

Effects of yeast inoculation on volatile compound production by grape marcs

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Abstract Grape marcs are a by-product of winemaking that can be used for production of Grappa, a traditional Italian spirit. This plant material needs to undergo a fermentation process before distillation to allow production of ethanol from residual sugar and volatile aromatic compounds. To study the possible effect of selected yeast inocula on the fermentation process and subsequent quality of the distillate, a laboratory-scale fermentation of grape marcs was performed. The inoculated yeasts proved to be good colonizers of grape pomace and contributed markedly to rapid sugar consumption, which was completed within 5 days. Fermentation products were analysed by gas-chromatography. A time course analysis performed to determine the impact of the inoculated strains on the main aromatic compounds that are concentrated in the product after distillation provided evidence of a positive effect of yeast inoculation.

Keywords Grappa · Fermentation · Gas chromatography · *Saccharomyces*

Introduction

Grappa is a traditional alcoholic beverage made in Italy (EEC 1989) by distillation of grape marc—a by-product of the winemaking process containing skins, seeds, and, in

some cases, stalks. After separation from must, grape pomace is usually pressed to drain out excess liquid and then delivered to the distillery. Sugars of marcs from red grapes are partially fermented to ethanol during the wine-making process, and such material can therefore be distilled within a few days. In the case of marcs from white grape varieties, complete fermentation occurs only following an appropriate period of storage, lasting from a few days to several weeks, inside concrete tanks, stainless steel containers or horizontal plastic tunnels that favour anaerobic conditions (Da Porto 2002). Sugar degradation and alcohol production are the main chemical processes occurring during this period, carried out mainly by the activity of yeasts. Nevertheless, a large number of other molecules with either positive or negative effects can develop from the activity of the indigenous microflora. To avoid off-flavours and production of toxic compounds from anomalous fermentation and bacterial spoilage that could affect the quality of the future distillate, distilleries have adopted technological approaches such as pH adjustment, temperature control, anaerobic environment setup and microbial population management, to avoid product spoilage. In most cases, bacterial proliferation is prevented by acidification treatment through addition of dilute sulphuric acid (Da Porto 2002). A seemingly promising approach for increasing spirit quality introduced recently is the inoculation of selected yeast strains into the marcs at the moment of storage; some producers inoculate pomace with commercial dried yeasts chosen from among those sold for winemaking. However, no studies on the exploitation of yeast strains isolated directly from grape marcs, which could possess traits peculiar to distillate production, have so far been reported.

This work represents the first report on the development of a yeast-guided fermentation process of grape pomace at laboratory scale. The effects of the yeast strain inocula were

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evaluated in terms of their ability to prevail on the resident microflora and to completely degrade sugars. A single gas-chromatographic run allowed determination of volatile compounds, both varietal and yeast-related, obtained from marc distillation. A first insight into the differences between the aromatic profile of inoculated and non-inoculated grape pomace and its evolution during storage is provided.

Materials and methods

Raw material

Prosecco grape marcs were collected, immediately after crushing, from the vineyards of the Oenological Institute "G.B. Cerletti" in Conegliano Veneto (Italy) and kept frozen until required.

Yeast strain growth and isolation conditions

Four strains of *Saccharomyces cerevisiae* were used to inoculate grape marcs, namely the commercial yeast strain FR95 from the product Blastosel FR95 (Perdomini, Italy) and the strains AR-1, NM-12 and TV-15, isolated from grape marcs of different origin. Yeasts were grown in YPD medium (yeast extract 10 g/l, peptone 10 g/l, dextrose 20 g/l) at 30°C for 24 h.

Grape marc fermentations were carried out in 100-ml Erlenmeyer flasks plugged with a one-hole stopper into which a J-shaped tube was inserted. Flasks were filled with 110 g grape marc, then heat treated for 20 min at 72°C in a water bath. Each flask was inoculated with 1×10^6 – 3×10^6 cells/g from 24-h cultures in YPD medium and incubated at 20°C for 8 days. Three separate replicates were performed.

For yeast isolation, 5 g grape pomace was collected from each flask, dispersed in 50 ml sterile PBS buffer (NaCl 8 g/l, KCl 0.2 g/l, Na_2HPO_4 1.44 g/l, KH_2PO_4 0.24 g/l) and, after appropriate decimal dilutions, plated on WL (Wallerstein Laboratory, Oxoid, Italy) medium containing 100 µg/ml chloramphenicol to contain bacterial growth and incubated at 30°C for 2 days.

