolation of 1200 yeast isolates. To this scope, serial dilutions of must and lees collected at the end of spontaneous fermentation were spread after on BIGGY agar. This selective medium allowed the isolation of 145 yeast colonies no or low H_2S producers. The above 145 isolates were identified by molecular analysis of yeast rDNA, and they confirmed to belong to the species *Saccharomyces cerevisiae.* Then, 36 isolates randomly selected were characterized at strain level using a PCR-based assay, relying on the amplification of interdelta regions. The molecular fingerprint allowed the identification of 15 different *S. cerevisiae* strains (not shown). One representative biotype for each strain/profile has been selected. For these strains (P1, P2, P5, P6, P9, P13, P14, P20, P25, P28, P26, P33, P34, P35 and P32) the fermentative performances in wine were further studied.

3.2. Lab-scale vinifications

These technological and oenological parameters were mainly considered for the selection of autochthonous yeast strains: (i) acetic acid < 0 .6 g/L, (ii) residual sugars < 2 g/L and (iii) absence of H_2S

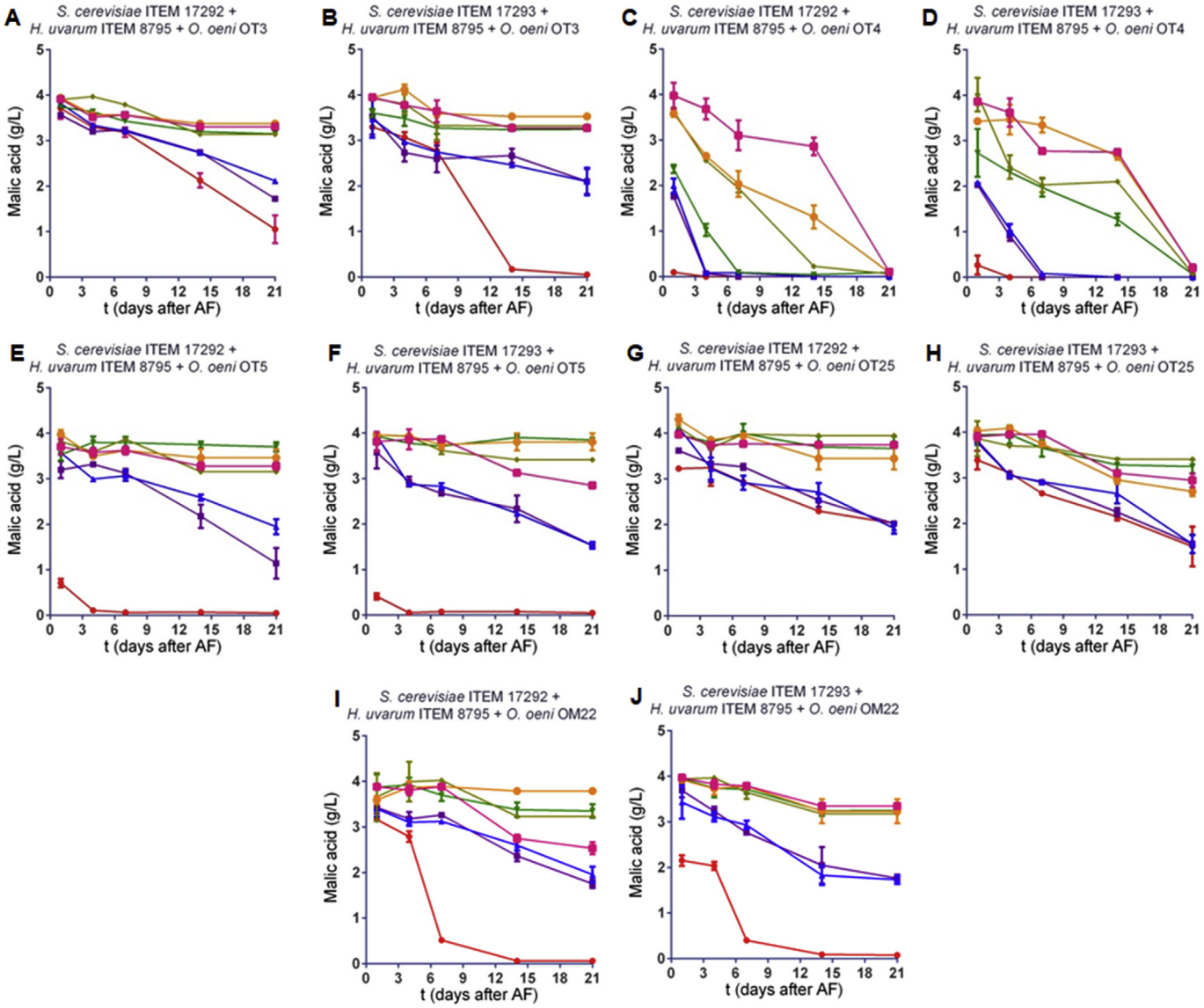

Fig. 1. L-malic acid consumption (g/L) by *O. oeni* strains (OT3, OT4, OT5, OT25, OM22) after AF, when were co-inoculated (●) or sequentially inoculated during AF, when ethanol content was 2% (■), 4% (▲), 6% (▼), 8% (♦), 10% (●) or 12% (■) (v/v).

