Use of fortified pied de cuve as an innovative method to start spontaneous alcoholic fermentation for red winemaking

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Abstract

Background and Aims: Some wineries, in order to promote the growth of yeasts able to ferment grape musts, traditionally produce wines using the 'pied de cuve' method. The aim of the present work was to study the performance of fortified pied de cuve (FPdC) prepared by addition of wine.

Method and Results: Two FPdCs were prepared with the addition of wine at 1.5 and 3% (v/v) of ethanol to the musts and allowed to spontaneously ferment. The FPdCs were then added to fresh bulk musts in order to accelerate the spontaneous alcoholic fermentation (AF). Interestingly, several *Saccharomyces cerevisiae* strains isolated during the pied de cuve preparation were detected at the highest concentration throughout AF. The chemical composition of the wines conformed to commercial regulations. The volatile organic compounds (VOCs) were mainly represented by diethyl succinate and ethyl lactate, as well as by hexanol and isoamyl alcohol. Principal component analysis of the chemical parameters, VOCs and sensory data showed that the use of experimental pied de cuve influenced positively the composition of the final wines.

Conclusion: The FPdC had no negative impact on the microbiological and chemical composition of wines, but it affected the diversity of the species *S. cerevisiae* present during AF and the sensory profile of the final wine.

Significance of the Study: This study provided evidence that FPdC accelerates spontaneous AF and influences the sensory profile of red wines.

Keywords: *fortified pied de cuve, lactic acid bacteria,* Saccharomyces cerevisiae, *spontaneous alcoholic fermentation, yeast*

Introduction

The alcoholic fermentation (AF) at the industrial level is undertaken mainly by yeast starter cultures inoculated directly into the grape must. This method ensures the commencement of AF and reduces the risk of off-flavours because the selected yeasts rapidly reach high concentration and dominate the microbial population during the entire vinification process (Fleet 2008). The microbial diversity of a fermentation undertaken by commercial or autochthonous starters does not represent the complexity and variability of the microbial populations that characterise the spontaneous AF. The latter is a heterogeneous microbiological process that involves a succession of non-*Saccharomyces* and *S. cerevisiae* species. Furthermore, during spontaneous AF, several *S. cerevisiae* strains develop to high concentration in must. Such microbial diversity exerts an important role, in terms of colour and flavour, for the final characteristics of the product (Fleet 2008). Several studies have shown that spontaneous AF improves the composition of the final product providing wines with unique regional characters (Fleet 2008). For these reasons, in recent years, the request for wines with typical sensory profiles has greatly increased, and the number of cellars producing wines by means of spontaneous AF has increased. The spontaneous AF, however, is still recognised as an uncontrolled biological process during which some spoilage yeast and/or bacterial strains can rapidly increase and negatively affect the composition of the final product. Thus, during the spontaneous AF the risk of off-odour and off-flavour generation is high.

In contrast, the use of the same starters to ferment musts obtained from different grape cultivars and/or from different geographical areas is often discussed because of their significant prevalence over the native microflora (Valero et al. 2005). As a result, excessive standardisation of the sensory profile of the final wines cannot be excluded. In recent years, besides starter cultures, yeast strains selected from indigenous populations have been the subject of several studies (Tofalo et al. 2009, 2014, Francesca et al. 2010) in order to maintain a certain typicity of the wines. A given yeast strain might be adapted to a specific climatic condition (Esteve-Zarzoso et al. 2000), as well as to the cellar environment where the AF occurs (Guzzon et al. 2011, Francesca et al. 2014a). Even though the use of selected autochthonous strains could significantly contribute to the expression of varietal characteristics of wines (Jolly et al. 2006, Zott et al. 2008), they have to encounter the indigenous microbial communities of grapes whose composition at species and/or strain level changes with the vintage (Fleet 2008).

Some wineries traditionally prepare wines by using the 'pied de cuve' method (Ubeda Iranzo et al. 2000, Clavijo et al. 2011, Li et al. 2012). This technique is based on the inoculation of a given amount of must, previously subjected to a partial AF, into

doi: 10.1111/ajgw.12166

a new must. Normally, when the fermented pied de cuve reaches an ethanol concentration of about 5% (v/v), the pied de cuve is added to must with a pied de cuve/new must ratio of 1:10. Thus, the pied de cuve method applies viable yeast cells to start a new fermentation and promotes the growth of yeasts with good fermentation characteristics.

Generally, the pied de cuve is inoculated with a commercial starter, in order to start the AF. In this way, the amount of starter inoculum is reduced and, overall, it is possible to transfer desirable oenological features of yeast strains from a successful fermentation to a new must (Li et al. 2012). If the pied de cuve is a spontaneously fermented AF, the use of pied de cuve might limit but not exclude the risks, such as growth of spoilage microorganisms, stuck AF and formation of off-flavours, related to the winemaking processes carried out without the inoculum of starter cultures.

The main objective of the present study was to develop a new method to produce a pied de cuve from a spontaneous AF. For this purpose, the experimental pied de cuve was obtained from a must with added wine and, subsequently, subjected to a spontaneous AF. The study was carried out with grapes of Nero d'Avola cultivar and microbiological, chemical and sensory parameters were evaluated. Because the experimental pied de cuve was 'supplemented' with wine, the term 'fortified pied de cuve' (FPdC) was adopted.

Materials and methods

Experimental winemaking and sample collection

The experimental winemaking consisted of three technological steps: (i) preparation of the base medium for FPdC by addition of wine to must; (ii) spontaneous fermentation for 3 days to obtain FPdC; and (iii) inoculation of FPdC into fresh must.

Grapes of the Nero d'Avola cultivar were used for all experiments. They were harvested in a vineyard located in Marsala (Trapani, Sicily, Italy) (37°45′18.00″N; 12°30′19.41″E). The experimental vinification took place at the cellar of the Centro di Ricerca per l'Innovazione della Filiera vitivinicola Ernesto del Giudice in Marsala. All experimental winemaking was undertaken in triplicate with the same lot of grapes (three vats for each trial) and during two consecutive vintages (2011 and 2012). All samples collected and the collection times are reported in Tables S1 and S2. All samples were transported at 4°C in a portable fridge and subjected to microbial and chemical analysis within 24-h collection.