Restriction analysis of mitochondrial DNA

Isolates from inoculated grape marcs were characterized by mitochondrial (mt)DNA analysis as described by Querol et al. (1992) with the following modification for yeast DNA extraction: a loopful of cells grown on YM plates was resuspended in 500 µl TE (50 mM Tris, 20 mM EDTA, pH 7.4) containing 0.3 g sterile glass beads (425–600 µm, Sigma, Milan, Italy). The suspension was vortexed vigorously for 3 min before adding 50 µl 10% SDS and incubating at 65°C for 30 min. After addition of 200 µl 5 M potassium

acetate, tubes were incubated on ice for 30 min and then centrifuged at 16,000 g for 5 min. The supernatant was transferred to sterile 1.5 µl Eppendorf tubes where 600 µl cold isopropanol were added. After incubation at room temperature for 5 min, tubes were centrifuged at 16,000 g for 10 min. The pellet was washed with 500 µl 70% ethanol, centrifuged for 5 min at 16,000 g, dried under vacuum at 37°C for 1 h and then resuspended in 50 µl sterile water. After addition of 1.5 µl RNase solution (10 mg/ml, Amersham Bioscience, Milan, Italy) and incubation at room temperature for 20 min, samples were stored at –20°C. Yeast DNA digestion was carried out in 15 µl volume using 300 ng DNA and 20 U *Hinf*I restriction endonuclease (Amersham Bioscience), which is known to have a large number of sites within yeast nuclear DNA and only a few in mtDNA (Querol et al. 1992). Restriction fragments were separated by 1% (w/v) agarose gel electrophoresis in 0.5× TBE (44.5 mM Tris-HCl, 44.5 mM boric acid, 1 mM EDTA, pH 8.3) and the resulting profiles were analysed with the pattern analysis software package GelComparII (<http://www.applied-maths.com/gelcompar/gelcompar.htm>), using the Dice coefficient (Dice 1945).

D1/D2 region sequencing

The D1/D2 domain was amplified using primers NL1 (5'-GCATAT CAATAA GCG GAG GAA AAG -3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G - 3') according to the protocol described by Kurtzman and Robnett (1998). Amplification products were checked for purity by agarose gel electrophoresis and then subjected to sequencing. Species were identified after BLASTN alignment (www.ncbi.nlm.nih.gov/BLAST) of the obtained sequences with those present in the GenBank public database. A minimum sequence similarity level of 98% was considered for taxonomic attribution.

Technological characteristics of selected yeasts

Fermentative vigour, in terms of the ability to start fermentation promptly, was determined routinely by the following method: yeast strains were inoculated at 1.5×10^6 cells/ml in flasks containing 100 ml synthetic must prepared as described by Delfini (1995) with 0.3 g/l $(\text{NH}_4)_2\text{SO}_4$, 0.3 g/l $(\text{NH}_4)_2\text{HPO}_4$, 200 g/l glucose, 0.2 g/l hydrolysed casein, 2 g/l malic acid, pH 3.5, and incubated without agitation at 25°C.

Fermentative vigour, in terms of the amount of glucose consumed after 48 h of incubation, was determined by measuring culture weight loss multiplied by the coefficient 2.118 (Delfini 1995). The level of variability among replicates, on the basis of hundreds of determinations performed, was always lower than 0.4 g/100 ml must. This value was therefore taken as a reference for experimental variation.

At the end of fermentations, a simple sensory evaluation was performed by a panel of five trained judges. Each panellist could give a positive, neutral or negative evaluation when the presence of fermentative aromas, no defect, or negative flavour (acetic acid and/or sulphur notes), respectively, were perceived. Each final score was expressed as the evaluation most frequently given by the panellists.

To evaluate hydrogen sulphide production, strains were grown on Biggy Agar medium according to Zambonelli (2003). After 48 h of incubation at 25°C, the intensity of the brown colour was considered proportional to H₂S production.

Sulphur dioxide resistance was determined in synthetic must at pH 3.2 with increasing SO₂ content. Potassium metabisulphite was added to obtain the following doses of SO₂: 0, 50, 100 and 200 mg/l. Cultures of 10⁷ cells/ml were used to inoculate 15-ml tubes containing synthetic must with different concentration of SO₂ to reach a final cell concentration of 10⁵ cells/ml. Strain resistance was evaluated by measuring the culture optical density at 600 nm after 48 h of incubation at 25°C.