production. The primary screening indicated that, among the 15 selected different biotypes, the P2, P5, P13, P20, P25, P26, P28, P33, P34 and P35 complied to the above criterions and they were further characterized. Table 1 and Table 2 describe their principal technological and chemical features of the obtained wines. The presence of higher alcohols produced by fermentation in must was evaluated (Table 3). The latter ranged from 51.79 mg/L (strain P34) to 59.66 mg/L (strain P26), indicating that all strains could positively contribute to the aromatic complexity of wine. The ethyl acetate values ranged between 12.54 mg/L for P13 and 22.79 mg/L for P20 (Table 3). Acetaldehyde concentrations ranged from 14.91 mg/L (strain P5) to 22.79 mg/L (strain P28). The amount of acetoin, produced by the tested strains, ranged from 1.62 mg/L for P26 to 4.96 mg/L for P34 (Table 3).

The fermented musts were also subjected to sensory analysis and the strains P25 and P28 obtained the maximum score with 12 and 13 points out of 15. The global evaluation of obtained data indicated that the P25 (ITEM 17292) and P28 (ITEM 17293) strains were those denoted by the best fermentative properties and they were chosen for the co-inoculation trials.

3.3. Malolactic activity of O. oeni strains in the mixture culture

The selected *S. cerevisiae* ITEM 17292 and ITEM 17293 strains were co-inoculated with *H. uvarum* ITEM 8795 in Negroamaro grape must and the five selected autochthonous *O. oeni* strains were further investigated for their ability to consume L-malic acid by co-inoculating (0%) or sequentially inoculating them during AF, when ethanol content was 2%, 4%, 6%, 8%, 10% or 12% (v/v) (Fig. 1). Results showed that ethanol level at the moment of bacterial inoculation was crucial for developing MLF. The strategy of co-inoculation with *S. cerevisiae* and *H. uvarum* was the best strategy for maintaining highest *O. oeni* populations and therefore for carrying out MLF in red must. Only OT3 *O. oeni* strain co-inoculated with *H. uvarum* ITEM 8795 and *S. cerevisiae* ITEM 17292 (Fig. 1A) and OT25 *O. oeni* strain co-inoculated with *H. uvarum* ITEM 8795 and *S. cerevisiae* ITEM 17292 (Fig. 1G) or ITEM 17293 (Fig. 1H) were not consuming all L-malic acid present in the red must after 21 days of the end of the AF. All strains of *O. oeni* exhibited malolactic activity when they were inoculate in an ethanol concentration up to 4%. We observed that *O. oeni* strains have more difficulties to initiate MLF with 6–12% of ethanol. Among the *O. oeni* strains, OT4 presented the highest malolactic activity, consuming completely the L-

