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SHORT COMMUNICATION

Aglianico and Fiano wines obtained with an autochthonous non-*Saccharomyces* yeast

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Abstract Non-Saccharomyces yeasts are microorganisms that play a prominent role in the fermentation dynamics, composition and flavour of wine. The principal aromatic compounds responsible for varietal aroma in wine are terpenes; of these, the monoterpenes represent the oenologically most important group in terms of volatility and odour, if they are present in a free form. The glycosidically bound forms can be converted into compounds with free odours by hydrolysis with the glycosidases produced by yeasts. We performed a screening of non-Saccharomyces yeasts present in the grapes and must of Aglianico and Fiano cultivars typical of South Italy (Irpinia), which have a high extra-cellular glycosidase activity. Among the species analysed was a strain belonging to *Rhodotorula* spp. that showed the highest glycosidase activity, an increased free terpene fraction and, simultaneously, little modification of the bouquet. The isolated yeast was subsequently utilized for experimental winemaking processes of Aglianico and Fiano wines. The results demonstrated that the obtained wines had a more intense floral aroma and some sweet and ripened fruit notes.

Keywords Non-Saccharomyces yeasts · cv. Aglianico · cv. Fiano · Varietal wine aroma · Aromatic compounds

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Introduction

Wine fermentation is a complex microbiological reaction involving the sequential development of various yeast strains that contribute to the flavour of wines. For many years, wines have been produced by natural fermentation carried out by yeasts that originate from both the grapes and the cellar. Several oxidative and apiculate yeasts (non-*Saccharomyces*) are predominant on the surface of grapes but after 3–4 days of fermentation, *Saccharomyces cerevisiae* "sensu stricto" predominates, subsequently playing the major role in alcoholic fermentation (Jackson 1994; Fleet 2003).

Nevertheless, the non-*Saccharomyces* yeasts do have a prominent role in determining the fermentation dynamics, composition and flavour of wine. The principal aromatic compounds responsible for varietal aroma of wine are the terpenes (others are nor-isoprenoids, C6 alcohols, aromatic compounds), which are metabolites derived from mevalonic acid and characterized by multiples of branched, five-carbon units resembling isoprene. The most important group, from an oenological point of view, is the monoterpenes (10-carbon compounds) because of their volatility and odour—if present in a free form. The glycosidically bound forms can be converted into the free-odour compounds by hydrolysis with glycosidases produced by the yeasts (Palmeri and Spagna 2007).

Among the several non-*Saccharomyces* yeasts obtained from grapes and must of Aglianico and Fiano cultivars typical of South Italy (Irpinia), we have isolated a strain that is characterized by a high extra-cellular β-glucosidase activity. This yeast was identified by morphological and biochemical methods as *Rhodotorula* spp., and it has been utilized for experimental winemaking processes to produce Aglianico and Fiano wines.

The purpose of the study reported here was to evaluate the effect of *Rhodotorula* spp. on the fermentations of Aglianico and Fiano musts and wines through the monitoring of oenological parameters and aromatic profiles.

Materials and methods

All chemicals used were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

Grape sampling, yeast isolation and identification

Grapes were sampled in vineyards located in South Italy near Avellino. The Aglianico vineyard is situated in the "Cappuccini" area, on a hilly terrain at 350 m a.s.l., with a south-west exposure. The soil has a sandy and silty texture, and the terrain has a moderately coarse, well-drained and deep soil skeleton. The density is 3,000 vines per hectare, and the simple Guyot farming system is employed. The Fiano vineyard is situated at "Le Torrette", a location close to that of Aglianico, on a very similar type of soil, albeit with a southwest–west exposure; the stocking density and cultivation methods are the same as those of the Aglianico vineyard.

The bunches were harvested from several plants within specific sub-areas, and the berries were then randomly collected and crushed in sterile bags. Samples (10 g) were blended in 90 ml Ringer solution (Oxoid, Hampshire, UK) in a stomacher (Laboratory Blender Stomacher 400; Seward Medical, London, UK) for 30 s at 230 rpm.