Screening of the β -glucosidase activity of whole cells was carried out using 4-methylumbelliferil- β -D-glucopyranoside (4-MUG) as substrate in black 96-well microtiter plates (Greiner Bio-One, Frickenhausen, Germany), with flat transparent bottom (200- μ l capacity), following the method reported by Fia et al. (2005) with some modifications. A 24-h-old culture on YPD was used to inoculate 10 ml synthetic must at pH 3.0, with a cell concentration of 2×10^6 cell/ml. After a 24 h incubation the test was performed in triplicate, at pH 3.5 in 0.05 mol/l sodium acetate buffer. The fluorescence emitted by 4-methylumbelliferone (4-MU) was detected using a spectrofluorimeter (Spectrafluor Tecan, Salzburg, Austria). To establish a relationship between fluorescence values emitted by 4-MU and enzymatic activity, a calibration curve was constructed by using increasing concentration of the reaction product 4-MU (0, 0.1, 1, 5, 10 and 50 μ M), prepared in sodium acetate.

Distillation of grape pomace and aromatic compound concentration

Volatile fermentation compounds were extracted from 100 g grape marc by distillation under current steam with Cazenave equipment up to a distillate volume of 40 ml. Aliquots of 20 ml from each distillate were added to 8 g ammonium sulphate and extracted with 7.5 ml dichloromethane.

Determination of sugar and alcohol content

Sugar content of marcs was determined by Fehling titration of aqueous extracts from pomace residues after distillation (De Rosa and Castagner 1994). Three independent

measurements were performed for each sample. Alcohol content of the distillate was determined with a commercial kit (Boehringer Mannheim/R-Biopharm, Mannheim, Germany).

Gas chromatography

Volatile and fermentation compounds produced in Prosecco must were determined in triplicate by gas-chromatography. External standard solutions A and B were used and chemicals were purchased from Sigma-Aldrich and Fluka (Milano, Italy) with a purity higher than 98%. Solution A was prepared in water by adding methanol, acetaldehyde, n-propanol, n-butanol, isobutanol, isoamyl alcohol, β -phenylethanol, 2-butanol, acetic acid, benzaldehyde, hexanal, trans-2-hexenal, and acetoin. Solution B was prepared in absolute ethanol and contained: ethyl acetate, isoamyl acetate, phenylacetate, ethyl caproate, ethyl caprylate, geraniol, linalol, nerol, and β -damascon. 2-Octanol was used as internal standard at 6 mg/ml concentration in ethanol. The analytical system used was a Trace GC2000 Thermo Finnigan gas chromatograph equipped with a fused silica capillary column Varian CP WAX 57 CB, and a split-splitless automatic injector coupled with a FID detector. Injector temperature 250°C; volume injected 2 μ l; flux of helium carrier gas of 1.8 ml/min. Oven temperature program: 5 min at 35°C, 2°C/min to 68°C, 2 min at 68°C, 5°C/min to 100°C, 2 min at 100°C, 3°C/min to 140°C, 2 min at 140°C, 5°C/min to 200°C, 5 min at 200°C, 8°C/min to 250°C, 1 min at 250°C.

Identification and quantitative analyses of the peaks of volatile compounds in the samples analysed were made by comparison of their retention time with those of pure standards and internal standards.

Results and discussion

Technological characteristics of selected yeasts

For grape inoculation, three yeast strains were chosen from a collection of 104 strains isolated from fermenting marcs in different years and storage conditions, together with the commercial *S. cerevisiae* strain FR95, added as a control. The yeasts were isolated during the ensilage period of grape marcs of different origin (from Moscato or Prosecco grape varieties), or marcs subjected to different treatments (e.g. acidification). All strains from the collection were characterised by mtDNA restriction analysis at strain level and subsequently evaluated for their technological properties by growing in synthetic must. Yeast strain AR-1 was isolated from Prosecco acidified grape marc, NM-12 from Moscato pomace and TV-15 from a pool of grape pomace of different varieties that underwent high temperature stress (35–40°C) during storage. All yeasts chosen were identified

genetically as *S. cerevisiae* by sequence analysis of the D1/D2 domains of 26 S rDNA (Kurtzman and Robnett 1998). Some oenological properties of the three selected strains are reported in Table 1. Although the commercial strains FR95 and NM-12 had higher fermentation vigour (sugar consumption during the first 48 h of fermentation), strain AR-1 was able to complete sugar transformation 4 days earlier and, together with TV-15, showed a better ability to transform varietal aromas present in raw material, linked to its high values of β -glucosidase activity. Concerning hydrogen sulphide production, TV-15 showed the lowest level among the strains chosen.

