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Effect of fungicides on the yeast population during spontaneous fermentation in the vinification of monastrell grapes

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ABSTRACT

The influence of six fungicides on indigenous yeasts of grape var. Monastrell, after performing two treatments (Good Agricultural Practices-GAP and Critical Agricultural Practices-CAP), was studied. Fungicide residues have been determined using a method of multi-residual extraction that uses QuEChERS and liquid chromatography in tandem with triple quadrupole mass spectrometry. Identification of yeast was carried out by PCR and subsequent sequencing. The fungicide residues are below the EU maximum residue limits (MRLs) established in wine grapes. At the beginning and during the fermentation, most of the treated samples show counts (CFU/mL) higher than the control test (20–30% more), even in the most unfavorable conditions (treated the same day of harvest). It is noteworthy the absence of *Hanseniaspora uvarum* and the presence of *Clavispora lusitaniae*, *Debaryomyces hansenii* and *Metschnikowia pulcherrima*. Nevertheless, no fungicide either under treatment inhibits fermentation or delays it. Moreover, the evolution of yeast populations found during fermentation follows the normal sequence of species. This research might be of interest for scientists and companies working on either basic or production aspects respectively of the winemaking process.

1. Introduction

Many intrinsic and extrinsic factors affect the occurrence and growth of microorganisms on the surface of grape berries, including rainfall, temperature, berry maturity, physical damage due to bird, insect, mould attack, and the application of agrichemicals such as fungicides and insecticides (Cadez, Zupan, & Raspor, 2010; Cordero-Bueso, Arroyo, & Valero, 2014; Grangeteau, David, Herve, Guilloux-Benatier, & Rousseaux, 2017; Zhou, Liu, Yuan, Deng, & Yu, 2020). The influence of winemaking technology like clarification of grape juice, sulfur dioxide, temperature and composition of the juice has also been reported (Barata, Malfeito-Ferreira, & Loureiro, 2012; Fleet, 1999).

The control of pests and diseases in the vineyard can lead to the presence of pesticide residues in grapes, and in some cases the remaining concentrations surpass the maximum residue limits (MLRs) established by current legislation. Pesticide residues remaining in the harvested grapes can be transferred to must and wine during the winemaking process, with the subsequent risk to the consumer (Ruediger, Pardon, Sas, Godden, & Pollnitz, 2004; Fernández, Oliva, Barba, & Cámara, 2005a, b; Oliva, Payá, Cámara, & Barba, 2007). Equally, these residues may directly or indirectly affect the fermentative process carried out by yeasts, especially in the case of fungicide residues. Some of them, the fewest, may negatively affect the population diversity (typification), others affect the growth and final counts of yeasts and therefore delay fermentation (Argabati, Canonico, Ciani, & Comitini, 2019; Coppola et al., 2011; Ganga & Martínez, 2004; González-Rodríguez et al., 2011; Kosel, Raspor, & Cadez, 2019; Noguerol-Pato, Torrado-Agrasar, González-Barreiro, Cancho-Grande, & Simal-Gándara, 2014; Regueiro, López-Fernández, Rial-Otero, Cancho-Grande, & Simal-Gándara, 2015). These effects could also alter the concentrations of certain compounds responsible for the sensorial quality of wine in general and the aroma in particular (Briz-Cid, Castro-Sobrino, Rial-Otero, Cancho-Grande, & Simal-Gándara, 2018; Noguerol-Pato, Gonzalez-Rodriguez, González-Barreiro, Cancho-Grande & Simal-Gándara, 2010; Oliva,

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Zalacaín, Payá, Salinas, & Barba, 2008). Monitoring spontaneous fermentation is a key issue for winemakers in order to obtain wines with distinct sensorial traits, which are generally recognized as having a fuller and rounder palate structure. Knowledge of the dynamics and occurrence of yeasts in wine fermentation is very important, especially as wine quality is a consequence of their diversity and attitudes. Hence, the need to characterize autochthonous yeasts isolated from spontaneous wine fermentations that are able to optimize the typical attributes of the vine variety (Bagheri, Bauer, & Setati, 2015; Ocon et al., 2010; Paraggio, 2004).