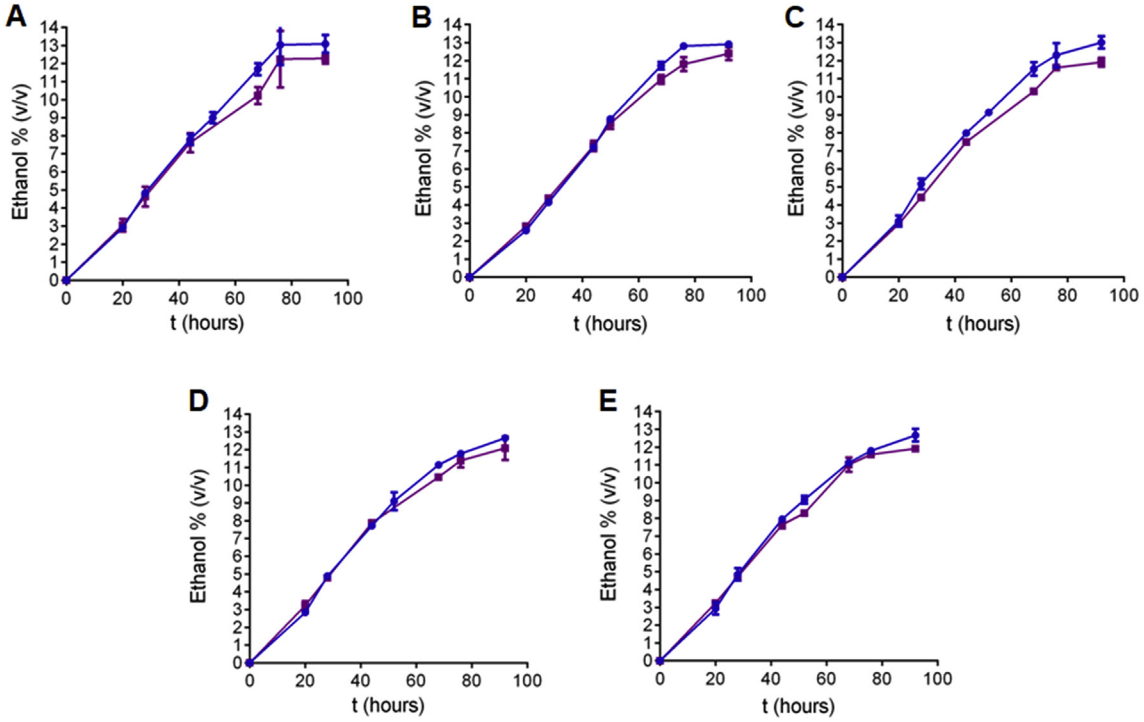

Fig. 2. Ethanol content (%, v/v) formation during the must fermentations carried out by the co-inoculation of *H. uvarum* ITEM 8795, *S. cerevisiae* ITEM 17292 (●) or *S. cerevisiae* ITEM 17293 (■), and A; *O. oeni* OT3, B; *O. oeni* OT4, C; *O. oeni* OT5, D; *O. oeni* OT25 and E; *O. oeni* OM22.

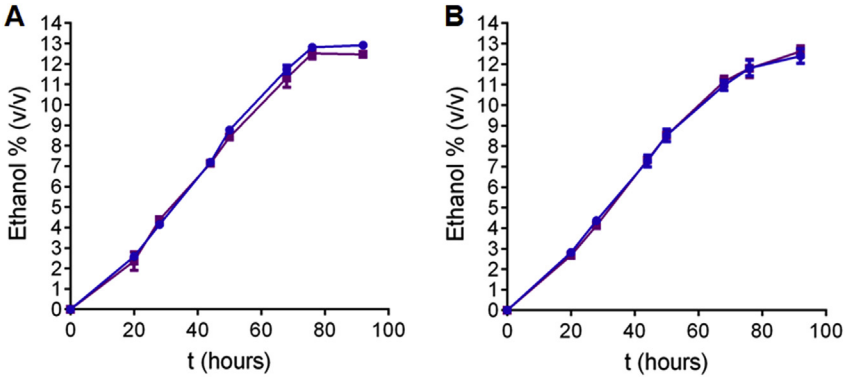

Fig. 3. Ethanol content produced during the must fermentations carried out by: A; *S. cerevisiae* ITEM 17292, *H. uvarum* ITEM 8795 and *O. oeni* OT4 co-inoculated (●) and sequentially inoculated when ethanol content was 12% (v:v) (■), and B; *S. cerevisiae* ITEM 17293, *H. uvarum* ITEM 8795 and *O. oeni* OT4 co-inoculated (●) and sequentially inoculated when ethanol content was 12% (v:v) (■).

malic acid in all the ethanol concentrations studied (Fig. 1C and D). When inoculated at ethanol concentrations up to 6% (v/v), *O. oeni* OT4 completed the MLF in less than 7 (Fig. 1C) or in 21 days (Fig. 1D), when the *S. cerevisiae* strains ITEM 17292 and ITEM 17293 were respectively used in the mixed starter formulation.

3.4. Kinetics of alcoholic fermentation in the multi-strain fermentations

In order to evaluate the effect of the inoculated microorganisms on the AF, the formation of ethanol was followed for 4 days. Significant differences ($P = 0.0020$) were found in ethanol formation depending on the *S. cerevisiae* stain used (Fig. 2). The ethanol concentration was 12.86% (v/v) and 12.12% (v/v), respectively when ITEM 17292 and ITEM 17293 were used in the co-inoculation tests. There were not significant no differences on the final ethanol concentration depending on the time of inoculation of the *O. oeni* strain. The concentration of ethanol in the produced wines was not influenced by the procedure adopted for the *O. oeni* OT4 strain inoculation, i.e. co-inoculation with *H. uvarum* and *S. cerevisiae* or inoculation at the end of the AF (Fig. 3). These findings were observed with all *O. oeni* strains used in the study (data not shown). Taken together, the above results indicated that the OT4 strain was the best-performing and it was chosen for the further co-inoculation assays.