Yeast-guided solid fermentations

Natural yeast and bacterial populations were present in grape marcs at the beginning of the storage period, from 10^5 to 10^6 CFU/g pomace, with the yeast genus *Hanseniaspora* being the most represented (Bovo et al. 2008). In order to avoid any influence of autochthonous microflora on the fermentation process, a thermal treatment for 20 min at 72°C of grape marcs was performed, allowing a reduction of 1–2 log units. Pasteurised grape marcs were then transferred to modified Erlenmeyer flasks, pressed to avoid the presence of air pockets and inoculated to obtain a yeast population of between 1 and 3×10^6 CFU/g pomace, similar to that for must. Part of the raw material was used to set up non-inoculated and non-pasteurised controls, with the aim of evaluating the contribution of natural yeasts to the aromatic profile obtained after distillation of fermented grape marcs.

Yeast dynamics and sugar consumption

At the beginning of fermentation (T0), and after 2 (T2), 5 (T5) and 8 (T8) days, quantitative analyses of yeast

population and reducing sugars concentration were performed (Fig. 1).

In both the inoculated grape pomace and in the untreated control, yeast populations reached stationary phase within 48 h, since no further increase in yeast concentration was observed after 2 days of incubation. Grape marcs inoculated with strains AR-1 and TV-15 reached the lowest yeast concentration, comparable to that of the control. Colony morphology on WL medium was characterised to detect the main yeast groups present at each sampling time (Cavazza et al. 1992). After 2 days of fermentation, the inoculated grape pomace contained a percentage of higher than 95% of colonies with typical *S. cerevisiae* morphology, i.e. white, light-green colour and smooth surface. This result was confirmed by mtDNA analysis on ten colonies collected from each inoculated sample. All the isolates analysed from marcs inoculated with AR-1 strain (Fig. 2), with NM-12, with TV-15 and with FR95 strains (data not shown) shared the electrophoretic profile of the inoculated strains, thus confirming their prevalence over the indigenous microflora. On the contrary, on WL medium control grape marcs contained, at the beginning of fermentation and after 2 days, 90% and 60% deep-green colonies, respectively, thus revealing a considerable presence of non-*Saccharomyces* yeast species.

The presence of 100% white colonies on WL medium after 5 and 8 days suggested the dominance of *Saccharomyces* strains at later timepoints. A remarkable decrease in sugar content occurred after just 2 days in all inoculated marc samples, indicating that fermentation was proceeding rapidly, as proven by complete sugar degradation within 5 days. In non-inoculated grape pomace, a slower fermentation dynamics was recorded, leading to sugar consumption after 8 days, linked to the slower development of the *S. cerevisiae* population.

Table 1 Oenological characteristics of yeast strains inoculated in grape pomaces for laboratory-scale fermentations. Fermentation performances were detected in synthetic must

Strain	Sugar transformation at 2 days ^a	Sugar transformation at 7 days ^a	Sugar transformation at the end of fermentation ^a	Days of fermentation	Sensory evaluation	H ₂ S production ^b	Resistance to SO ₂ 100 mg/l ^c	β -glucosidase ^d
NM-12	7.1	18.8	20.2	15	Positive	High	Sensitive	1.7±0.1
AR-1	6.3	17.4	20.0	11	Neutral	High	Resistant	3.7±1.4
TV-15	4.8	16.9	20.0	15	Neutral	Medium	Resistant	5.8±0.9
FR95	7.1	17.4	19.3	15	Positive	High	Resistant	Not detected