Many ecological studies in different wine regions of the world have identified the main species of yeast that develop during fermentation (Baffi et al., 2011; Capece, Pietrafesa, & Romano, 2011; del Real, Lairon-Peris, Barrio, & Querol, 2017; Di Maro, Ercolini, & Coppola, 2007; Kraková et al., 2012, Alonso; Nisiotou & Nychas, 2007; Terpou et al., 2019; Zhang et al., 2010). However, little has been reported about the typification of species in yeast populations during spontaneous fermentations of fungicide treated grapes. An extensive review shows that some authors such as Comitini and Ciani (2008) reported that the use of fungicides of organic synthesis can drastically modify the yeast flora, favouring the colonisation of non-fermenting and ubiquitous yeasts, such as *A. pullulans* and *Cryptococcus spp*. Cus and Raspor (2008) reported that pyrimethanil inhibits the initial growth of *H. uvarum* and hypothesize that this may have a decisive influence on the more rapid replacement of that species by *S. cerevisae*. Zara et al. (2011) showed that the use of fenamidone, indoxacarb, pyraclostrobin and deltamethrin has no effect on *Saccharomyces sp*. Indigenous microflora, but it does have a partial effect on the non-*Saccharomyces* population even at concentrations of ½ MRL.

The aims of the study are to obtain a complete picture of the species composition and the dynamic changes in the main yeast population involved in the spontaneous fermentation of Monastrell grape treated with fungicides under two types of applications: Good Agricultural Practices (GAP) respecting the PHI (Pre-Harvest Interval) and Critical Agricultural Practices (CAP) a second treatment of each fungicide at the same dose 2 h before harvest.

2. Materials and methods

2.1. Materials and reagents

The fungicides used in this study (famoxadone, fenhexamid, kresoxim-methyl, fluquinconazole, quinoxyfen and trifloxystrobin) were provided by Dr. Ehrenstorfer (Augsburg, Germany) standard quality analytics with a purity > 98%. The plant-products used in the field applications were: Equation pro GR 22.5 (famoxadone at 22.5% w/w), Teldor WG (fenhexamid 50% w/w), Castellan GD (fluquinconazole 25% w/w), Stroby WG (kresoxim-methyl 50% w/w), Arius SC (quinoxyfen 25% w/v) and Flint WG (trifloxystrobin 50% w/w) at application dosage at application dosage of 0.045, 0.450, 0.028, 0.045, 0.034 and 0.034 kg a.i./ha, respectively.

Liquid chromatography quality acetonitrile were obtained from Scharlau (Barcelona. España); formic acid and ammonium formate of 95% purity; magnesium sulfate anhydrous, of 97% purity and sodium chloride, of 99.5% purity were purchased from Fluka (Buchs. Switzerland); disodium citrate sesquihydrate and dehydrate trisodium citrate of 99% purity was obtained from Sigma Aldrich (St. Louis, USA); milli-Q water was produced by a Millipore de Purification Pak system (Billerica, USA); PCR kit (buffer, nucleotide mix, DNA Polymerase and DNA solution) and PCR kit purification were purchased from Roche (Madrid, Spain).

2.2. Field trials

The study was performed with red grapes (*Vitis vinifera* var. *Monastrell*) from an experimental plot in Jumilla, Murcia (SE Spain). Cultivation references were 2.5 × 2.5 m^2. Grapes were obtained in perfect nutritional state and excellent physiological conditions. Seven experimental plots of 225 m^2 were marked out on the farm (one control and six for the individual treatments with the fungicides under study).

The first treatment was performed in accordance with GAP, respecting the PHI (Pre-Harvest Interval) of each product: 14 days for fenhexamid, 21 days for fluquinconazole, 35 days for kresoxim-methyl and 28 days for famoxadone, quinoxyfen and trifloxystrobin. After the PHI, and following harvesting of the grapes for winemaking, a new application was made, again followed by harvesting 2 h later. This harvest was performed under CAP, i.e. in the most unfavorable conditions, on the same day as the application.

2.3. Winemaking

Using the thirteen samples of harvested grapes (one untreated control, six with treatment under GAP and six treated under CAP conditions), microvinifications (15 kg of grapes) were performed in triplicate, without addition of selected yeasts. 80 mg/L of SO_2 were added to the crushed grapes, maintained in dynamic maceration for 10 days at controlled temperature of 26–28 °C. After mechanic pressing at pilot scale and the subsequent fermentation (5 days), the wine was decanted and clarified using 40 g/hl of bentonite and 8 g/hl gelatine mixture. The wines were then filtered through a nylon fiber of 0.45 μm diameter.

2.4. Fungicide residues analysis

Fungicides were extracted from the matrix following a QuEChERS multiresidue method that uses acetonitrile as an extraction solvent (Martínez et al., 2015). The obtained extract was acidified with formic acid and then directly injected into the liquid chromatograph. Liquid chromatography with tandem mass spectrometry triple quadrupole (LC-MS/MS QqQ) analyses were performed following the chromatographic conditions described by Cermeño, Martínez, Oliva, Cámara & Barba (2016). The limits of quantification (LOQ) were 0.01 mg/kg for all the fungicides.

