The making of Sicilian SO_2-free wines

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ABSTRACT

Sulphites are widely used as preservatives (for their antioxidant power) and as antimicrobial agents; however they can be a health risk factor. During the 2012 vintage we fermented Sicilian white (*cv* Grillo) and red (*cv* Nero d'Avola) grapes, using commercial *Saccharomyces cerevisiae* yeast strains, with and without SO_2. Care was taken to avoid (as much as possible) any contact of the musts (before) and of the wines (afterward) with oxygen; to this aim inert gases (nitrium, argon and CO_2) were utilized throughout. In both Grillo wines, after the cold static clarification, the amount of non-*Saccharomyces* yeasts and of lactic bacteria was markedly reduced (although less effectively in the SO_2-free wines). After inoculation and during alcoholic fermentation (AF) the ratio between *Saccharomyces* and non-*Saccharomyces* yeasts was always above 100. White sulphite-free wines underwent malolactic fermentation (MLF), unlike sulphite wines. During AF of Nero d'Avola wines, the ratio between *Saccharomyces* and non-*Saccharomyces* yeasts was always at least 20. At the end of MLF higher yeast concentrations were found in sulphite-free wines, while the concentration of lactic bacteria was similar. A slight reduction in total polyphenols was noted in sulphite-free wines of both *cv*. Duo-trio tests were performed and statistically significant differences were found by the assessors only between the two kind of white wines. However no preference emerged toward either product. Therefore our vinification protocol allowed us to produce wines without the use of sulphites, which were as appealing as those made with the use of SO_2.

RIASSUNTO

I solfiti sono usati ampiamente come agenti conservanti (dato il loro potere antiossidante) e antimicrobici; tuttavia possono costituire un fattore di rischio per la salute.

Durante la vendemmia del 2012, abbiamo condotto delle fermentazioni di uve bianche (*cv* Grillo) e nere (*cv* Nero d'Avola) inoculando ceppi di lievito *Saccharomyces cerevisiae* commerciali, con e senza l'aggiunta di solfiti. Particolari precauzioni sono state prese per limitare quanto più possibile il contatto dei mosti, prima, e dei vini, poi, con l'ossigeno; per questo nel corso di tutta la procedura sono stati utilizzati gas inerti (azoto, argon e anidride carbonica). Nei vini Grillo, dopo la chiarifica statica a freddo, la quantità di lieviti non-*Saccharomyces* e di batteri lattici si è ridotta nettamente (anche se in modo meno marcato in quelli senza solfiti). Comunque dopo l'inoculo e durante la fermentazione alcolica (FA) il rapporto tra lieviti *Saccharomyces* e non-*Saccharomyces* è stato sempre superiore a 100.

I vini senza solfiti sono andati incontro alla fermentazione malo-lattica (FML), mentre i secondi no. Durante la FA dei vini Nero d'Avola, il rapporto tra lieviti *Saccharomyces* e non-*Saccharomyces* è

stato sempre almeno 20. Alla fine della FML sono state trovate concentrazioni più alte di lieviti nei vini senza solfiti,mentre le concentrazioni di batteri lattici erano simili. Nel corso di test "duo-trio", sono state notate differenze statisticamente significative solo tra i due tipi di vini bianchi; tuttavia non è emersa una preferenza verso un tipo di vino o verso l'altro. Perciò, con l'impego del nostro protocollo di vinificazione, senza l'uso dei solfiti è stato possibile ottenere dei vini gradevoli tanto quanto quelli fatti con uso di solfiti.

INTRODUCTION

Small amounts of sulfites (under 10 mg/l) are normally present in wines, as natural by-product of the fermentation of grape sugars by yeasts. Extra amounts can however be added during wine-making: to clean equipment (*e.g*. to prevent *Brettanomyces* spoilage); to the uncrushed grapes, to prevent unwanted wild yeast strains and bacteria proliferation (this however can have large effects on grape transcription patterns, Giraud et al., 2012); to control oxygen contacts. Addition of sulfites can also happens at bottling, to protect wines during shipping and storage (Ribéreau-Gayon et al., 1998).

Sulfites are generally added to wines at levels that are lower than most other foods and beverages. Nonetheless these compounds have been indicated as possible contributors to the onset of asthmatic or allergic reactions (Vally and Thompson, 2003) and several studies have highlighted that wines can be major source of sulfite intake in the adult population (*e.g.* Bemrah et al., 2012; Mischek and Krapfenbauer-Cermak, 2012; Vandevijvere et al., 2010). In winemaking technology, sulfites have also been indicated as possible responsible for protein haze (Pocock et al., 2007). Therefore their utilization has been questioned and efforts are made in order to develop winemaking practices which reduce or avoid their utilization. This however demands a special attention to the microbiological aspects of the fermentation process: microbes have to be carefully monitored and controlled so that their contribution can be understood and the outcome of a fermentation can be predicted and possibly reproduced. Other important considerations have also to be made concerning oxidative processes which need to be prevented.

Here we present the results of winery fermentations of two Sicilian autochthonous grape cultivars, "Grillo" (white) and "Nero d'Avola" (red), with and without sulfites, during the 2012 vintage.