Preparation and monitoring of FPdC

The grapes were manually harvested and stemmer-crushed. The bulk must was divided into four steel vats (30 kg each), representing four experimental trials (A, B, C and D), and potassium metabisulfite (0.05 g/kg) was added. Trial A received an addition of Nero d'Avola wine [average composition for vintages 2009 and 2010: pH 3.40; ethanol 13.1% (v/v), total acidity 5.85 g/L (tartaric acid); total SO_2 87 mg/L; free SO_2 10 mg/L] to a final ethanol concentration of the must of 1.5% (v/v). Trial B received a larger volume of Nero d'Avola wine than trial A to reach a final ethanol concentration of the must of 3% (v/v). Trial C had no added wine. Trial D, with no added wine, was inoculated (0.15 g/kg) with a commercial strain of *S. cerevisiae* (NDA 21, Springer Oenologie, Marcq-en-Baroeu, France) in dried form [about 8.00×10^{12} colony-forming units (CFU)/g], commonly used to ferment commercial Nero d'Avola wines in the Marsala area. Trials A–C were spontaneously fermented. The AF of all trials took place at 26°C for 3 days. The contents of each vat was mixed (three times per day), only after the cap had formed, in order to facilitate the contact between liquid and solid phase of the musts.

Winemaking

The four FPdCs were transferred into 100-L stainless steel vats, which were filled with fresh grape must (approximately 80 kg) to achieve a ratio of 1:10 FPdC:fresh must. Diammonium phosphate and diammonium sulfate (1:1) (15 g/hL) were also added as activators of the AF. The AF took 7 days during which the cap was manipulated as above.

At the end of AF the contents of each vat were hydraulically pressed. The resulting liquid phase (about 50 L) was transferred into new stainless steel vats where the ageing phase, including the spontaneous malolactic fermentation (MLF), took place at 23°C. At the end of MLF (day 34 of ageing), in order to avoid contact between the wine and oxygen, all vats were sealed by nitrogen gas and stored for 2 months at 16 ± 1°C. At the end of ageing (day 90), the wines were filtered (5.0-μm pore size filter) and bottled. The final wines were stored at 16°C and 80% RH for 2 months. Samples for analysis were collected at all stages from grape harvest to wine bottling.

Microbiological analysis

Samples collected during wine production were serially diluted in Ringer's solution (Sigma-Aldrich, Milan, Italy). The surfaces of cellar equipment (stemmer-crusher, hydraulic press and steel tanks) were analysed following the methodology of the International Organization for Standardization (2004); the surface of grape berries was analysed according to Settanni et al. (2012). All samples were analysed in duplicate for total yeast (TY) on Wallerstein Laboratory (WL) nutrient agar, presumptive *Dekkera*/*Brettanomyces* spp. on *Dekkera*/*Brettanomyces* differential medium, mesophilic lactic acid bacteria (LAB) rods on de Man–Rogosa–Sharpe agar, coccus LAB on glucose M17 agar, acidophilic LAB on medium for *Leuconostoc oenos* agar and acetic acid bacteria (AAB) on Kneifel agar medium as reported by Francesca et al. (2014b). All media and the supplements used were supplied from Oxoid (Thermofisher, Basingstoke, England).

Yeast isolation and identification

Yeasts were isolated from WL medium. At least three colonies per morphology were randomly collected from the agar plates, purified to homogeneity after several subculturing steps onto WL, and at least three isolates (from each sample) sharing the same morphology were subjected to the genetic characterisation.

The InstaGene Matrix kit (Bio-Rad Laboratories, Hercules, CA, USA) was used to extract DNA according to the manufacturer's instructions. All selected isolates were initially grouped by restriction fragment length polymorphism (RFLP) analysis of the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene as reported by Esteve-Zarzoso et al. (1999). Subsequently, five isolates representative of each group were subjected to an additional enzymatic restriction targeting the 26S rRNA gene as reported by Settanni et al. (2012). The 26S rRNA gene was amplified with the primers NL1 and LR6 (Baleiras-Couto et al. 2005). One isolate per group was identified at species level by sequencing the D1/D2 region of the 26S rRNA gene to confirm the preliminary identification obtained by RFLP analysis. The D1/D2 region was amplified by using primers NL1 and NL4 (O'Donnel 1993) and polymerase chain reaction (PCR) products were visualised as described by Settanni et al. (2012); DNA was sequenced at Primmbiotech (Milan, Italy). The identity of the sequences was determined by

BlastN search against the NCBI non-redundant sequence database located at http://www.ncbi.nlm.nih.gov.

Strain typing of S. cerevisiae *isolates*

All isolates belonging to the species *S. cerevisiae* were further characterised at the intra-specific level by employing two techniques: interdelta analysis with primers delta12 and delta21 (Legras and Karst 2003) and microsatellite multiplex PCR based on the analysis of polymorphic microsatellite loci, that is SC8132X, YOR267C and SCPTSY7 (Vaudano and Garcia-Moruno 2008). The PCR products were analysed and visualised as reported by Settanni et al. (2012).

Chemical analysis

Conventional parameters. Titratable acidity (TA), pH, volatile acidity (VA), reducing sugars, ethanol, glycerol, malic acid, lactic acid and dry extract were determined by means of a Winescan (FOSS, Hillerød, Denmark) calibrated following EEC 2676 standard procedure (European Commission 1990, Sannino et al. 2013). Total and free SO_2 were measured in accordance with the official methods described by the European Commission (1990). Anthocyanin and total flavonoid concentration was determined by spectrophotometry (Di Stefano 1980). All chemical analyses were carried out in triplicate.

Volatile organic compounds. Free volatiles were determined with an Agilent GC 6890 GC coupled to an Agilent 5973 mass-selective detector (Agilent Technologies, Santa Clara, CA, USA) according to the method reported by Corona (2010). Volatile organic compounds were identified by comparison of the mass spectra and GC retention times with those of the pure commercial standard compounds or others prepared in our laboratory. The concentration of the compounds was estimated by comparing their peak areas with those of the respective internal standards with known concentration. All analyses were carried out in duplicate.

Sensory evaluation

The sensory profiles of the experimental wines were evaluated by a descriptive method (Ente Nazionale Italiano di Unificazione 2003). Twelve judges were trained in preliminary sessions using different samples of Nero d'Avola commercial wines in order to develop a common vocabulary for the description of the sensory attributes of the experimental wine samples, as well as to familiarise them with scales and procedures. Each attribute was extensively described and explained to avoid any doubt about the relevant meaning. The standards used to define descriptors were chosen according to Noble et al. (1987).