2.5. Yeast enumeration, isolation and identification

Wine samples were taken at 4 points during vinifications: after grape crushing (Must), on maceration day 3 (Must I), day 6 (Must II) and vinification day 15 (Wine). Samples were taken from the middle of the fermentation vessels. Samples were serially diluted with sterile peptone water; for the enumeration of yeast, 0.1 mL of each dilution was spread in triplicate onto plates of GPYA (glucose-peptone-yeast extract agar) culture medium. Plates were incubated at 26 °C during 48 h for colony development.

A total of 10 randomly picked colonies from each plate were used for yeast identification. Identification was performed in triplicate and the frequency percentage of each species was expressed as function of the 10 colonies isolated. The results were expressed as the average percentage of the triplicate and its standard deviation. Isolates were frozen at −80 °C in GPY broth for further identification. PCR was performed according to the method described by Esteve-Zarzoso, Belloch, Uruburu & Quero (1999) with slight modifications. Cells were directly collected from a fresh yeast colony using a microbiological loop and suspended in 20 μl of PCR water. This solution ("DNA solution") was heated at 95 °C for 15 min. ITS1 (5′TCCgTAggTgAACCTgCgg) and ITS4 (5′TCCTCCgCTTATTgATATgC) primers were used to amplify the region of the rDNA repeat unit it included the 5.8S rRNA gene and the two non-coding regions designated the internal transcribed spacers (ITS1 and ITS2) (White et al., 1990). PCR was performed in a final volume of 50 μl containing 31.5 μl water, 5 μl PCR buffer, 2 μl PCR nucleotide mix, 1.5 μl of the each reverse and forward primer, 0.5 μl Taq DNA Polymerase and 5 μl of the extracted DNA solution.

After an initial 5 min denaturation at 95 °C, the reactions were run

Table 1
Fungicide residues (mg/kg ± SD, n = 3) during winemaking under GAP and CAP.

Steps winemaking	Famoxadone		Fenhexamid		Fluquinconazole		Kresoxim-methyl		Quinoxyfen		Trifloxystrobin	
	GAP	CAP	GAP	CAP	GAP	CAP	GAP	CAP	GAP	CAP	GAP	CAP
Must 0 days	0.10 ± 0.02	0.11 ± 0.03	0.51 ± 0.09	1.28 ± 0,16	<0.01	0.02 ± 0.02	<0.01	0,11 ± 0.03	0.08 ± 0.02	0.29 ± 0.08	<0.01	0.05 ± 0.02
Must I 3 days	0.10 ± 0.01	0.10 ± 0.02	0.46 ± 0.07	1.22 ± 0.08	<0.01	<0.01	<0.01	0.10 ± 0.02	0.04 ± 0.01	0,19 ± 0.05	<0.01	<0.01
Must II 6 days	0.09 ± 0.01	0.10 ± 0.02	0.43 ± 0.06	1.11 ± 0.13	<0.01	<0.01	<0.01	0.09 ± 0.03	<0.01	0,09 ± 0.02	<0.01	<0.01
Wine 15 days	0.07 ± 0.01	0.08 ± 0.01	0.36 ± 0.09	1.01 ± 0.09	<0.01	<0.01	<0.01	0,09 ± 0.01	<0.01	<0.01	<0.01	<0.01

Table 2
Significative differences with control in final counts of yeast, density and % ethanol (%SD, n = 3) during winemaking of Monastrell grapes treated under GAP and CAP.