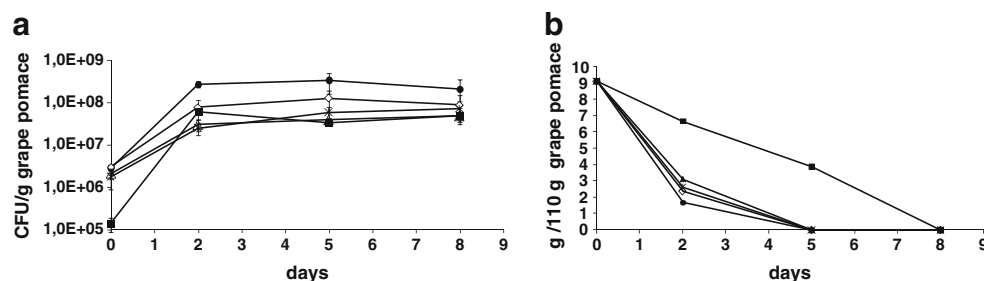
^a Expressed as g/100 ml synthetic must

^b Relative to the intensity of brown colour on Biggy agar medium

^c Determined in synthetic must on the basis of growth after 48 h in comparison with a control without sulphur dioxide by measuring the culture optical density at 600 nm

^d Expressed as μ mol of substrate consumed

Fig. 1 Dynamics of yeast population (a) and sugar consumption (b) in grape marcs. ■ Control heat-treated and non-inoculated; ● inoculated with strain FR95; ◇ inoculated with strain NM-12; ▽ inoculated with strain TV-15; * inoculated with strain AR-1



As regards pH, the initial value of unfermented grape pomace was 4.4 and no relevant variation was observed during the period of fermentation, with any decrease always comprising between 0.2 and 0.3 units.

Aromatic compounds produced during fermentation

After 2, 5 and 8 days of fermentation, inoculated and control grape marcs were submitted separately to distillation by current steam extraction. The resulting water–alcohol solutions had an ethanol content of approximately 10% (v/v), therefore a preliminary extraction step with organic solvent was necessary to concentrate volatile compounds. Previous studies have shown that different solvents may be used to efficiently extract the various molecules recovered from wine or distillate. Ortega et al. (2001) proved that dichloromethane was the most efficient solvent to resolve a wide range of compounds. In this work, the levels of the most important aromas formed during alcoholic fermentation (higher alcohols, esters, acetaldehyde, acetoin) and some varietal compounds commonly found in Grappa (hexanal, *trans*-2-hexenal, geraniol, linalool, nerol, β -damascone) were determined by gas chromatography.

Higher alcohols are secondary products derived mainly from yeast nitrogen metabolism, and are quantitatively the most abundant group of volatile compounds in distilled alcoholic drinks (Nykänen 1986), contributing significantly

to their sensory properties (Silva et al. 1996). Good fermenting yeasts, such as *S. cerevisiae*, generally produce high levels of secondary alcohols, whereas yeasts common in the vineyard, such as *Hanseniaspora uvarum*, usually do not (Romano et al. 2003). Figure 3 reports the kinetics of n-propanol, n-butanol, β -phenylethanol and isoamyl alcohol production during the storage period. Our data show that the total amount of fusel alcohols is higher in inoculated grape marcs, thus indicating the strong impact of the introduced yeasts. In all inoculated marcs, a relevant increase was observed after 5 days, when relevant changes in metabolism are likely to occur following sugar exhaustion. Strains TV-15 and FR95 stand out for the increase in n-butanol production and for the high concentration of β -phenylethanol after 8 days, respectively.

Esters constitute the most interesting class of flavour volatile compounds in alcoholic beverages because of their fruity and flowery notes (Nykänen 1986; Verstrepen et al. 2003). Concentrations of the main esters produced by yeasts, namely ethyl acetate (solvent-like aroma), isoamyl acetate (banana), phenylacetate (flowery), and medium-chain fatty acid ethyl esters such as ethyl caproate and ethyl caprylate (fruity), were determined (Fig. 4). Generally, an increase in concentration was observed with time, with the highest values reached after 8 days of storage. Inoculated grape marc generally showed greatest amounts with respect to the control, as observed for higher alcohols. As regards single esters, ethyl acetate, considered one of the most important esters because of its unpleasant flavour, seemed to increase slightly throughout the whole period, both in inoculated and in control marcs. On the contrary, the concentrations of isoamyl acetate, phenylacetate and ethyl caprylate increased strongly after 5 days of fermentation, as was also the case for higher alcohols production. Strain AR-1 is a good producer of ethyl caprylate, and FR95 releases the highest quantities of phenylacetate as well as its precursor β -phenylethanol.