The samples were serially diluted, and 0.1 ml of each dilution was spread onto plates of different media (Oxoid, Hampshire, UK): YPD (that allows the growth of yeasts, moulds and bacteria), WL (a complete medium in which the colonies develop a specific color allowing an initial differentiation) and lysine medium (a selective medium that does not allow the growth of the *Saccharomyces* spp. genus). The plates were incubated at 28°C for 5 days and, after viable counts, the morphologically different colonies from the different media were purified by repetitive streaking on YPD-agar. All yeast isolates were preserved on YPD-agar slants, stored at 4°C and subcultured every 3 months.

The yeasts were grouped according to colony colour and morphology on WL, as well as by their microscopic and biochemical characteristics.

The API 20 C AUX kit (Bio-Merieux SA, Marcy l'Etoile, France), was used for yeast identification on the basis of the ability of the microorganism to metabolize specific substrates represented by different types of sugars. Microorganism identification was achieved at the genus and species level by the interpretation of an analytical index provided by the manufacturer.

In order to unambiguously identify the yeasts at the species level, we sequenced the 26 S rDNA gene D1/D2 region consisting of a 600-bp fragment by PCR using yeast colonies grown on YPD-agar plates. The commonly used standard primers, namely, NL1 and NL4 (Esteve-Zarzoso et al. 2000; Arroyo-Lòpez et al. 2006), have the following sequences: 5′-GCATATCAATAAGCGGAGGAAAAG-3′ and 5′-GGTCCGTGTTTCAAGACGG-3′, respectively. Sequencing of the PCR products was carried out using the Sanger method with a 3730 Genetic Analyzer DNA Sequencer (Applied Biosystems, Foster City, CA).

Technological cycles of wine production

Aglianico grapes (300 kg) were harvested at 22.6 °Brix, destemmed and crushed. The must was treated with 10 g hl^{-1} of potassium metabisulfite (MBK) and, for the control fermentations, inoculated with 20 g hl^{-1} (corresponding to about 7×10^6 cells ml^{-1}) commercial *Saccharomyces cerevisiae* starter yeast, Oenoferm Structure (Erbslöh Geisenheim AG, Geisenheim, Germany), previously activated in warm water for 20 min.

For experimental fermentations with the *Rhodotorula* spp. WLR12, the tanks were initially inoculated with 2×10^6 cells ml^{-1} of the selected indigenous yeast and 20 g hl^{-1} of the commercial yeast starter. Duplicate fermentations were carried out in 70-l stainless steel tanks at 26–28°C, and several parameters, including content of reducing sugars, total acidity, pH and acetic acid concentration, were monitored.

Maceration lasted 12–18 days with a regular pumping over and punching down of the skins three times per day. Upon completion of the alcoholic fermentation process, the must was racked and pressed in a pneumatic press, giving approximately 45 l of wine, which was then left to settle spontaneously for 3-4 days at 18°C in a stainless steel tank, prior to the addition of 6 g hl^{-1} MBK for 10 days in an air-free environment. The wine was cold settled for 4 months at 10°C.

Fiano grapes (300 kg) were harvested at full maturation (21.5 °Brix), destemmed and crushed. After pressing, 2 g hl^{-1} of pectic enzymes (Trenolin Opti DF; AG Erbslöh Geisenheim) and 10 g hl^{-1} of MBK were added. The must

was immediately cooled to 10°C and submitted to static decantation for 24 h. For control fermentations, inoculations were carried out by adding 20 g hl^{-1} (corresponding to about 6×10^6 cells ml^{-1}) of commercial *Saccharomyces bayanus*, Oenoferm Freddo (AG Erbslöh Geisenheim) rehydratated in warm water for 20 min, as described by the manufacturer. For the experimental fermentations with the *Rhodotorula* spp. WLR12 strain, the tanks were initially inoculated with 2×10^6 cells ml^{-1} of the selected indigenous yeast and 20 g hl^{-1} of the commercial yeast starter. Duplicate fermentations were carried out in 70-l stainless steel tanks at 10–12°C, and several parameters, including reducing sugars, total acidity, pH and acetic acid concentration, were monitored.