Fungicide	Must (o days)						Must I (3days)					
	GAP			CAP			GAP			CAP		
	Log CFU/ml	Density (g/ml)	Ethanol (%v/v)	Log UFC/ml	Density (g/ml)	Ethanol (%v/v)	Log CFU/ml	Density (g/ml)	Ethanol (% v/v)	Log CFU/ml	Density (g/ml)	Ethanol (% v/v)
Famoxadone	3.5 ± 0.4*	1.102 ± 0.004	–	3.8 ± 0.5*	1.103 ± 0.004	–	7.5 ± 0.3	1.046 ± 0.009	4.72 ± 0.31*	9.5 ± 0.3*	1.039 ± 0.007	5.74 ± 0.23*
Fenhexamid	3.6 ± 0.3*	1.099 ± 0.006	–	6.0 ± 0.6*	1.101 ± 0.003	–	7.6 ± 0.5	1.043 ± 0.012	4.50 ± 0.22*	9.5 ± 0.5*	1.042 ± 0.011	4.95 ± 0.16*
Fluquinconazole	3.3 ± 0.5*	1.097 ± 0.008	–	3.7 ± 0.5*	1.100 ± 0.005	–	7.4 ± 0.4	1.037 ± 0.007	4.43 ± 0.17	9.6 ± 0.5*	1.033 ± 0.006*	6.29 ± 0.22*
Kresoxim-m	3.7 ± 0.4*	1.104 ± 0.002	–	3.3 ± 0.2*	1.102 ± 0.008	–	7.6 ± 0.4	1.049 ± 0.010	3.99 ± 0.33	9.3 ± 0.4*	1.043 ± 0.003	4.94 ± 0.26*
Quinoxyfen	2.5 ± 0.6	1.099 ± 0.003	–	4.5 ± 0.4*	1.103 ± 0.007	–	7.9 ± 0.7	1.045 ± 0.011	4.18 ± 0.15	9.5 ± 0.2*	1.041 ± 0.008	4.74 ± 0.15*
Trifloxistrobin	5.2 ± 0.7*	1.104 ± 0.009	–	3.8 ± 0.3*	1.101 ± 0.004	–	7.6 ± 0.5	1.039 ± 0.005	5.48 ± 0.26*	9.5 ± 0.5*	1.036 ± 0.005	5.93 ± 0.19*
Control	2.6 ± 0.5	1.100 ± 0.004	–	2.6 ± 0.5	1.100 ± 0.002	–	7.8 ± 0.6	1.046 ± 0.013	4.16 ± 0.29	7.8 ± 0.6*	1.046 ± 0.013	4.16 ± 0.29
Famoxadone	8.0 ± 0.4*	1.019 ± 0.006	10.37 ± 0.16*	8.7 ± 0.6*	1.015 ± 0.011	9.73 ± 0.12*	7.8 ± 0.3*	0.993 ± 0.001	14.52 ± 0.16*	7.5 ± 0.6*	0.993 ± 0.000	14.40 ± 0.07*
Fenhexamid	8.3 ± 0.5*	1.016 ± 0.005	9.97 ± 0.09*	8.5 ± 0.4*	1.026 ± 0.006	7.58 ± 0.19*	5.2 ± 0.6	0.933 ± 0.000	13.81 ± 0.18	6.9 ± 0.3*	0.994 ± 0.001	14.11 ± 0.09
Fluquinconazole	7.6 ± 0.4	1.021 ± 0.009	9.53 ± 0.07	9.2 ± 0.4*	1.013 ± 0.005	9.64 ± 0.08	6.9 ± 0.4*	0.944 ± 0.001	13.52 ± 0.09*	8.1 ± 0.5*	0.994 ± 0.002	13.96 ± 0.17
Kresoxim-m	8.1 ± 0.2*	1.014 ± 0.007	9.92 ± 0.15*	8.7 ± 0.5*	1.013 ± 0.003	8.91 ± 0.13	7.5 ± 0.4*	0.944 ± 0.001	14.35 ± 0.09	8.2 ± 0.4*	0.993 ± 0.001	14.25 ± 0.09
Quinoxyfen	7.7 ± 0.5	1.018 ± 0.010	10.13 ± 0.21*	9.4 ± 0.5*	1.012 ± 0.007	9.91 ± 0.12*	6.7 ± 0.5*	0.993 ± 0.000	13.72 ± 0.012	8.8 ± 0.2*	0.993 ± 0.000	14.06 ± 0.10
Trifloxistrobin	7.3 ± 0.4	1.019 ± 0.003	9.69 ± 0.16	9.0 ± 0.6*	1.013 ± 0.006	9.78 ± 0.07*	7.0 ± 0.3*	0.994 ± 0.001	14.15 ± 0.08	7.2 ± 0.3*	0.993 ± 0.001	13.92 ± 0.14
Control	6.9 ± 0.5	1.020 ± 0.010	9.30 ± 0.12	6.9 ± 0.5*	1.020 ± 0.010	9.30 ± 0.12	5.6 ± 0.6	0.994 ± 0.001	13.96 ± 0.12	5.6 ± 0.6	0.994 ± 0.001	13.96 ± 0.12

*Higher than Control ($p < 0.05$).

for 35 cycles: denaturation was at 94 °C for 1 min, annealing at 55.5 °C for 2 min and extension at 72 °C for 2 min followed by final 10 min extension at 72 °C. A 10 μl sample of the PCR product was migrated on 4% agarose gel. PCR products were purified using PCR purification kit. Purified PCR products were directly sequenced using the ABI prism 3730 DNA analyzer (Applied Biosystems, Foster city, USA). Chromas 2.1 was used for reading and editing the DNA sequences. Blast analysis of sequences was performed by *Saccharomyces* Genome Database and Fungal Genomes Search (Wu-Blast 2).