In addition to the main fermentative aromas contributed by yeasts, some aromatic substances of plant origin that greatly affect Grappa flavour were investigated to verify the influence of yeast growth on their concentrations. Hexanal and *trans*-2-hexenal, two aldehydes that confer a herbaceous and fat note (Flamini 2005), were detected at low levels. Values ranging from 5 to 10 μ g/ml were found in all samples

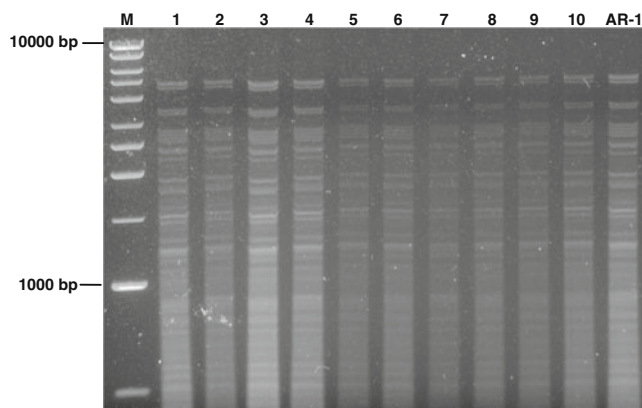
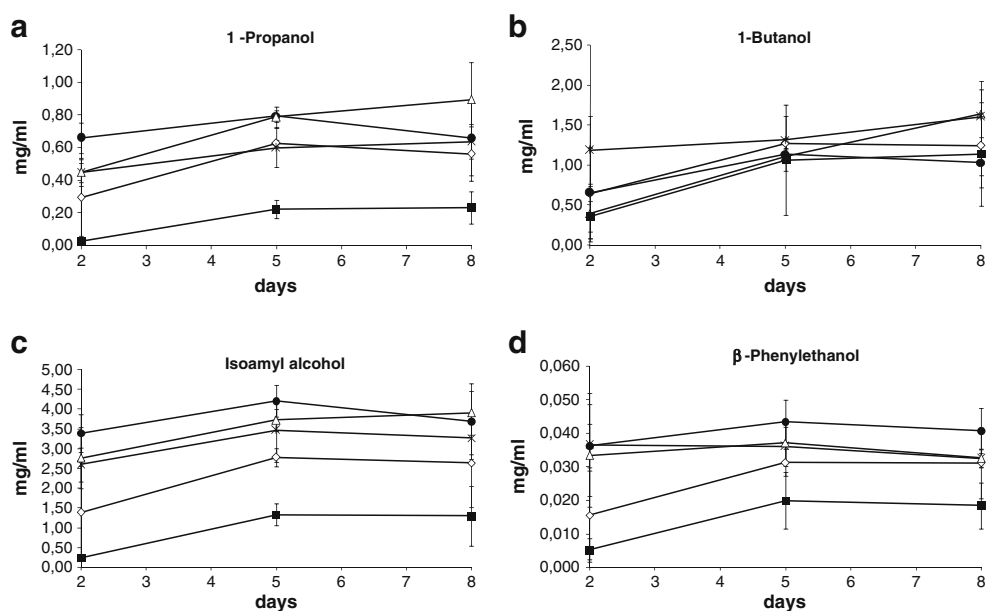


Fig. 2 Agarose gel electrophoresis of *Hinf*I mitochondrial DNA (mtDNA) analysis of isolates from marc fermentation inoculated with AR-1 strain after 2 days. Lanes: 1–10 Marc isolates, AR-1 AR-1 strain, M 1-kb molecular ladder (Amersham)