3.5. Dynamics of yeast and bacterial population

After 24 h of fermentation, *H. uvarum* underwent a slight yeast concentration decrease and then increased fast (Fig. 4). *S. cerevisiae* ITEM 17292 reached the maximum yeast population after 24 h of the inoculation, increasing from 1.00×10^6 CFU/mL to 5.55×10^7 CFU/mL. In this case *H. uvarum* ITEM 8795 presented a maximum concentration of 1.00×10^7 CFU/mL after 60 h of the inoculation, however after 72 h of incubation, the population of this yeast descended drastically (Fig. 4A). When the *H. uvarum* strain was co-inoculated with *S. cerevisiae* ITEM 17293, it reached its maximum concentration after 48 h with a population of 3.30×10^7 CFU/mL. The strain of *O. oeni* OT4 showed a similar trend in both trials: reached a population higher than 1.00×10^7 CFU/mL after 168 h of the inoculation (Fig. 4C and D) and kept constant until the end of the fermentation. Moreover, after 168 h of incubation *O. oeni* OT4 inoculated with 6% (v/v) of ethanol showed a cell viability of 2.40×10^7 CFU/mL in combination with *S. cerevisiae* ITEM 17292 while with *S. cerevisiae* ITEM 17293 was 7.50×10^5 CFU/

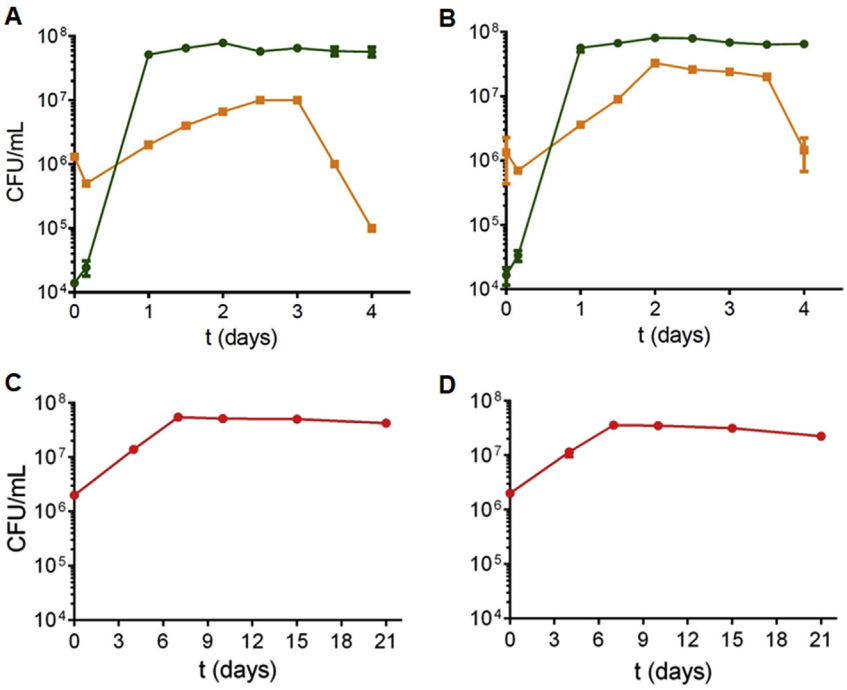

Fig. 4. Viable cell count (CFU/mL) of: **A**; *S. cerevisiae* ITEM 17292 (●) and *H. uvarum* ITEM 8795 (■), and **B**; *S. cerevisiae* ITEM 17293 (●) and *H. uvarum* ITEM 8795 (■) co-inoculated with *O. oeni* OT4 in red must. **C**; *O. oeni* OT4 (●) co-inoculated with *S. cerevisiae* ITEM 17292 and *H. uvarum* ITEM 8795 and, **D**; *O. oeni* OT4 (●) co-inoculated with *S. cerevisiae* ITEM 17293 and *H. uvarum* ITEM 8795. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 4
Concentration of major chemical compounds in wines obtained by the pilot-scale vinifications.

Trial	Alcohol	Sugars	TA	VA	pH	Malic acid	Lactic acid	Tartaric acid	Glycerol
A	11.89 ± 0.56	1.25 ± 0.12	7.56 ± 0.56	0.36 ± 0.07	3.19 ± 0.36	2.75 ± 0.56	0.26 ± 0.06	4.19 ± 0.10	9.50 ± 0.60
B	11.80 ± 0.10	1.24 ± 0.07	7.17 ± 0.10	0.35 ± 0.05	3.24 ± 0.26	0.16 ± 0.05	1.83 ± 0.14	4.19 ± 0.25	9.05 ± 0.87
C	11.97 ± 022	1.23 ± 0.16	7.41 ± 0.76	0.35 ± 0.10	3.19 ± 0.24	2.7 ± 0.76	0.19 ± 0.05	4.2 ± 0.14	9.61 ± 0.87
D	12.26 ± 0.84	1.29 ± 0.23	7.02 ± 0.48	0.27 ± 0.08	3.27 ± 0.26	0.19 ± 0.04	1.97 ± 0.16	4.14 ± 0.15	9.12 ± 0.56
E	11.70 ± 0.17	1.19 ± 0.17	7.82 ± 0.86	0.33 ± 0.08	3.20 ± 0.28	2.78 ± 0.55	0.26 ± 0.05	4.12 ± 0.26	9.31 ± 0.67
F	11.85 ± 0.54	1.19 ± 0.07	7.52 ± 0.66	0.41 ± 0.06	3.31 ± 0.15	0.13 ± 0.04	1.99 ± 0.07	3.93 ± 0.24	9.25 ± 0,38
G	11.99 ± 0.11	1.22 ± 0.34	7.35 ± 0.10	0.41 ± 0.12	3.27 ± 0.18	2.76 ± 0.85	0.04 ± 0.02	4.14 ± 0.20	9.42 ± 0.33
H	12.56 ± 0.10	1.29 ± 0.41	7.62 ± 0.77	0.29 ± 0.07	3.21 ± 0.16	0.18 ± 0.04	1.89 ± 0.15	4.11 ± 0.33	9.03 ± 0.94