After 4 days, musts were aired and 15 g hl^{-1} of activators (VitaDrive, Combi Vitamon; Erbslöh AG Geisenheim) were added. Upon completion of alcoholic fermentation, an additional 6 g hl^{-1} of MBK was added and the wine cold stabilized for a 3-month period at 10°C.

Sugar analysis

Sugars were evaluated by densitometric analysis using a Babo mustimeter. A sample of must or wine was placed in a glass cylinder and the densitometric apparatus slowly immersed in the solution. The value on the scale at the flotation level enables evaluation of the sugar content (w/w), i.e., scale value × density = sugar content (w/v).

Determination of alcohol content

The alcohol content of all the wines was determined using a Malligand ebulliometer (Tecnolab; Belpasso, Italy).

Acetic acid and total acidity analysis

Acetic acid was determined by an enzymatic method kit (Paramedical Srl, Pontecagnano, Italy). Briefly, in the presence of acetic acid, ATP and NAD^+, citrate synthase and acetyl-CoA synthetase enzymes, through a coupled reaction, produce NADH. The concentration of NADH can then measured by absorbance at 340 nm. Total acidity was measured using a titrimetric method.

Total polyphenol analysis

Total polyphenol content of wines was determined using the Folin-Ciocalteu reagent (Di Stefano et al. 1989). The reaction mixtures contained 1 ml of wine sample (adequately diluted in distilled water), 250 µl of carbonate-tartrate solution (200 g l^{-1} Na_2CO_3, 12 g l^{-1} $Na_2C_4H_4O_6 \cdot 2H_2O$ in water) and 25 µl of Folin–Ciocalteu's reagent. After a 30-min reaction, the absorbance was measured at 720 nm, and the total polyphenol concentration was calculated from a calibration curve using gallic acid as the standard. The results were expressed as milligrams of gallic acid equivalents (GAE) l^{-1}.

Total antioxidant capacity of wine

The antioxidant capacity of wines was evaluated according to a modified method based on the ferric reducing antioxidant power (FRAP) assay (Arnous et al. 2002; Makris et al. 2007) that measures the ability of the antioxidants contained in a sample to reduce ferric-tripyridyltriazine (Fe^{3+}-TPTZ) to a ferrous form (Fe^{2+}) that absorbs light at 594 nm (Benzie and Strain 1996). Wine (0.05 ml), diluted 1:10 with distilled water, and 0.05 ml of ferric chloride (3 mM in 5 mM citric acid) were mixed thoroughly and incubated for 30 min in a water bath at 37°C. The mixture was then added to 0.90 ml of 1 mM TPTZ solution in 0.05 N HCl and, after 10 min, the absorbance was read at 620 nm. Total antioxidant capacity of the samples was calculated from a calibration curve established utilizing known amounts of ascorbic acid. The results were expressed as ascorbic acid equivalents (AAE mM).

HS-SPME-GC wine aroma analysis

Prior to analysis, wine samples were cooled to 4°C to minimize the loss of highly volatile compounds. About 20 ml of each wine was placed into a 100-ml glass vial containing 3 g of NaCl (saturation level) and 0.5 µl isooctane as an internal standard. The vials were subsequently sealed with PTFE-silicone septa (Supelco, Bellefonte, PA) and mixed by magnetic stirring. Each analysis was undertaken in duplicate using different vials. Polydimethylsiloxane (PDMS; 100 mm) (Arthur and Pawliszyn 1990) was the fiber used for the extraction of the volatile components, in the headspace (HS) condition. The sample vials were equilibrated for 30 min at 40°C in a thermostated bath followed by fibre exposure to the HS for 20 min. The exposure was performed by inserting the stainless steel needle through the vial septum and pushing the fiber into the sample HS to collect the analytes. The fiber was then drawn into the needle and the solid phase microextraction (SPME) device removed from the vial and inserted into the injection port of the gas chromatography (GC) for thermal desorption. The analytes from the fiber removal procedure was carried out in the splitless mode at 240°C for 5 min. During the injection process, the fiber was maintained for 10 min in splitless mode.