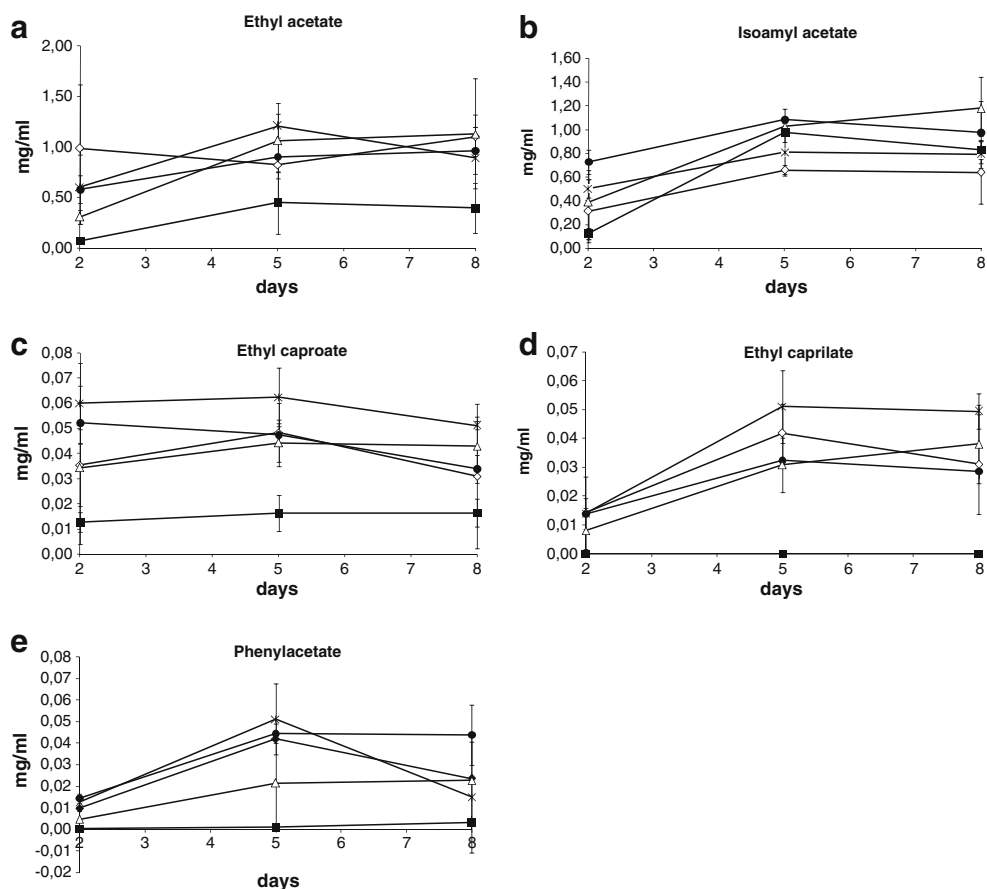
Fig. 3 Higher alcohols production during marc storage. **a** 1-Propanol, **b** 1-Butanol, **c** Isoamyl alcohol, **d** β -phenylethanol. ■ Control heat treated and non-inoculated; ● inoculated with strain FR95; ◇ inoculated with strain NM-12; △ inoculated with strain TV-15; * inoculated with strain AR-1



analysed after 8 days, and these values did not seem to be affected by the presence of inoculated yeasts. Moreover, some varietal grape compounds present in the Prosecco cultivar were considered: linalool, nerol and geraniol were

the most prevalent terpenols, and β -damascone was prevalent among the norisoprenoid compounds (Calò et al. 2001), with concentrations very close to the detection limit of gas-chromatography. In particular, yeast inoculation seems to

Fig. 4 Ester production during marc storage. **a** Ethyl acetate, **b** isoamyl acetate, **c** ethyl caproate, **d** ethyl caprilate, **e** phenylacetate. ■ Control heat-treated and non-inoculated; ● inoculated with strain FR95; ◇ inoculated with strain NM-12; △ inoculated with strain TV-15; * inoculated with strain AR-1



negatively influence the concentration of geraniol, which, after 8 days, was 3–4 $\mu\text{g/ml}$ in all inoculated grape marcs, while reaching 12 $\mu\text{g/ml}$ in the non-inoculated control.

Other molecules that are often detrimental to the quality of the spirit were taken into account. Acetaldehyde, which gives an unpleasant taste to the distillate if present at concentrations above 100–150 mg/100 ml anhydrous alcohol (Di Stefano and Ciolfi 1982), and which is generally ascribed to non-*Saccharomyces* yeast metabolism, was found in slightly higher concentration in control grape marcs, where a non-*Saccharomyces* microflora had developed during the first period of storage. In fact, its concentration in uninoculated grape pomace was 0.12 mg/ml after 5 days, while all the inoculated grape marcs showed values ranging from 0.05 to 0.07 mg/ml. A similar trend was observed for acetoin, highly produced by non-*Saccharomyces* yeasts such as *Hanseniaspora* (Romano et al. 1996). In the non inoculated control, a strong increase, up to 40 $\mu\text{g/ml}$ after 5 days, was observed when native microflora was still fermenting sugars into alcohol, while in the inoculated marc the level was 3–5 $\mu\text{g/ml}$ when the fermentative activity of inoculated yeasts was completed.