On the basis of frequency of citation (>60%), 16 descriptors were included in the analysis: colour intensity, odour intensity, odour complexity, off-odours, fresh fruits, dried fruits, flowers, aromatic herbs, spices (odour), sweet, hot (tactile in mouth), acid, astringent, bitter, taste complexity and off-flavour (taste). The wine samples were randomly evaluated by assigning a score between 1.00 (absence of sensation) and 9.00 (extremely intense) in individual booths under incandescent white light.

Statistical analysis

Statistical analyses were conducted using STATISTICA software (StatSoft, Tulsa, OK, USA). Microbial data were analysed with a generalised linear model that included the effects of samples; the Student *t*-test was used for mean comparison. The post-hoc Tukey's method was applied for pairwise comparison. Statistical significance was attributed to P values of <0.05.

In addition, a principal component analysis (PCA) of values of chemical conventional parameters and VOCs was carried out. Statistical analysis was performed using XLSTAT 2006, version 2006.6 (Addinsoft, Paris, France). The resulting scores from the sensory analysis were averaged and compared. The ANOVA test (STATISTICA software) was applied to identify significant differences among the wine attributes.

Results

Microbiological analysis

The viable counts of the microbial groups during both FPdC preparation and the AF are reported in Table 1. The TY count detected on grape berries and in must just after crushing was about 5 log CFU/(g or mL) in both vintages. After the addition of wine into must, the TY count in trials A and B decreased slightly, but they reached a count higher than 8 log CFU/mL after 3 days of AF. A similar count was shown by the trial D inoculated with the starter culture. In contrast, at day 3, trial C that was neither supplemented with wine nor inoculated with starter, showed the lowest TY count in both vintages. *Dekkera/Brettanomyces* spp. population, as well as LAB and AAB, were not detected during FPdC preparation (Tables S1,S2).

After the inoculation of FPdCs into new grape musts, the TY count significantly increased in all trials. At day 2 of AF, the trials A and B reached the same TY count (about 8 log CFU/mL) estimated for the trial D. At the same time, the trial C showed the lowest TY count in both vintages. At the end of AF (day 7), no statistical difference was found among the trial ferments. During the ageing of wines from all trials and in both vintages, the TY counts decreased to about 4 log CFU/mL (day 34) and were not detected at bottling.

The LAB population reached a detectable level at the beginning of ageing (day 3) (Table S1) and had significantly increased at day 10 of ageing on all media tested; the lowest counts were detected for trial C on glucose M17 medium during both vintages. At bottling no LAB were estimated in all trials.

Yeast was found on cellar equipment surfaces at low count around 2.4 log CFU/cm^2. The highest count was detected on the stemmer-crusher surfaces (around 2.6 log CFU/cm^2). Lactic acid bacteria were not detected on cellar equipment surfaces. *Dekkera/Brettanomyces* spp. and AAB were not detected during the entire period of winemaking and after filtration of the wines (Tables S1,S2). All microbial groups reported above were not detected in wines added to bulk must to produce FPdC.

Isolation, identification and distribution of yeasts

A total of 4543 yeasts was isolated from count plates, purified to homogeneity and grouped on the basis of colony appearance on WL medium. There were 50 colonies per morphology that were selected and subjected to molecular identification. After the restriction analysis of the 5.8S-ITS region and 26S rRNA gene, the isolates were clustered into nine groups (Table 2). The isolates belonging to all groups were directly identified at species level by comparison of the restriction bands with those available in literature (Esteve-Zarzoso et al. 1999, Sannino et al. 2013, Francesca et al. 2014a). The identification of the isolates was concluded by sequencing of the D1/D2 domain of 26S rRNA gene that confirmed the presence of the species *Aureobasidium pullulans*, *Candida zemplinina*, *Hanseniaspora guilliermondii*, *Hanseniaspora uvarum*, *Issatchenchia terricola*, *Metschnikowia pulcherrima*, *Pichia guilliermondii*, *Rhodotorula mucillaginosa* and *S. cerevisiae* (Table 3).

The distribution of yeast species in both vintages and the corresponding counts estimated per each sample are reported in

Table 1. Total yeast count of samples collected during the pied de cuve preparation and winemaking process of Nero d'Avola wines during the 2011 and 2012 vintages.

Samples	Total yeast count†							
	Trial A		Trial B		Trial C		Trial D	
	2011	2012	2011	2012	2011	2012	2011	2012
Pied de cuve preparation								
Grape berries	4.60 ± 0.1a	5.19 ± 0.2a	4.60 ± 0.1a	5.19 ± 0.2a	4.60 ± 0.1a	5.19 ± 0.2a	4.60 ± 0.1a	5.19 ± 0.2a
Must	5.44 ± 0.1a	5.78 ± 0.2a	5.44 ± 0.1a	5.78 ± 0.1a	5.44 ± 0.1a	5.78 ± 0.1a	5.44 ± 0.1a	5.78 ± 0.1a
Wine addition‡/starter inoculum§	5.22 ± 0.1a	5.30 ± 0.1a	5.02 ± 0.1a	5.11 ± 0.1a	n.s.	n.s.	7.13 ± 0.1b	7.33 ± 0.2b
Alcoholic fermentation								
Day 1	7.93 ± 0.1c	7.81 ± 0.1b	7.55 ± 0.1c	7.53 ± 0.1b	5.81 ± 0.1a	6.03 ± 0.1a	7.47 ± 0.1bc	7.91 ± 0.1b
Day 3	8.23 ± 0.1c	8.63 ± 0.1b	8.90 ± 0.1b	8.88 ± 0.3b	7.41 ± 0.1a	6.97 ± 0.1a	8.56 ± 0.1bc	8.56 ± 0.2b
Winemaking process								
Grape berries	4.78 ± 0.1a	5.74 ± 0.1a	4.78 ± 0.1a	5.74 ± 0.1a	4.78 ± 0.1a	5.74 ± 0.1a	4.78 ± 0.1a	5.74 ± 0.1a
Must	5.77 ± 0.1a	6.12 ± 0.1a	5.77 ± 0.1a	6.12 ± 0.1a	5.77 ± 0.1a	6.12 ± 0.1a	5.77 ± 0.1a	6.12 ± 0.1a
Inoculum of pied de cuve	7.20 ± 0.1b	7.51 ± 0.1b	7.16 ± 0.1b	7.40 ± 0.1b	6.57 ± 0.1a	6.74 ± 0.2a	6.74 ± 0.1a	7.77 ± 0.2b
Alcoholic fermentation								
Day 1	7.91 ± 0.1b	7.60 ± 0.2a	7.97 ± 0.1b	7.21 ± 0.2a	7.09 ± 0.1a	7.00 ± 0.2a	7.80 ± 0.1b	7.77 ± 0.2a
Day 2	8.13 ± 0.1b	8.28 ± 0.1b	8.00 ± 0.1b	8.33 ± 0.1b	7.18 ± 0.2a	7.09 ± 0.1a	8.16 ± 0.1b	8.12 ± 0.1b
Day 4	8.47 ± 0.1b	8.50 ± 0.1b	8.12 ± 0.1b	7.99 ± 0.2ab	7.61 ± 0.1a	7.78 ± 0.1a	8.14 ± 0.1b	8.21 ± 0.2ab
Day 7 – racking	8.06 ± 0.2a	8.22 ± 0.1a	8.01 ± 0.1a	8.41 ± 0.1a	7.81 ± 0.1a	7.80 ± 0.2a	7.97 ± 0.1a	8.30 ± 0.2a
Bottling	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Results indicate average values ± standard deviation of three plate counts. Different letters indicate a significant difference among the trials for the same sample and the same medium ($P < 0.05$). n.d., not detected (value < detection limit of method); n.s., not sampled. †Log CFU/g for grape berries and log CFU/mL for must and wine samples; ‡only for trial A and trial B; §only for the trial C. CFU, colony-forming units.