All aroma standards were purchased from Sigma (St. Louis, MO) and were of the highest purity available. The analyses were performed using a Agilent 6890 GC coupled with an Agilent 5973 mass spectrometer (MS) (Agilent

Table 1 Values of oenological parameters of the Aglianico fermentations

Starter	Fermentation	Residual sugars (g l^{-1})	Total acidity (g l^{-1})	pH	Alcohol volume %	Acetic acid (g l^{-1})
Commercial yeast	Start	nd	12.50	3.05	nd	nd
	End	1.70	7.61	3.34	13.60	0.30
Commercial yeast+ WLR12	Start	nd	11.10	3.01	nd	0.30
	End	2.40	8.36	3.23	13.00	0.33

nd, Not determined

All data are the means of duplicate fermentations, each analyzed in duplicate

Technologies, Santa Clara, CA) and a DB-WAXetr column (id: 30×0.25 mm ; film thickness: 0.25 μm) (J & W Scientific, Folsom, CA). Helium was used as the carrier gas, with a flow rate of 1.5 ml min^{-1}, an injector temperature of 250°C and an oven temperature scheduled from 40°C (held for 6 min) to 180°C at 5°C min^{-1} (held for 3 min), then at 7°C min^{-1} to 240°C (held for 5 min). Detection was by MS on the total ion current obtained by electron impact at 70 eV, and the masses were scanned from 29 to 300 m/z. The components were identified by comparison with the spectra of the National Institute of Standards and Technology and Wiley library (Cabredo-Pinillos et al. 2004; Genovés et al. 2005; Genovese et al. 2007).

Statistical analysis

Statistical analyses were carried out using the Microsoft Excel software package (Microsoft Corp, Redmont, WA)

Results and discussion

In order to assess the influence of indigenous yeasts on industrial and biochemical parameters of Aglianico and Fiano wines, we performed several fermentations using commercial yeasts in monoculture and in combination with the isolated non-*Saccharomyces* yeast *Rhodotorula* spp. WLR12, which had been identified by morphological and biochemical analysis and by the sequence of the 26 S rDNA D1/D2 region. This non-*Saccharomyces* yeast was chosen for its unique extra-cellular β-glucosidase activity and good fermentative potential. The fermentations of Aglianico and Fiano were carried out in duplicate. The different combinations allowed us to study the interaction among these two strains and their influence on the final quality of the wine (Fleet, 2003). Interesting data were obtained with both fermentations.

The fermentation process of the Aglianico wine was particularly tumultuous using the *Rhodotorula* spp. WLR12 strain in combination with commercial yeasts, with the fermentation completed in only 12 days from start to finish. The final values of sugar content, total acidity, pH and alcohol volume remained constant throughout the fermentation period. Conversely, the control fermentation carried out utilizing only the commercial strain *S. cerevisiae*, Oenoferm Freddo had a normal course and ended after 18 days from the start. Analysis of the main oenological parameters (Table 1) of the two fermentations of Aglianico wine showed that both the control and experimental processes reached completion, based on a residual sugar content of about 2 g l^{-1}.

The results of the winemaking process also demonstrated a good compatibility between WLR12 and the commercial starter. It has been reported that the use of a mixed culture, due to nutrient depletion, secretion of killer toxins or production of lytic enzymes, may block the fermentation process (Pérez-Nevado et al. 2006). Total acidity was higher in the WLR12 mixed fermentation than in the control process, and the volumetric alcohol value was only slightly lower in the mixed fermentation than in the control one.