2.5. Statistical study

In all cases, the calculation of the descriptive parameters (mean and standard deviation) was carried out with IBM SPSS statistics 24.0.

3. Results and discussion

3.1. Fungicide residues

Residue trends of target fungicides in grape wine-making process are shown in Table 1. The result showed that the six target fungicides dissipated to different degrees during the fermentation process. For all fungicides, the residues are below the maximum residue limits (MRL) for wine grapes since for wine they have not been established in UE: famoxadone (2 mg/kg), fluquinconalzole (0.01 mg/kg), trifloxystrobin (3 mg/kg), fenhexamid (5 mg/kg), kresoxim-methyl and quinoxyfen (1 mg/kg) (EU Pesticide database, 2020). After 6 days of winemaking (must II and wine) the residues of fluquinconalzole, trifloxystrobin, kresoxim-methyl and quinoxyfen are <0.01 mg/kg; while those of famodaxone and fenhexamid decrease to values close to LOQ. Famoxadone, fluquinconalzole and trifloxystrobin in the different stages of winemaking do not show differences between the GAP and CAP treatments. However, the concentrations of fenhexamid, kresoxim-methyl

Table 3
Distribution of yeast species (% SD, n = 3) during spontaneous fermentations of Monastrell musts treated under GAP.

Sampling day	Control				Famoxadone				Fenhexamid				Fluquinconazole			
	0	3	6	15	0	3	6	15	0	3	6	15	0	3	6	15
C. lusitaniae	74.±3	–	–	–	80 ± 0	–	–	–	70 ± 0	–	–	–	72 ± 2	–	–	–
M. pulcherrima	16 ± 2	–	–	–	10 ± 0	–	–	–	19 ± 2	–	–	–	12 ± 2	–	–	–
D. hansenii	4 ± 2	–	–	–	10 ± 0	–	–	–	11 ± 2	–	–	–	10 ± 0	–	–	–
Metschnikowia sp.	6 ± 2	–	–	–	–	–	–	–	–	–	–	–	6 ± 2	–	–	–
R. glutinis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
C. tropicalis	–	20 ± 0	–	–	–	–	–	–	–	30 ± 0	–	–	–	20 ± 0	–	–
K. delphensis	–	–	–	–	–	16 ± 2	–	–	–	14 ± 2	–	–	–	–	–	–
Saccharomycete sp.	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
S. cerevisiae	–	50 ± 0	100 ± 0	100 ± 0	–	84 ± 2	100 ± 0	100 ± 0	–	–	100 ± 0	100 ± 0	–	50 ± 0	100 ± 0	100 ± 0
S. paradoxus	–	30 ± 0	–	–	–	–	–	–	–	46 ± 2	–	–	–	30 ± 0	–	–
S. bayanus	–	–	–	–	–	–	–	–	–	10 ± 0	–	–	–	–	–	–

Sampling day	Kresoxim-m				Quinoxyfen				Trifloxystrobin			
	0	3	6	15	0	3	6	15	0	3	6	15
C. lusitaniae	62 ± 2	–	–	–	60 ± 0	–	–	–	66 ± 2	–	–	–
M. pulcherrima	26 ± 3	–	–	–	28 ± 2	–	–	–	16 ± 2	–	–	–
D. hansenii	4 ± 2	–	–	–	6 ± 2	–	–	–	12 ± 2	–	–	–
Metschnikowia sp.		–	–	–	7 ± 3	–	–	–	7 ± 3	–	–	–
R. glutinis	8 ± 2	–	–	–	–	–	–	–	–	–	–	–
C. tropicalis	–	20 ± 0	–	–	–	20 ± 0	–	–	–	–	–	–
K. delphensis	–	–	–	–	–	–	–	–	–	–	–	–
Saccharomycete sp.	–	–	–	–	–	10 ± 0	–	–	–	–	–	–
S. cerevisiae	–	50 ± 0	100 ± 0	100 ± 0	–	46 ± 2	100 ± 0	100 ± 0	–	100 ± 0	100 ± 0	100 ± 0
S. paradoxus	–	30 ± 0	–	–	–	24 ± 2	–	–	–	–	–	–
S. bayanus	–	–	–	–	–	–	–	–	–	–	–	–

and quinoxyfen after grape crushing (must) are higher in CAP.