TA; total acidity. VA; volatile acidity. Values are expressed in g/L. The ethanol concentration is expressed in g/100 mL. Results are the mean of three injections of each replicate (n = 9); the standard deviation values (±) are indicated. No significant differences were detected at $p < 0.05$.

mL, indicating the connection of the cell viability with the malolactic activity. *O. oeni* OT3, OT5, OT25 and OM22 after 168 h of inoculation only presented populations above 1×10^6 CFU/mL when were inoculated simultaneously to *S. cerevisiae* and *H. uvarum* (data not shown), explaining the reduced malolactic activity of these strains when were inoculated from 2% (v/v) of ethanol up to 12% (v/v).

3.6. Pilot-scale vinifications

In order to evaluate the fermentation performance and interactions of mixed cultures at winery-scale, selected yeast strains of *S. cerevisiae* ITEM 17292 and ITEM 17293, *H. uvarum* (ITEM 8795) and the selected bacteria *O. oeni* (OT4), the following pilot-scale vinifications were carried out: Trial A: ITEM 17292; Trial B: ITEM 17292 + OT4; Trial C: ITEM 17292 + ITEM 8795; Trial D: ITEM 17292 + ITEM 8795 + OT4; Trial E: ITEM 17293; Trial F: *S. cerevisiae* ITEM 17293 + OT4; Trial G: ITEM 17293 + ITEM 8795; Trial H: ITEM 17293 + ITEM 8795 + OT4.

The principal chemical parameters were analyzed by FT-IR (Table 4). In all the obtained fermented musts, volatile acidity, expressed as acetic acid, was quite low ranging from 0.27 g/L (trial D) to 0.41 g/L (trial H). The lower values of VA were detected in trial D (ITEM 17292 + *H. uvarum* + *O. oeni*) and trial H (ITEM 17293 + *H. uvarum* + *O. oeni*). A decrease in malic acid concentration coupled to increase of lactic acid content was achieved, 0.16 g/L in trial B 0.19 g/L in trial D, 0.13 g/L in trial F and finally 0.18 g/L in trial H. The values of total acidity, tartaric acid and glycerol did not differ in the eight fermentations, indicating that the technique of co-inoculation does not adversely affect the chemistry of the wine compared to the classical inoculation procedures (Table 1).

The GC-MS assay allowed the identification and quantification of 22 different volatile compounds (Table 5). The higher concentrations of alcohols were detected in trial D (59.04 mg/L), trial B (46.59 mg/L), trial C (39.07 mg/L) and trial H (32.56 mg/L). The esters were detected in higher concentrations in the same samples (A-B-C-H), while the acids content ranged from 1.0 mg/L (trial G) to 2.72 mg/L (trial H). Among esters, isoamyl acetate, ethyl lactate, ethyl octanoate, ethyl decanoate, diethyl succinate and mono ethyl succinate showed significant differences among the wines analyzed. When compared with the other obtained wines, the concentrations of these molecules was higher in the samples B, C and H. Moreover, the wine samples B, D, and H showed the higher amounts of hexanoic (ranging from 0.35 to 0.40 mg/L), octanoic (ranging from 0.54 to 0.60 mg/L) and decanoic (ranging from 0.27 to 0.36 mg/L), acids.

The Principal Component Analysis (PCA) was performed on the concentrations of molecules detected by GC-MS in the produced wines (Fig. 5). Indeed, the wines from the trials D and H, both obtained by employing the *Saccharomyces*/non- *Saccharomyces*/*O. oeni* mixed starter, were located in the third and in the fourth quadrant, both areas characterized by high concentrations of volatiles respect to the others trials (E-F-G) located in the first quadrant. The wine from trial D

Table 5
Concentration of selected volatile compounds determined by GC-MS in wine obtained by the pilot-scale vinifications.