the Table 3. During the preparation of the FPdC, just after the addition of wine into the must, among non-*Saccharomyces* populations, the species *H. guilliermondii*, *H. uvarum* and *C. zemplinina* were detected in trial B, whereas only *C. zemplinina* was found in trial A. During the AF, *S. cerevisiae* species were detected at the highest count in trials A and B, both for FPdC and winemaking. In contrast, the yeast population of trial C was mainly represented by *H. guilliermondii*, *H. uvarum* and *M. pulcherrima*. From day 4 of the AF onwards, in trial C the presence of *S. cerevisiae* was detected.

During wine ageing, *H. uvarum* and *P. guilliermondii* were mainly isolated. As expected, the yeast population of trial D was mainly represented by *S. cerevisiae* at all sampling points and in both vintages. *Aureobasidium pullulans*, *H. guilliermondii* and *I. terricola* were the species mainly isolated from the surfaces of equipment, whereas no *S. cerevisiae* isolates were found.

Typing and distribution of S. cerevisiae *strains*

A total of 1164 isolates belonging to the species *S. cerevisiae* were investigated at strain level. The interdelta analysis was able to separate the isolates into 84 groups (47 and 37 from the 2011 and 2012 vintages, respectively) (Figure 1). The microsatellite multiplex PCR estimated only 67 (40 and 27 from the 2011 and 2012 vintages, respectively) different profiles (data not shown).

During FPdC preparation, trial A showed the greatest number of strains; in contrast, no strain was found in trial C. Only two strains (9 and 29) were isolated from the bulk must (2011 vintage). After addition of FPdC to the new musts, the highest number of strains was found for trial A (25 and 18 from the 2011 and 2012 vintages, respectively) followed by the trial B (22 and 13 from the 2011 and 2012 vintages, respectively). The number of strains estimated from trial C was significantly lower than that detected in the other trials.

Some strains [i.e. 11, 30, 15 and 22 (2011 vintage), as well as 49, 79, 61 and 68 (2012 vintage)] were isolated during FPdC preparation and they also dominated the yeast population during the winemaking process. As expected, the commercial starter (8) inoculated in the pied de cuve of trial D was most frequently isolated during the entire winemaking process.

Chemical analyses of conventional parameters

Table 3 shows the composition of the samples collected during the preparation of FPdC; on day 3 of the AF, the highest ethanol concentration was reached by trial D. At the same time, trials A and B reached an ethanol concentration of about 4.5% (v/v); in contrast, trial C showed the lowest ethanol concentration [about 2.5% (v/v)], in both vintages. Furthermore, in trial C the VA also increased up to the highest value of about 0.5 (g/L of acetic acid) on day 3 of the AF. The VA of all other trials was about 0.3 g/L (acetic acid). The concentration of malic acid did not change, and lactic acid was not detected in all trials.

Table S3 (2011 vintage) and Table S4 (vintage 2012) show the composition of the must during the winemaking process. In all trials, the reducing sugars were mostly converted to ethanol by day 7 of AF; they were not detectable at bottling. At day 1 of the AF, trial A showed the highest ethanol concentration (12.9 and 13.3% (v/v) for the 2011 and 2012 vintages, respectively).

During the monitored vinifications, the values of pH and VA slowly increased up to 3.3 and 0.4 g/L (acetic acid), respectively. Trial C showed the highest values of VA 0.6 g/L (acetic acid) at bottling during the 2012 vintage. The amount of glycerol greatly increased during the experiments reaching the highest value at the bottling phase of trial B (8.0 and 8.7 g/L for the 2011 and 2012 vintages, respectively). The malic acid amount was almost completely converted into lactic acid during the ageing of wine in all trials and in both vintages. The trend of the concentration

Table 2. Molecular identification of yeasts and distribution of yeast species during the pied de cuve preparation and winemaking process of Nero d'Avola wines during the 2011 and 2012 vintages.

Strain	Species (% identity)†	Accession number	Distribution			
			Pied de cuve preparation		Winemaking process	
			2011 Vintage	2012 Vintage	2011 Vintage	2012 Vintage
NDLCT8	*Aureobasidium pullulans* (99)	KM609503	GB(4)‡; M(5); HP ,SC(2)	GB(4); SC(2)	F1[C,D(7)]; SC,ST(2)	GB(4); F1[D(7)]; ST(2)
NDLCT76	*Candida zemplinina* (99)	KM609504	M(5)	M(5); AW[A(5)]; F3[A(8),C(6)]		M(6); F1[A,C,D(7)]
NDLCT65	*Hanseniaspora guilliermondii* (99)	KM609511	GB(4); M(5); AW[A,B (5)]	M(5); HP(2)	GB(4); M(5); F1[A,C,D (7)]; SC, HP(2)	F1[A,C(7),B(8)]; SC,HP(2)
NDLCT79	*Hanseniaspora uvarum* (99)	KM609505	M(5); AW[A(5)]; F3[A,B(8),C(7)]	GB(4); M(5); AW[A(5)]; F1[A(7),C(6)]; F3[A(8),C(6)]	M(5);	M(6); F7[A(8), C(7)]; A3[A(6),B(7)]
NDLCT5	*Issatchenchia terricola* (99)	KM609506	GB(4); ST(2)	GB(5); ST(2)	GB(4); SC,HP(2)	GB(5); M(6)
NDLCT26	*Metschnikowia pulcherrima* (97)	KM609509	GB(4); M(5)	GB(4); M(5)	GB(4); M(5); F1[C(7)]	GB(5); M(6); F1[A,C(7)]
NDL405	*Pichia guilliermondii* (98)	KM609507	GB(4); M(5); AW[A,B(5)]	GB(4); M(5)	M(5); A17[A,C,D(5)]	M(6); ST(2)
NDL373	*Rhodotorula mucillaginosa* (99)	KM609508		M(5)		GB(5); M(6)
NDL350	*Saccharomyces cerevisiae* (98)	KM609510	IS[D(6)]; F1[A,B,D(7)]; F3[A,B,D(8)]	IS[D(7)]; F1[A,B,D(7)]; F3[A,B,D(8)]	M(5); IP[A,B(7),D(6)]; F1[A,B,D(7)]; F7[A,B(8),C,D(7)]; A3[A,B,D(7),C(6)]; A17[A,B,C,D(5)]; A34[A,B,D(4),C(3)]	IP[A,B(7),D(6)]; F1[A,B,D(7)]; F7[A,B,D(8),C(7)]; A3[A,D(6),B,C(7)]; A17[A,B,C,D(5)]; A34[A,B,C(4),D(3)]