3.2. Yeast population

The alcoholic fermentation of the musts proceeded to the completion of the fermentation of must sugars (final density of 0.993 g/ml) 15 days after grape crushing. The behavior of the yeasts throughout the fermentation are represented in Table 2, together with the evolution of the density and the content in ethanol for the different tests, in the sampled stages. At the beginning of the spontaneous fermentation, the fresh grape juices obtained from samples treated under GAP exhibited total yeast counts ranging from 2.5 log CFU/ml in the case of quinoxyfen samples to 5.2 log CFU/ml in trifloxystrobin. A maximum population was exhibited on day 6 for famoxadone, fenhexamid, fluquinconazole and kresoxim-methyl, while samples from quinoxyfen and trifloxystrobin showed the highest counts on day 3. Finally, in the last stage of the process, viable cells decreased to values ranging from 5.2 log CFU/ml for fenhexamid to 7.8 log CFU/ml in the case of famoxadone samples. Samples treated under CAP exhibited initial and final yeast counts higher than GAP samples; all CAP samples reached maximum population on day 3.

In both cases, GAP and CAP treatments, yeasts showed the typical growth pattern of microorganisms grown in batch culture. This was characterized by an exponential phase, a stationary phase and a decline or death phase. The characteristics of these phases could be influenced by the toxic effect related to the increasing ethanol content of the must, the depletion of fermentable sugars, oxygen availability, cell-cell contact, quorum sensing and space limitation (Nissen & Arneborg, 2003; Nissen, Nielsen, & Arneborg, 2003).

It is noteworthy that the final counts of the treated samples under both GAP and CAP were higher than the control, except in the quinoxyfen assay under GAP (day 0) and fenhexamid under GAP (day 15), highlighting the fact that, under the most unfavorable conditions, all samples are superior to the control counts. Table 2 shows significant differences for the assays ($p < 0.05$) with respect to the control in the total yeast count in the initial stage (Must, 0 day) and the final fermentation stage (Wine, 15 days); the samples marked previously as exceptional are the only ones not to maintain significant differences with the control assay. These results are in agreement with those obtained by Cadez et al. (2010) in their studies with other fungicides: iprodione, pyrimethanil and fludioxonil + cyprodinil. These authors found higher counts in treated grapes than in untreated control samples, indicating that this effect could be explained by the chemical composition of the preparation of fungicides, as well as the active principle, containing a number of other chemicals such as sulfur compounds, salts of calcium, nitrogen compounds, etc., that can be used by yeasts living in the grape berries. The density and ethanol values are indicative that the presence of these fungicides does not affect the development of the fermentation.

3.4. General yeast dynamics

In crushed must, yeast populations showed high homogeneity (Tables 3 and 4 sampling day 0). *Clavispora*, *Metschnikowia* and *Debaryomyces* were the main genera within non-*Saccharomyces* yeasts. Thus, the species *Clavispora lusitaniae*, *Metschnikowia pulcherrima* and *Debaryomyces hansenii* were found in all crushed must samples, both control and treated, regardless of the fungicide tested. In all samples, both control and treated (GAP and CAP), the evolution of the flora during fermentation followed the usual sequence of populations. Thus, in the early stages of fermentation, non-*Saccharomyces* yeasts are predominant and are later gradually replaced by *Saccharomyces* species. Thus, in all

Table 4
Distribution of yeast species (%±SD, n = 3) during spontaneous fermentations of Monastrell musts treated under CAP.

Sampling day	Control				Famoxadone				Fenhexamid				Fluquinconazole			
	0	3	6	15	0	3	6	15	0	3	6	15	0	3	6	15
C. lusitaniae	74 ± 3	–	–	–	90 ± 0	–	–	–	62 ± 2	–	–	–	80 ± 0	–	–	–
M. pulcherrima	16 ± 2	–	–	–	6 ± 2	–	–	–	20 ± 0	–	–	–	8 ± 2	–	–	–
D. hansenii	4 ± 2	–	–	–	4 ± 2	–	–	–	18 ± 2	–	–	–	9 ± 1	–	–	–
Metschnikowia sp.	6 ± 2	–	–	–		–	–	–		–	–	–	3 ± 0	–	–	–
R. glutinis	–	–	–	–		–	–	–		–	–	–		–	–	–
C. tropicalis	–	20 ± 0	–	–		–	–	–		–	–	–		26 ± 2	–	–
K. delphensis	–	–	–	–		–	–	–		–	–	–		–	–	–
Saccharomycete sp.	–	–	–	–		–	–	–		–	–	–		–	–	–
S. cerevisiae	–	50 ± 0	100 ± 0	100 ± 0		100 ± 0	100 ± 0	100 ± 0		100 ± 0	100 ± 0	100 ± 0		74 ± 2	100 ± 0	100 ± 0
S. paradoxus	–	30 ± 0	–	–		–	–	–		–	–	–		–	–	–
S. bayanus	–	–	–	–		–	–	–		–	–	–		–	–	–