	Trial A	Trial B	Trial C	Trial D	Trial E	Trial F	Trial G	Trial H
	mg/L ± sd							
ALCOHOLS								
2-Methyl-1-propanol	0.49 ± 0.11^{b}	0.99 ± 0.23^{b}	0.56 ± 0.18^{b}	0.87 ± 0.24^{b}	0.04 ± 0.01^{a}	0.020 ± 0.01^{a}	0.019 ± 0.04^{a}	1.33 ± 0.22^{a}
Isoamylalcohols	13.88 ± 3.67^{a}	24.95 ± 5.62^{b}	19.21 ± 5.55^{b}	26.40 ± 5.18^{b}	7.20 ± 2.11^{a}	9.20 ± 2.55^{a}	7.40 ± 1.87^{a}	15.95 ± 4.16^{b}
1-Hexanol	0.29 ± 0.07^{a}	0.64 ± 0.12^{b}	0.27 ± 0.12^{a}	0.44 ± 0.13^{b}	0.02 ± 0.01^{a}	0.020 ± 0.011^{a}	0.019 ± 0.04^{a}	0.63 ± 0.22^{b}
3-Hexen-1-ol (E)	0.54 ± 0.11	0.67 ± 0.22	0.74 ± 0.21	0.81 ± 0.25	0.46 ± 0.18	0.94 ± 0.26	0.22 ± 0.08	0.67 ± 0.21
3-Hexen-1-ol (Z)	0.011 ± 0.06	0.03 ± 0.01	0.014 ± 0.04	0.02 ± 0.01	nd	nd	nd	0.024 ± 0.09
1-heptanol	nd	nd	0.28 ± 0.10	0.56 ± 0.17	nd	nd	nd	0.74 ± 0.21
Methyonol	0.03 ± 0.01^{a}	nd	0.033 ± 0.011^{a}	0.76 ± 0.23a	nd	nd	nd	1.65 ± 0.37^{b}
Phenylethanol	14.91 ± 4.52^{b}	19.31 ± 4.94^{b}	17.96 ± 5.38^{b}	29.17 ± 4.56^{c}	8.04 ± 2.77^{a}	9.06 ± 2.10^{a}	8.70 ± 2.56^{a}	11.56 ± 4.38^{a}
TOTAL	**30.15**	**46.59**	**39.07**	**59.04**	**15.77**	**19.24**	**16.34**	**32.56**
ESTERS								
Isoamyl-acetate	3.77 ± 0.95^{a}	2.85 ± 0.74^{a}	4.11 ± 0.65^{a}	5.28 ± 1.56^{b}	2.11 ± 0.54^{a}	2.96 ± 0.16^{a}	3.11 ± 0.25^{a}	3.85 ± 0.94^{a}
Ethyl-hexanoate	0.028 ± 0.011	0.05 ± 0.02	0.02 ± 0.01	0.08 ± 0.02	nd	nd	nd	0.09 ± 0.03
Ethyl-lactate	0.14 ± 0.05	1.38 ± 0.17	0.11 ± 0.03	1.18 ± 0.44	0.22 ± 0.08	2.76 ± 0.94	0.01 ± 0.01	2.13 ± 0.76
Ethyl-octanoate	0.07 ± 0.02	0.11 ± 0.04	0.05 ± 0.02	0.072 ± 0.013	nd	nd	nd	0.13 ± 0.03
3-Hydroxy-ethylbutanoate	nd	0.04 ± 0.02	0.012 ± 0.04	0.025 ± 0.010	nd	nd	nd	nd
Ethyl-decanoate	0.94 ± 0.34^{a}	0.83 ± 0.14^{a}	0.77 ± 0.26^{a}	2.67 ± 0.34^{c}	0.95 ± 0.26^{a}	1.76 ± 0.38^{b}	1.88 ± 0.25^{b}	2.46 ± 0.84^{c}
Diethyl-succinate	0.45 ± 0.12^{a}	0.07 ± 0.02^{a}	0.40 ± 0.17^{a}	0.67 ± 0.28^{b}	0.22 ± 0.07^{a}	0.32 ± 0.08^{a}	0.21 ± 0.06^{a}	0.69 ± 0.19^{b}
Phenyl-acetate	0.18 ± 0.06	0.24 ± 0.10	0.21 ± 0.06	0.22 ± 0.06	nd	0.01 ± 0.01	0.01 ± 0.01	0.19 ± 0.05
Monoethyl-succinate	2.37 ± 0.94^{b}	3.84 ± 0.84^{b}	4.42 ± 1.45^{b}	6.04 ± 2.67^{c}	1.09 ± 0.27^{a}	1.13 ± 0.16^{a}	1.07 ± 0.16^{a}	3.11 ± 0.83^{b}
TOTAL	**7.94**	**9.41**	**10.12**	**16.24**	**4.60**	**8.94**	**6.28**	**12.66**
ACIDS								
2-Methyl propanoic acid	0.52 ± 0.18^{a}	0.45 ± 0.17^{a}	0.77 ± 0.23^{a}	0.95 ± 0.34^{a}	0.77 ± 0.15^{a}	0.65 ± 0.18^{a}	0.47 ± 0.12^{a}	1.56 ± 0.27^{b}
Hexanoic acid	0.30 ± 0.08^{b}	0.40 ± 0.16^{b}	0.29 ± 0.11^{a}	0.36 ± 0.12^{a}	0.01 ± 0.01^{a}	0.02 ± 0.05^{a}	0.01 ± 0.01^{a}	0.35 ± 0.08^{a}
Octanoic acid	0.49 ± 0.16	0.60 ± 0.23	0.46 ± 0.13	0.56 ± 0.25	0.41 ± 0.07	0.53 ± 0.17	0.41 ± 0.16	0.54 ± 0.12
Decanoic acid	0.18 ± 0.05	0.36 ± 0.14	0.18 ± 0.04	0.27 ± 0.08	0.13 ± 0.04	0.21 ± 0.06	0.10 ± 0.03	0.27 ± 0.08
TOTAL	**1.49**	**1.89**	**1.70**	**2.14**	**1.31**	**1.41**	**1.00**	**2.72**
TERPENS								
Citronellol	0.76 ± 0.17	n.d.	n.d.	1.56 ± 0.34	n.d.	n.d.	n.d.	0.73 ± 0.21