†According to BlastN search of D1/D2 26S rRNA gene sequences in NCBI database. ‡The number reported in parenthesis refers to the highest count (log cycle) of detection. A, trial A; B, trial B; C, trial C; D, trial D; GB, grape berries; M, must; AW, addition of wine into must; IS, inoculum of starter; IP, inoculum of pied de cuve into must during winemaking process; F1, F3, F7 refer to the first, third and seventh day of alcoholic fermentation, respectively; A3, A17 and A34 refer to day 3, 17 and 34 of ageing; B, bottling; SC, stemmer-crusher; HP, hydraulic press; ST, stainless steel tank.

Table 3. Chemical composition during the preparation of the pied de cuve of Nero d'Avola wines during the 2011 and 2012 vintages

Parameters	2011 Vintage					2012 Vintage				
	Must	Alcoholic fermentation				Must	Alcoholic fermentation			
		Day 3 (A)†	Day 3 (B)	Day 3 (C)	Day 3 (D)		Day 3 (A)	Day 3 (B)	Day 3 (C)	Day 3 (D)
pH	3.3 ± 0.1	3.2 ± 0.1	3.2 ± 0.1	3.2 ± 01	3.3 ± 0.1	3.2 ± 0.1	3.3 ± 0.1	3.4 ± 0.1	3.1 ± 0.1	3.3 ± 0.1
TA	7.7 ± 0.1	7.5 ± 0.1	7.3 ± 0.1	7.5 ± 0.1	7.7 ± 0.1	7.6 ± 0.1	7.6 ± 0.1	7.4 ± 0.1	7.4 ± 0.1	7.5 ± 0.1
VA	0.1 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
RS	216 ± 4	130 ± 2	163 ± 2	179 ± 4	109 ± 3	260 ± 5	185 ± 1	216 ± 3	211 ± 4	148 ± 4
EtOH	n.d.	4.7 ± 0.2	4.4 ± 0.1	2.5 ± 0.3	6.0 ± 0.4	n.d.	4.8 ± 0.2	4.6 ± 0.1	2.7 ± 0.3	5.8 ± 0.3
GL	n.d.	5.5 ± 0.1	5.1 ± 0.1	4.2 ± 0.2	6.0 ± 0.1	n.d.	5.0 ± 01	5.1 ± 0.3	3.9 ± 0.2	4.7 ± 0.2
MA	1.6 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.3 ± 0.2	1.3 ± 0.1
LA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
DE	148 ± 4	155 ± 3	150 ± 4	147 ± 4	151 ± 3	177 ± 5	194 ± 4	187 ± 5	159 ± 3	190 ± 4
FL	1304 ± 78	1297 ± 60	1300 ± 32	1304 ± 78	1288 ± 10	1412 ± 51	1387 ± 77	1362 ± 41	1328 ± 52	1374 ± 37
AN	454 ± 21	398 ± 17	400 ± 14	454 ± 21	382 ± 16	495 ± 25	421 ± 31	456 ± 52	427 ± 30	442 ± 19
Total SO_2	n.d.	46 ± 0.2	46 ± 0.4	13 ± 0.3	47 ± 0.3	n.d.	52 ± 0.3	57 ± 0.1	24 ± 0.4	50 ± 0.6
Free SO_2	n.d.	22 ± 0.3	21 ± 0.2	4 ± 0.2	24 ± 0.8	n.d.	28 ± 0.2	30 ± 0.8	6 ± 0.5	27 ± 0.7

Results indicate average values ± standard deviation of three replicates. n.d., not detected (values < detection limit). †The letter in parenthesis refers to trials A, B, C, and D. AN, anthocyanins (mg/L malvidin-3-glucoside); DE, dry extract (g/L); EtOH, ethanol (% w/v); FL, flavonoids (mg/L); GL, glycerol (g/L); LA, lactic acid (g/L); MA, malic acid (g/L); RS, reducing sugar (g/L); TA, total titratable acidity (g/L tartaric acid); Total SO_2 and Free SO_2 (mg/L); VA, volatile acidity (g/L acetic acid).