Sampling day	Kresoxim-m				Quinoxyfen				Trifloxystrobin			
	0	3	6	15	0	3	6	15	0	3	6	15
C. lusitaniae	64 ± 2	–	–	–	68 ± 2	–	–	–	60 ± 0	–	–	–
M. pulcherrima	24 ± 2	–	–	–	20 ± 0	–	–	–	21 ± 2	–	–	–
D. hansenii	6 ± 2	–	–	–	8 ± 2	–	–	–	12 ± 2	–	–	–
Metschnikowia sp.		–	–	–	4 ± 2	–	–	–	7 ± 3	–	–	–
R. glutinis	6 ± 2	–	–	–		–	–	–		–	–	–
C. tropicalis		–	–	–		–	–	–		20 ± 0	–	–
K. delphensis		–	–	–		–	–	–		–	–	–
Saccharomycete sp.		–	–	–		–	–	–		–	–	–
S. cerevisiae		64 ± 3	100 ± 0	100 ± 0		100 ± 0	100 ± 0	100 ± 0		44 ± 2	100 ± 0	100 ± 0
S. paradoxus		36 ± 2	–	–		–	–	–		26 ± 2	–	–
S. bayanus		–	–	–		–	–	–		10 ± 0	–	–

tested fermentations, *Saccharomyces cerevisiae* was identified as the sole species from Must I in the case of famoxadone, fenhexamid and quinoxyfen in CAP and trifloxystrobin in GAP, and from Must II for the rest of active ingredients assayed. These results are in agreement with other authors show that, in grapes and in the early stages of fermentation, non-*Saccharomyces* species dominate the process and are gradually replaced by species of the genus *Saccharomyces* with higher fermentative power and greater tolerance to ethanol (Pérez-Nevado, Albergaria, Hogg & Girio, 2006; Nisiotou & Nychas, 2007; Jensen, Umiker, Arneborg, & Edwards, 2009; Francesca et al., 2010, Parapouli, Hatziloukas, Drainas, & Perisynakis, 2010; Hall, Durall, & Stanley, 2011, Moreira et al., 2011, Barbosa et al., 2018). Del Castillo (1992) reported higher ethanol tolerance of Saccharomyces species was directly related to the higher ergosterol content in these species membrane. It is known that high non-*Saccharomyces* populations can influence wines' chemical compositions as well as the kinetics of growth and metabolism of *Saccharomyces spp* (Baffi et al., 2011; Ocon et al., 2010; Ubeda-Iranzo, Díaz-Hellín Chacon-Ocana & Briones, 2015). *C. lusitaniae* was the main non *Saccharomyces* species in control samples, constituting up to 74.44% of the population of the grape yeast. Besides this *Clavispora* strain, *M. pulcherrima* and to a lesser extent, *D. hansenii* and *Metschnikowia sp*, were also identified in grapes, with 4.44% and 5.56% of population isolates respectively. The analysis of musts reveals a dramatic population decline of non *Saccharomyces* species. Thus, in Must I, 80% of the population belongs to the genus *Saccharomyces* (50% *S. cerevisiae* and 30% *S. paradoxus*). On the other hand non *Saccharomyces* microbiota was reduced to the species *Candida tropicalis*. In the remaining samples, Must II and Wine, the only species found is *S. cerevisiae*.

In the samples treated with the different fungicides under GAP (Table 3), grape yeast population consists exclusively of non *Saccharomyces* yeasts, where *C. lusitaniae* is the dominant species (60%–80% of the population), followed by *M. pulcherrima* (10%–27.78%) and *D. hansenii* (4.44%–12.22%). *Metschnikowia sp*. showed the same values in fluquinconazole, quinoxyfen and trifloxystrobin treatments, although these disappeared for the rest of tests. The disappearance of the non *Saccharomyces* population in musts was evident; indeed, between 55.56% and 100% of the population in Must I was made up of species of the *Saccharomyces* genus, with the lowest level for fenhexamid samples and the highest for trifloxystrobin. Non *Saccharomyces* yeasts were represented by *Kluyveromyces delphensis* and *Candida tropicalis* with percentages ranging from 14.44% to 30%: These species were absent in trifloxistrobin samples. In the remaining samples (Must II and Wine) *S. cerevisiae* is the only yeast present.