The mixed fermentation with WLR12 also differed from the control in having a higher quantity of lees on the bottom

Table 2 Values of the oenological parameters of the Fiano fermentations

Starter	Time	Residual sugars (g l^{-1})	Total acidity (g l^{-1})	pH	Alcohol volume %	Acetic acid (g l^{-1})
Commercial yeast	Start	nd	8.10	3.36	nd	nd
	End	1.50	5.50	3.70	12.65	0.11
Commercial yeast + WLR12	Start	nd	7.85	3.35	nd	nd
	End	2.0	7.50	3.26	12.40	0.38

All data are the means of duplicate fermentations, each analyzed in duplicate

of the fermentation tanks. This is an important feature because at the end of fermentation the wine is opalescent or turbid, and it is necessary to prevent extended contact with suspended particles (yeast, mucilage, curdled protein, bacteria) in order to avoid any alteration in the wine's taste or aroma and an increase in volatile acidity. To this end, immediate separation of these substances is critical, and fining agents (gelatine, bentonite, casein, egg albumin, carbon, sparkolloids, silica sol and arabic gum) have to be used. Natural precipitation of these compounds is slow and takes many months, but some yeasts belonging to the genus *Rhodotorula* are able to secrete polysaccharides similar to sparkolloids and arabic gum (Takita et al. 2001). The *Rhodotorula* spp. WLR12 is probably able to produce certain polysaccharides (not yet characterized), which could account for the precipitation of lees and act as fining agents on the suspended particles present in wine during fermentation.

The results were different in Fiano, where the fermentation with *Rhodotorula* spp. WLR12 resembled that of the control, and the duration of the winemaking process was the same in both processes. Both fermentations reached completion when the residual sugar value was around 2 g l^{-1} (Table 2).

The experimental Fiano wine obtained in the mixed fermentation with *Rhodotorula* spp. WLR12 had a higher total acidity and a lower alcoholic grade than the control, but the increase in total acidity was higher than that in Aglianico. In addition, during the fermentation of Fiano with WLR12, the pH value decreased significantly compared to the control where, in contrast, the pH increased.

We also determined polyphenol content and antioxidant activity in the experimental wines obtained. The results are shown in Table 3. Total polyphenol content was higher in the mixed fermentation with the *Rhodotorula* spp. WLR12 strain than in the control, where only commercial *S. cerevisiae* was used.

Increases in polyphenol content and antioxidant activity were observed in both the Aglianico and in Fiano mixed fermentations performed with the WLR12 strain. The presence of a higher polyphenol concentration provides an additional health benefit to wines as these compounds have been shown to be effective against reactive oxygen species, which are compounds shown to have a beneficial effect on cardiovascular diseases, certain types of cancer, neurological disorders, chronic kidney disease, inflammation hypertension and diabetes. The high polyphenol content was accompanied by a higher antioxidant activity in the experimental wines with *Rhodotorula* spp. WLR12.

The volatile fractions of the Aglianico and Fiano wines, obtained both with the autochthonous strain of *Rhodotorula* spp. WLR12 and with the commercial *S. cerevisiae* strain as control, were characterized by several components (Tables 4 and 5). A comparison of Tables 4 and 5 reveals differing concentrations of a number of compounds in the

Table 3 Polyphenol content and antioxidant activity in wines

Wine	Polyphenols[a]		Antioxidant activity[b]	
	Control	WLR-12	Control	WLR-12
Aglianico	2.04±0.04	2.36±0.04	16.2±0.4	18.4±0.1
Fiano	0.27±0.004	0.30±0.01	0.44±0.01	0.55±0.01

All data are the means ± standard error of triplicate analyses

[a]Values are expressed as mg l^{-1} of gallic acid equivalents (GAE)

[b]Values are expressed as mmol l^{-1} ascorbic acid equivalents (AAE)

Table 4 Concentration of volatile compounds identified in Aglianico wine

Compound	Concentration (μg l^{-1})		
	Grape	Control	WLR-12
Terpinolene	nf	nf	1.1±0.2
Nerol	1.5±0.2	3.5±0.3	6.5±0.7
Vitispirane	nf	1.3±0.2	1.9±0.3
α-Ionone	1.2±0.3	2.4±0.3	4.5±0.4
β-Damascenone	5.4±1.1	2.6±0.4	12.1±1.7
Geraniol	2.6±0.4	4.7±0.5	7.6±0.7
Citronellol	3.1±0.4	4.2±0.7	25.7±1.9
Linalool	6.3±1.2	9.6±0.7	31.1±1.5
α-Terpineol	7.4±1.6	14.9±1.5	63.2±3.2
Σ Terpenes	27.50	43.20	153.70