Each value is expressed in mg/L. Results are the mean of three injections of each replicate (n = 9); the standard deviation values (±) are indicated. Different upper letters in row means significant differences at $P < 0.05$.

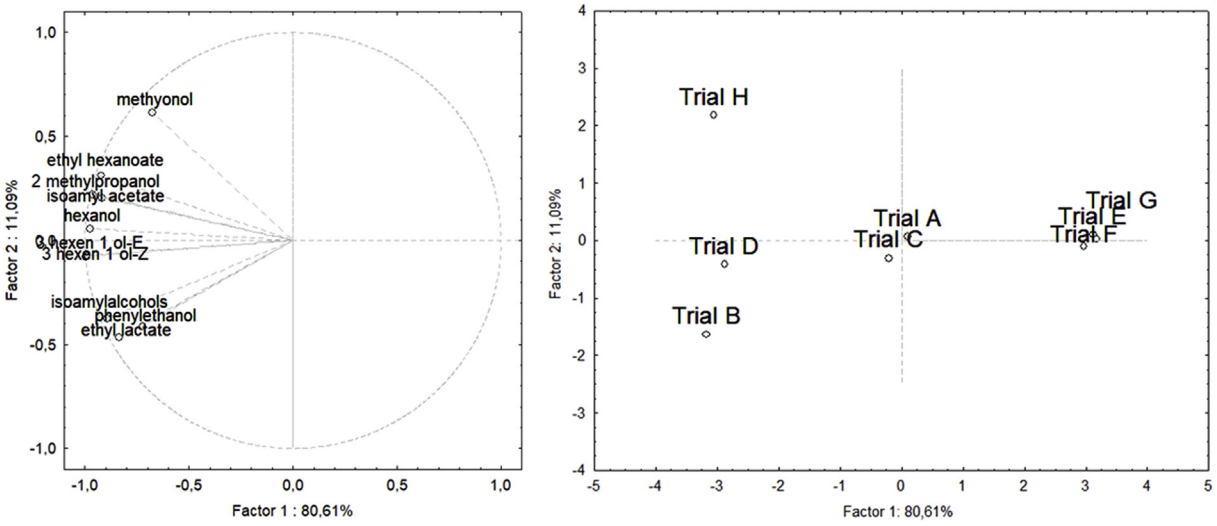

Fig. 5. Principal Component Analysis (PCA) performed employing the data obtained by the GC-MS analysis of the wines obtained by the pilot-scale vinifications.

showed in particular high values of isoamylalcohols, phenylethanol and ethyl lactate, while wines from the vinification H showed high values of isoamylacetate, ethyl hexanoate, 2-methylpropanol and 1-hexanol.

Taken together, the obtained outcome indicated that the *Saccharomyces*/non-*Saccharomyces*/*O. oeni* mixed starter formulations, detained the technological and enological features required for their possible use as industrial starter.

4. Discussion

Two autochthonous *S. cerevisiae* strains (ITEM 17292 and ITEM 17293) were selected using the procedure described by Tufariello et al. (2019). The two selected *S. cerevisiae* strains were always able to dominate the fermentation process and to obtain a final product with an adequate chemical composition. These strains were used for the co-inoculation trials to develop a mixed starter culture with non-*Saccharomyces* yeasts and LAB.

The addition of non-*Saccharomyces* yeast species as part of mixed starter formulations, has been indicated as a way to simulate the spontaneous fermentations (Petruzzi et al., 2017; Suzzi et al., 2012; Tristezza et al., 2016b), thus conferring particular organoleptic characteristics to wines without increasing the risks for wine quality and