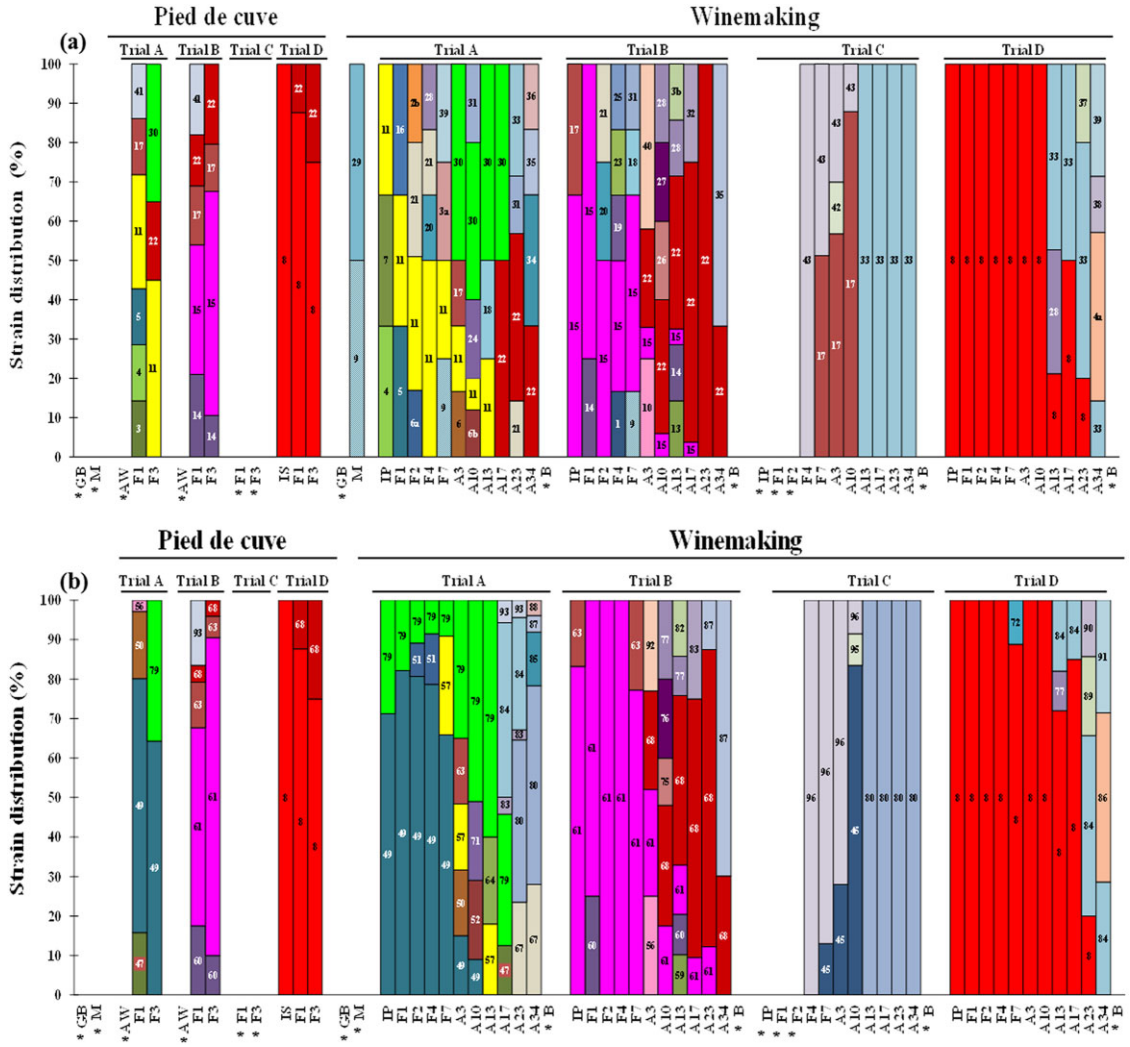

Figure 1. Distribution of *Saccharomyces cerevisiae* strains during the pied de cuve preparation and winemaking process of Nero d'Avola wines during the (a) 2011 and (b) 2012 vintages. The numbers within the figure refer to strain codes; strains belonging to *Saccharomyces cerevisiae* were not isolated from samples marked with *. A3, A10, A13, A17, A23 and A34 refer to the wines on day 3, 10, 13, 17, 23 and 34 of ageing; AW, addition of wine into must; B, refers to wine at bottling; GB, grape berries; F1, F2, F3, F4, F7 refer to the wines on day 1, 2, 3, 4 and 7 of alcoholic fermentation; IP, inoculum of pied de cuve into must during winemaking process; M, must.

of phenolic substances was similar in all experimental trials. Both anthocyanins and flavonoids showed a consistent decrease from the beginning of AF until bottling (Tables 3, S4).

Effect of winemaking on VOCs

The results of VOC analyses carried out on the samples collected during the winemaking process are reported in Table S5 (2011 vintage) and Table S6 (2012 vintage). The esters were mainly represented by diethyl succinate, ethyl lactate, ethyl 2-hydroxy-3-phenylpropionate and isoamyl acetate that reached the highest concentration at bottling of both trials A and B. At the same time, the trial D showed also a high concentration of diethyl malate and ethyl lactate. Furthermore, both trials A and D showed a high concentration of ethyl hexanoate and isoamyl acetate on day 7 of the AF.

The main compounds within the class of alcohols were hexanol and isoamyl alcohol that reached the highest concentration in trial C at bottling. *cis*-3-Hexenol was estimated at high concentration in both trials A and B, in particular at bottling during the 2011 vintage.

The concentration of the fatty acids octanoic and hexanoic was highest at the bottling phase of trials A and B. Decanoic and isovaleric acids were estimated at high concentration in trials A and C, respectively. During the AF, the methanol concentration was similar in all experimental trials, with the exception of trial C that had the highest concentration both during ageing and at bottling.

PCA of conventional chemical compounds and VOCs

The PC1 and PC2 components, selected from the PCA analysis, explained 72.09 and 26.15% (2011 vintage) and 38.98 and 22.43% (2012 vintage) of total variance, respectively (Figure 2). The descriptors that mainly contributed to the PC1 components were ethyl 2-hydroxy-3-phenylpropionate, ethanol, glycerol, total SO_2, hexanoic acid, isoamyl alcohol, lactic acid, monoethyl succinic acid, free SO_2 (2011 vintage) and glycerol, ethyl 2-hydroxy-3-phenylpropionate, total SO_2, lactic acid, hexanoic acid, isoamyl alcohol, monoethyl succinic acid, free SO_2, ethyl acetate (2012 vintage) of which the loading values were 0.956, 0.951, 0.937, 0.842, 0.830, 0.785, 0.752, 0.749, 0.714 (2011 vintage) and 0.953, 0.956, 0.842, 0.759, 0.830, 0.785, 0.749, 0.714, 0.702 (2012 vintage), respectively. In contrast, F2 components were represented mainly by hexanol, *cis*-3-hexenol, ethyl decanoate, ethyl 9-decanoate, decanoic acid, TA, malic acid (2011 vintage) and *cis*-3-hexenol, hexanol, decanoic acid, ethyl 9-decanoate, 2-phenylethyl acetate, lactic acid (2012