Methanol, which has well-known severe effects on human health, as its ingestion in large doses can produce blindness or even death, is generated by native plant pectin methyl esterases (Zocca et al. 2007). An increase in methanol concentration was detected during days 5–8 of storage: in this period, the concentration increased from 0.05 mg/ml to 0.20 and 0.30 mg/ml, respectively, in the case of NM-12 and FR95, the most vigorous strains, whose growth might have influenced the plant matrix, favouring conditions for plant pectin methyl esterase activity. Considering that the distillates contained 10% ethanol, the concentrations of acetaldehyde, methanol and

acetoin found were much lower than European and US legal limits (1 g per 100 ml of alcohol anhydrous, and 280 mg/100 ml of 40% alcohol, respectively). Figure 5 reports the kinetics of acetaldehyde, methanol and acetoin production during the storage period.

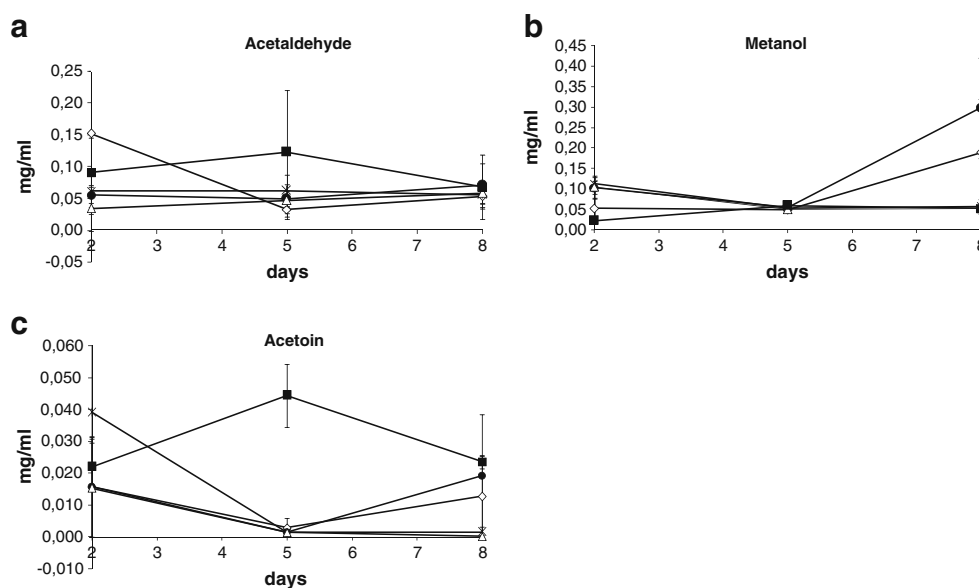
Finally, no relationship was found between yeast activity and the presence of 2-butanol, considered a marker of marc quality, since its presence appears linked to undesired side fermentations (Masino et al. 2009). We detected concentrations from 10 to 15 $\mu\text{g/ml}$, well below the European legal limit (EC regulation 1576/89; EEC 1989).

Conclusions

The results from this study demonstrate that inoculation of yeast strains has a great impact on fermentation of grape marc during storage. Firstly, as all yeast strains introduced were able to prevail over the autochthonous microflora, sugar consumption was faster than in the non-inoculated control, terminating fermentation at least 3 days earlier. Nevertheless, the main differences were found in the aroma profile drawn from gas chromatography analysis of the distillates. In fact, in the inoculate grape marc, a greater number of molecules of fermentative origin (higher alcohols and esters) were produced compared to the control. The only ester found at the same level in all marcs was isoamyl acetate, whose production is linked mainly to non-*Saccharomyces* yeasts, such as *Pichia* and *Hanseniaspora* (Plata et al. 2003; Rojas et al. 2001). In the case of uninoculated grape marc we demonstrated that, during the first 3 days of storage, yeast microflora comprised mostly non-*Saccharomyces* species.

Our results suggest the use of yeast inoculation not only for vinification but also for improving the quality of Grappa

Fig. 5 **a** Acetaldehyde, **b** methanol, and **c** acetoin production during marc storage. ■ Control heat-treated and non-inoculated; ● inoculated with strain FR95; ◇ inoculated with strain NM-12; △ inoculated with strain TV-15; * inoculated with strain AR-1



spirit. In such cases, yeast addition could lead not only to the increased development of aroma molecules, but also to the control of spoilage microorganisms that could greatly affect product quality.

Further studies are needed to better understand the role of yeast strains in emphasising differences in the quality of the distillate, even taking into account the prolonged incubations of marcs.

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