nf, Not found

All data are the means ± standard error of triplicate analyses

Table 5 Concentration of volatile compounds identified in Fiano wine

Compound	Concentration (μg l^{-1})		
	Grape	Control	WLR-12
para-Cymene	nf	1.0±0.2	6.9±1.1
Terpinolene	nf	nf	1.5±0.2
Nerol	2.0±0.3	3.2±0.4	8.4±1.3
β-Myrcene	nf	1.1±0.2	2.1±0.4
β-damascenone	1.1±0.2	1.9±0.2	2.8±0.4
Geraniol	4.6±0.6	4.5±0.5	9.7±1.3
Citronellol	5.2±1.0	8.2±1.4	29.8±2.4
Linalool	6.1±0.9	15.7±1.8	49.5±2.1
α-Terpineol	1.4±0.3	13.7±1.3	119±6.2
Σ terpenes	20.40	49.30	229.70

All data are the means ± standard error of triplicate analyses

Fiano and Aglianico wine samples, particularly for nor-isoprenoid molecules in the grape and wine samples. It is noteworthy that there is a general and important enrichment in the volatile fraction of wines obtained with WLR12 compared to the control wine. In the Aglianico wine obtained with *Rhodotorula* spp. WLR12, the content of terpenes increased by approximately threefold relative to the control; in the Fiano wine, the increase was more than fourfold.

The levels of nor-isoprenoid compounds in the controls werre similar to those in the Aglianico and Fiano experimental wines, with the predominant compounds being linalool and α-terpineol. In the experimental wines obtained with *Rhodotorula* spp. WLR12, the most abundant terpenes were β-damascenone citronellol, linalool and α-terpineol in the Aglianico wine and nerol, geraniol, linalool, citronellol and α-terpineol in the Fiano wine. The most abundant terpene in both wines was α-terpineol, which accounted for about one half of the total volatile compounds in terms of quantity.

Conclusion

The aromatic profile of a wine consists of a qualitatively and quantitatively complex chemical pattern. Flavour production is affected by several factors, such as soil, climate, vines, ripeness, yeast strain, winemaking process and ageing effect. More than 1,000 compounds have been identified as contributors to wine aroma, including a wide range of compound classes, such as hydrocarbons, alcohols, esters, aldehydes, ketones, acids, ethers, lactones, sulphur and nitrogen compounds. In recent years, a new group of compounds, the nor-isoprenoids, have been object of significant interest due to their substantial contribution to wine aroma, despite the fact that they are present at very low concentrations.

The purpose of our study was to evaluate the influence of the yeast strain *Rhodotorula* spp. (WLR12) on the kinetics of must fermentation and on the corresponding aromatic profiles of Aglianico and Fiano wines by monitoring the whole fermentation process. To this end, we performed a trial wine production in the experimental cellar to evaluate the performance of this interesting non-*Saccharomyces* yeast strain and its potential application in wine industry. We observed that the fermentation process using a mixed starter in the Aglianico samples was characterized by an extremely rapid fermentation relative to the corresponding control fermentation, inoculated only with *S. cerevisiae.*

Based on the initial organoleptic analysis, the Fiano wine was considered to be characterized by a marked acidic taste and an intense, aromatic and pleasant flavour, rich of grapefruit and fruity notes, while the Aglianico was considered to be an astringent and acidic young wine, characterized by an intense flavour rich in cherry, viola and berries notes. These properties enhanced the flavour and taste of both wines when compared with their counterparts produced with conventional starter cultures. The HS–SPME technique allowed the detection of trace aroma compounds involved in the characterization of the qualitative aroma profile of the wines under study. In conclusion, we believe that the use of the *Rhodotorula* spp. yeast strain increases the amount of terpenes in the volatile fraction of Fiano and Aglianico wines, resulting in a flavour more intense than that of the control wines produced by monoculture fermentation.

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