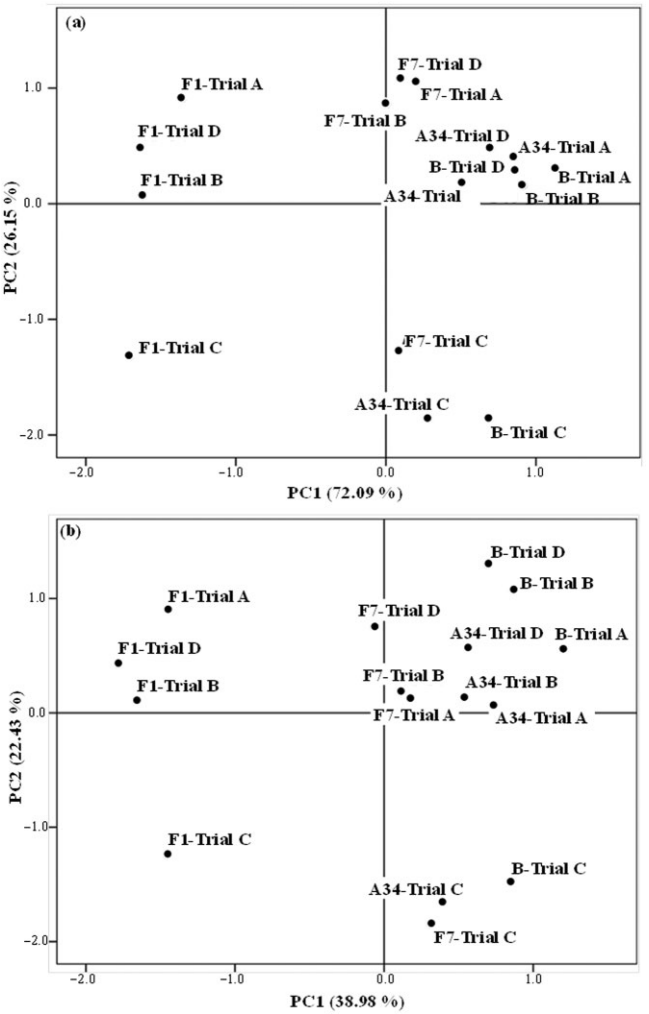

Figure 2. Score plot for the components PC1 and PC2 of samples collected during winemaking process in (a) 2011 and (b) 2012 vintages. F1 and F7 refer to the wines on day 1 and day 7 of the alcoholic fermentation, respectively; A34 refers to the wines on day 34 of ageing; B, refers to the wines at bottling.

vintage) of which the loading values were 0.930, 0.888, 0.772, 0.771, 0.738, 0.330, 0.294 (2011 vintage) and 0.887, 0.847, 0.766, 0.694, 0.647, 0.128 (2012 vintage), respectively.

With the exception of the samples collected at day 1 of the AF, most wines were located in the quadrant characterised by positive values both of PC1 and PC2 components. In detail, the samples of trial C at day 7 of the AF, as well as at day 34 of ageing and at bottling, were clearly separated from the others during both vintages. At bottling, wines from trials A and B were located in the same area of the control (trial D) and clearly separated from the others during both vintages.

Sensory analysis

The experimental wines were evaluated by a panelist expert in sensory analysis of wine (Table 4). Samples fermented with pied de cuve with added wine (trials A and B) differed significantly ($P < 0.05$) from trials C and D. The main difference was estimated in terms of odour intensity and complexity, taste complexity, as well as for the odours of flowers and dried fruits. Trial D showed values of fresh fruits (odour) significantly higher than that of the trials A, B and C.

Discussion

The scope of the present work was to apply new methodology for the preparation of pied de cuve and to evaluate the effect of the inoculum addition to the bulk must on the microbial, chemical and sensory composition of the red wine Nero d'Avola obtained by spontaneous fermentation. The results of the microbial analyses clearly showed a substantial concentration and a high strain diversity of the *S. cerevisiae* population during the vinification carried out with FPdC. Furthermore, several strains were isolated first from FPdC and, subsequently, found at the highest concentration during the complete winemaking process. These results suggested FPdC as a valuable method to favour the growth of *S. cerevisiae* strains with winemaking characteristics that are able to promote the complete spontaneous AF. To our knowledge, no study has been carried out on the monitoring of yeast and LAB populations during winemaking based on the use of an FPdC to promote the spontaneous AF. Within the published literature, however, little information (Ubeda Iranzo et al. 2000, Clavijo et al. 2011, Li et al. 2012) is available on the use of pied de cuve during the winemaking process.

In the present study, three FPdCs (trials A, B and C), which varied in the amount of wine added into the new must, were followed. According to the literature (Ubeda Iranzo et al. 2000, Li et al. 2012), trial D was added as a control trial, based on the common use of a commercial starter to prepare the pied de cuve. During the preparation of FPdC, the addition of wine into must did not inhibit the growth of yeast during the AF; only just after the wine addition, a decrease of less than 0.5 log cycle was measured in trials A and B. Subsequently, the yeast counts of both experimental trials were superimposable to that of trial D. Interestingly, yeast counts of trials A and B were significantly higher than those of trial C.

During the entire winemaking process of trials A and B, *S. cerevisiae* was the main species found; except in must and on day 1 of the AF, when it was detected at the same microbial count as *Hanseniaspora* spp. and *M. pulcherrima*, it dominated the yeast population. All the non-*Saccharomyces* species isolated in the present work are commonly associated with the wine environment, mostly with the beginning of the AF (Sannino et al. 2013, Francesca et al. 2014b). During the wine ageing of trials A and B, among non-*Saccharomyces*, only *H. uvarum* and *P. guilliermondii* were found at high count. Although, *P. guilliermondii* does not represent a species commonly used as a starter or co-starter for wine fermentation, our work is not the first to report the isolation of *P. guilliermondii* during winemaking. This species was already isolated within the first month of wine ageing (Sannino et al. 2013) at a high concentration of ethanol. Recently, Francesca et al. (2014a) found *P. guilliermondii* at co-dominant concentration with *S. cerevisiae* during both the AF and wine ageing. Interestingly, *P. guilliermondii* was found after the transfer and/or clarification of wine that might increase the rate of oxygen ingress thus favouring the growth of this species. In the present work, *P. guilliermondii* was isolated within 14 days from transfer of wine.

In trial D the yeast population was mainly represented by non-*Saccharomyces* species as already reported for spontaneous AF (Zott et al. 2008, Sannino et al. 2013). As expected, the AF of trial D was mainly undertaken by the commercial starter inoculated into must.