safety often associated with uncontrolled vinifications (Berbegal, Spano, Tristezza, Grieco, & Capozzi, 2017; Capozzi et al., 2015). The performance of MLF by LAB is highly affected by the physicochemical intrinsic properties of wine, such as pH, ethanol, SO_2 and by yeast metabolism (Petruzzi et al., 2017). Alcoholic fermentation in wine undergoes deep chemical changes enhanced by ethanol concentrations over 4% (v/v) and can inhibit the growth of most LAB (Balmaseda, Bordons, Reguant, & Bautista-Gallego, 2018). In our study, all strains showed better malolactic activity when *O. oeni* were co-inoculated (0% ethanol v/v) with the selected yeasts or inoculated up to 4% of ethanol. Indeed, only *O. oeni* OT4 consumed all L-malic acid when inoculated with an ethanol concentration above 4% v/v. Moreover, the obtained evidences indicated that the duration of MLF was reduced by the co-inoculation of yeasts and all the *O. oeni* strains investigated. Interaction with yeasts can be from inhibitory, to neutral of stimulatory depending on the release of nutrients by yeasts, and on the ability of yeasts to produce metabolites that can affect LAB (Alexandre, Costello, Remize, Guzzo, & Guilloux-Benatier, 2004). One of the main strategies to mitigate the possible inhibitory interactions that have been proposed is the co-inoculation of yeast and *O. oeni* (Izquierdo-Cañas, Pérez-Martín, Romero, Prieto, & Herreros, 2012).

Our findings confirmed data of previous studies (Ciani et al., 2016; Maturano et al., 2018; Tristezza et al., 2016b), by showing that grape musts co-inoculated with the mixed starter cultures presented less ethanol content that when single cultures of *S. cerevisiae* were employed. Besides, *H. uvarum* ITEM 8795 grew better in combination with *S. cerevisiae* ITEM 17293 than with *S. cerevisiae* ITEM 17292. Contrariwise, the *O. oeni* OT4, with best malolactic activity in grape must, presented a higher L-malic consumption rate and cell viability when *S. cerevisiae* ITEM 17292 was used. Lactic acid bacteria have complex nutrient requirements and so, their development depends on the nutrients consumption by the yeasts during the AF (Ivey, Massel, & Phister, 2013). In accordance with the results of Curiel, Morales, Gonzalez, and Tronchoni (2017), *O. oeni* OT4 showed lower malolactic activity and growth in fermentation trials where *H. uvarum* ITEM 8795 showed higher population. The outcome achieved by the lab-scale tests were validated by carrying out pilot-scale vinification trials. It is interesting to highlight that the presence of fermentable sugars did not affect the values of the volatile acidity, as reported in previous studies (Liu, Lu, Duan, & Yan, 2016; Trani et al., 2016 the use of yeast/bacteria mixed inoculums for the management of the MLF, not affected by the addition of the non-*Saccharomyces* starter strain and it had a positive influence on fermentation lenght and on aroma composition of wine (Muñoz, Beccaria, & Abreo, 2014). In fact, highly considerable was the effect of the mixed starter formulation on the aroma pattern of produced wines, compared to those obtained by inoculation of the *S. cerevisiae* starter alone. Recent investigations have highlighted the variation of the biochemical profile of wine produced by different LAB inoculation procedures (Abrahamse & Bartowsky, 2011; Izquierdo-Cañas et al., 2012). Our data suggested, in accordance to literature (Antalick, Perello, & de Revel, 2013), that yeast/LAB co-inoculation could enhance the fruity aroma, thereby increasing the level of esters.

Among alcohols identified, other higher alcohols shows higher values standing out 2-isoamyl alcohols and 2-phenyletanol. The higher alcohols increase were significantly higher when the fermentation was carried out by mix composed by *Saccharomyces*/non-*Saccharomyces*/*O. oeni* strains and they were significant different when one of the two *S. cerevisiae* strains (ITEM 17292 or ITEM 17293) were used.

The combination of the three different microbial starters was responsible for the high esters production, contributing to improve wine flavor with fruity notes. In fact, the wines obtained by the pilot-scale trials D and H showed a higher concentration of hexanoic-octanoic and decanoic acids, which during the storage or aging could undergo to the esterification with the higher alcohols, thus increasing the fruity aroma (Francis & Newton, 2005). Total alcohol and acid concentrations were found to be higher in wines produced by *Saccharomyces*/non-*Saccharomyces*/*O. oeni* co-inoculation, these compounds being responsible for fruity, sweet, winery and acid sensory notes in wine.

In conclusion, the proposed approach can be very effective for the preparation of mixed starter culture formed by *Saccharomyces*, non-*Saccharomyces* yeasts and LAB. These mixed starter cultures represent a value solution to improve the specific attributes of typical regional wines. At the best of our knowledge, this investigation firstly illustrates the preparation and validation of a non-*Saccharomyces*/*Saccharomyces*/*O. oeni* mixed starter formulation that could be successfully adopted for the industrial production of typical Apulian red wines.

Acknowledgements

This research was partially supported by the Apulia Region in the framework of the Project DOMINA APULIAE (POR Puglia FESR – FSE 2014-2020-Azione 1.6. –InnoNetwork; Project code AGBGUK2). The authors thank Mr. Giovanni Colella for technical assistance and Prof. H. Smith for text proofreading.

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