In samples of Must I, Must II and Wine treated with famoxadone, fenhexamid and quinoxyfen under CAP (Table 4), *S. cerevisiae* was the only species identified. In kresoxim-methyl samples *S. paradoxus* was identified (35.56%) while the remaining population consisted of *S. cerevisiae*. Finally, fluquinconazole and trifloxystrobin samples still showed non *Saccharomyces* strains in Must I, represented by *Candida tropicalis* (20%–25.56%). However, population of Must II and Wine samples consisted of *S. cerevisae* as the sole species. Prevalence of *C. lusitaniae* is unusual in samples of grapes and wine. But, despite the lack of information about its oenological ability, Mingorance-Cazorla, Clemente-Jiménez, Martínez-Rodríguez, Las Heras-Vázquez, and Rodríguez-Vico (2003) suggest that this species is able to generate a good product of low alcoholic degree. This presence has also been described by other authors, e.g. Longo, Cansado, Agrelo, and Villa (1991), who identify this species from rainy vintage grapes. The authors also suggest that in these conditions, the presence of *Clavispora lusitaniae* along with other oxidative species may be indicative of poor sanitary quality of the grapes in which fungal growth is, moreover, favored by rain. These hypotheses are confirmed by Nisiotou, Spiropoulos, and Nychas (2007), who identified the species in the flora of grapes affected by *Botrytis*. In our case, the grapes were in healthy conditions, but the

month before the harvest was quite rainy in the area. Predominance of *Metschnikowia* species observed at the beginning of the fermentation may be due to the high presence of this genus on the berries' surface, demonstrated by other studies (Barbosa et al., 2018; Raspor, Milek, Polanc, Smole-Možina, & Čadež, 2006; Ribereau-Gayon, Dubourdieu, Donèche, & Lonvaud, 2006, pp. 1–49). Therefore, the presence of this species at the initial phases of the fermentation in all samples is indicative that the fungicides studied have no influence on this yeast.

Our results show that *S. cerevisiae* could be detected mainly in the middle phase (sampling day 3 or 6) and that it dominated at the end of the alcoholic fermentation (sampling day 15). This indicates that the fungicides studied have no effect on this species behavior throughout spontaneous fermentation; results that are in agreement with previous studies (Milanovic, Comitini, & Ciani, 2013; Zara et al., 2011). Therefore, *S. cerevisae* represented a significant part of the microbiota of the fermentation but only a small contribution to the overall broad species diversity found in this study. The absence of *H. uvarum* is in agreement with previous studies by Cus and Raspor (2008) who found inhibitory effect of pyrimethanil over *H. uvarum* even at concentrations of 1 mg/L. The effect was significant in the second half of the exponential growth, which confirmed its reduced growth in spontaneous wine fermentation. On the other hand, Comitini and Ciani (2008) showed that the use of fungicides of organic synthesis applied on grapes during ripening favored the presence of non fermenting yeast, such as *A. pullulans*, and reduced the presence of fermenting yeast *H. uvarum*.

4. Conclusions

No fungicide, either under GAP or CAP inhibits fermentation or delays it since the counts found in the treated samples are not, in any case, lower than those found in the control. On the other hand, in the identification of non *Saccharomyces* flora, the absence of *H. uvarum* and the presence of *C. lusitaniae*, *D. hansenii* and *M. pulcherrima* is noteworthy, with their appearance in all plots and under all treatments. Finally, the evolution of populations found during fermentation follows the normal sequence of species, with a progressive decrease of non *Saccharomyces* species, and complete disappearance in samples of Must II and Wine. For all fungicides, the residues during wine-making process are below the maximum residue limits (MRL) established in wine grapes.

Pesticides

Famoxadone (PubChem CID: 213032); fenhexamid (PubChem CID: 213031); kresoxim-methyl (PubChem CID: 6112114); fluquinconazole (PubChem CID: 86417); quinoxyfen (PubChem CID: 3391107) and trifloxystrobin (PubChem CID: 11664966).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

José Oliva: Conceptualization, Methodology, Resources, Validation, Writing - original draft, Writing - review & editing, Visualization, Supervision. **Francisco Girón:** Methodology, Validation, Investigation, Writing - original draft. **José M. Cayuela:** Validation, Investigation, Resources. **Juana Mulero:** Validation, Investigation, Resources. **Pilar Zafrilla:** Validation, Resources. **Miguel Ángel Cámara:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Supervision, Project administration.

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