Although trials A and B were spontaneously fermented, a low diversity of species was found. This observation could be explained by the specific conditions due to the ethanol concentration of must after the addition of wine during the pied de cuve preparation. This condition resulted in a selection of yeast species and, at the same time, did not negatively affect the diversity of yeast strains. The results obtained by interdelta analysis showed that the number of strains found during the

Table 4. Sensory scores of Nero d'Avola wines prepared by different pied de cuve methods during the 2011 and 2012 vintages.

Descriptors	2011 Vintage				2012 Vintage			
	Trial A	Trial B	Trial C	Trial D	Trial A	Trial B	Trial C	Trial D
Colour intensity	7.0a	7.3a	6.9a	7.2a	7.1a	7.2a	7.0a	7.2a
Odour								
Intensity	7.7a	7.5b	7.0ac	7.2ac	7.6ab	7.8b	7.2a	7.4ab
Complexity	7.4b	7.2a	7.0a	6.8a	7.7c	7.4bc	7.2b	6.6a
Fresh fruits	6.1a	6.0a	6.0a	6.3a	6.4a	6.2a	6.3a	6.8a
Dried fruits	3.5b	3.9b	3.0a	2.9a	3.0a	3.1a	2.9a	2.7a
Flowers	3.7b	3.5b	3.2b	2.7a	3.6b	3.6b	3.4ab	3.0a
Aromatic herbs	2.1bc	2.4bc	2.2bc	1.7a	2.3b	2.4b	1.8a	1.6a
Spices	2.7b	3.0b	2.1a	2.0a	2.7bc	3.0b	2.1a	2.0a
Off-odours	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Taste								
Sweet	1.3a	1.3a	1.2a	1.2a	1.3a	1.4a	1.3a	1.2a
Hot	3.3a	3.5a	3.3a	3.4a	3.5a	3.5a	3.5a	3.5a
Acid	3.5a	3.6a	3.5a	3.4a	3.3a	3.3a	3.5a	3.4a
Astringent	6.5a	6.4a	6.7a	6.6a	6.2a	6.1a	6.5a	6.8a
Bitter	3.7a	3.5a	3.9a	3.9a	3.1a	3.2a	3.5a	3.3a
Complexity	6.5b	6.3b	5.9a	5.5a	6.8b	6.4b	6.1ab	5.7a
Off-flavours	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Different letters indicate a significant difference between maceration times for the same sample for $P \leq 0.05$.

winemaking process of both trials with FPdC was significantly higher than that commonly reported in the literature for conventional spontaneous AF.

Furthermore, the number of *S. cerevisiae* strains estimated in the trials A and B was significantly higher than that of trial C. In both years, the strains selected during the preparation of FPdC dominated the microbial population of the AF and wine ageing. Thus, the use of FPdC could represent an innovative strategy to start the AF with a significant number of *S. cerevisiae* strains.

Furthermore, no *S. cerevisiae* strain was found in common between the two vintages. *Saccharomyces cerevisiae* species, however, are commonly recognised as the main technological yeast able to ferment must. Thus, its dominance during winemaking could represent a guarantee of quality for the final product both in microbiological and chemical terms. In fact, the presence of several *S. cerevisiae* strains during winemaking is reported to improve the complexity of the sensory profile of wine (Fleet 2003).

Our work also described, for the first time, the effect of the microbial population on composition of wines prepared by FPdC. Yeast activity was clearly observed by sugar consumption and ethanol production by day 7 of AF. The VA concentration of trial A and B was observed to be low probably due to the presence of yeast strains producing a low concentration of acetic acid. A high concentration of acetic acid in wine is responsible for the generation of the off-flavours and makes the product unmarketable. Wines prepared by spontaneous fermentation are frequently characterised by high VA concentration due to the proliferation of spoilage yeasts (Wang and Liu 2013).

The impact of LAB activity on wine composition was also revealed by the results of the chemical analysis. Lactic acid bacteria reached detectable levels during wine ageing, which agrees with the data reported in literature (Bae et al. 2006, Yanagida et al. 2008, Francesca et al. 2010); during this phase the degradation of malic acid by MLF is clearly shown (Francesca et al. 2014b). The production of lactic acid is desired in terms of the sensory profile of red wines because it contributes positively to fullness and roundness sensations (Nurgel and Pickering 2005). Also, these data showed that the use of FPdC in vinification could improve the quality of wine because of the activity of yeasts and LAB.

Conclusions

Our work provided an overview on the microbial ecology of wines prepared by using the FPdC, as well as on the spontaneous AF associated with the pied de cuve method. The addition of ethanol into pied de cuve, before the beginning of the spontaneous AF, favoured the development of several *S. cerevisiae* strains during the vinification process. The high strain diversity of the *S. cerevisiae* population, as well as its annual variability, may affect positively the quality of final wine.

All aspects of the composition of the experimental wines were in agreement with those reported for the production regulations of commercial wines, and undesired off-odours and off-flavours were not detected. In addition, data obtained by VOCs and sensory analysis showed that wines produced by FPdC, in particular that of trial A, were characterised by the highest scores of sensory intensity and complexity. Thus, an addition of wine to must of 1.5% (v/v) ethanol is suggested for pied de cuve preparation. Although this work was undertaken in triplicate and during two consecutive vintages, further investigations in other cellars and with other grape cultivars are being prepared to extend the knowledge of the effect of FPdC on wine composition.

Acknowledgements

The authors wish to thank Dr Ciro Sannino and Dr Alida Milazzo for their significant support with the microbiological and chemical analyses; Dr Vito Falco [Centro per l'Innovazione della Filiera Vitivinicola E. del Giudice, Marsala (TP)] and Mr Franco Rallo [Agrichimica S.n.c. di Rallo Francesco & C, Marsala (TP)] for their oenological support during the winemaking process.

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Manuscript received: 26 September 2014
Revised manuscript received: 10 February 2015
Accepted: 22 February 2015

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site: http://onlinelibrary.wiley.com/doi/10.1111/ajgw.12166/abstract

Table S1. Microbial load of samples collected during the pied de cuve preparation and winemaking process of Nero d'Avola wines during the 2011 vintage.

Table S2. Microbial load of samples collected during the pied de cuve preparation and winemaking process of Nero d'Avola wines during the 2012 vintage.

Table S3. Chemical parameters during the winemaking process of Nero d'Avola wines during the 2011 vintage.

Table S4. Chemical parameters during the winemaking process of Nero d'Avola wines during the 2012 vintage.

Table S5. Concentration of volatile organic compounds during the winemaking process of Nero d'Avola wines during the 2011 vintage.

Table S6. Concentration of volatile organic compounds during the winemaking process of Nero d'Avola wines during the 2012 vintage.