MATERIALS AND METHODS

About 1600 Kg of "Grillo" (18 ^{o}Babo; total Acidity 5.9 g/L; pH 3.2) and "Nero d'Avola" (NdA) grapes (18.7 ^{o}Babo; total Acidity 7.07 g/L; pH 3.4) were delivered to the IRVOS "G. Dalmasso" winery in Marsala (TP-Italy). Grapes were left at 4^{o}C for 48h, de-stemmed and then crushed. From each cultivar approx 1000L of must were divided into four aliquots and put in stainless steel containers, avoiding turbulence and maintaining low temperatures. At all times musts contact with oxygen was avoided by using inert gases (nitrogen, argon and CO_2). At each step samples were taken for microbiological and chemical analyses (sugar consumption, APA, pH etc). Temperature was constantly monitored. Equipment was carefully sanitized and microbiological controls were performed.

Grillo grapes were placed in stainless steel containers for cold static clarification (4^{o}C, 48h). Pectolytic enzymes (2 g/hL) and PVPP (20 g/hL) were added.

For each cultivar, two aliquots were added with SO_2 (5g/hL, "control" A and B), while two aliquots were left without ("No-SO_2", A and B). Nephelometric Turbdity Units were determined.

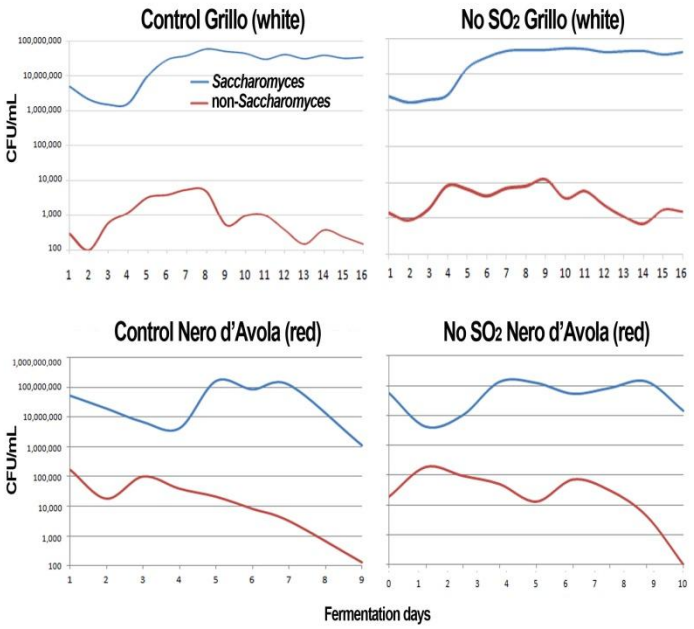
Grillo musts were fermented with Zymaflor X5 (Laffort); NdA musts were fermented with NDA21 (Biospringer), following manufacturer's directions; 60 mg/hL of thiamine were added. Fermentations took 13 days (at 16^{o}C) and 8 days (at 25^{o}C) for white and red musts respectively.

At the end of Grillo AF, lees samples were also taken and cryopreserved for downstream molecular analyses. Musts were divided in 8 aliquots of approximately 100L each. 3 g/hL of SO_2 were added to control aliquots. During fining, "no-SO_2" Grillo musts underwent MLF, which lasted 5 days. Samples were taken afterward for analysis. Wines were subjected to "battonage". Before bottling

wines underwent protein and tartaric stabilization; wines were filtered (0.45 μm); SO_2 (28mg/hL) was added to control wines.
During the AF of NdA musts, punching down of the caps and pumping over the dregs were performed. At the end musts were pressed, wines and lees samples were taken. Lactic bacteria (Laffort) were inoculated to induce MLF (which lasted 8 days). Each must aliquot was divided in two; test aliquots were added with 3 g/hL of SO_2. Wine fining was conducted at 15-16^{o}C.
Microbiological controls were performed before and after cold static clarification; during and at the end of AF on white wines; during and at the end of AF and after MLF on red wines. Samples for microbiological controls were also taken at bottling. At every step musts were mixed and samples were taken with sterile equipments. Must samples were kept in sterile stainless steel containers and delivered cold to the microbiological laboratory. Lees samples were taken at the end of all alcoholic fermentations and kept frozen until downstream molecular analyses.
Microbiological controls were performed and colony morphology were defined as in (Cavazza and Poznanski, 1998; Cavazza et al., 1992; Pallmann et al., 2001; Romancino et al., 2008).
Molecular controls were performed following the protocols of (Di Maio et al., 2012). Chemical analyses of the wines were performed according to (OIV, 2006; Squadrito et al., 2007). All measurements were performed in triplicate, using a Enotech Steroglass apparatus (code SQRQ053586; Steroglass-Italy), and following the instructions provided. Free and total SO_2 were determined following the official OIV methods using a " Solfotech" (Bullio, Milano, Italy) apparatus. Sensory analyses were performed according to (ISO 5495, 2005; UNI EN ISO 10399, 2004). For each session 30 assessors were interrogated.

RESULTS AND DISCUSSION

In No-SO_2 Grillo musts, a significant reduction of the microbial contamination could be induced upon static clarification, although this was less effective than that seen in control musts: non *Saccharomyces* yeasts were reduced by approx 70% (*vs* 88% of controls; initial contamination, 5000 CFU/mL); lactic bacteria were reduced by approximately 82% (*vs* 92%; intial contamination, 9400 CFU/mL).
During both Grillo and Nero d'Avola AF, the evolution of the microbial populations was similar between Control and No-SO_2 wines. This can be seen in fig 1 (right) where one fermentation per kind is shown.



Differently from control Grillo wines, in both no-SO_2 Grillo wines (A and B) MLF was carried out and completed, probably due to lactic bacteria which persisted in the musts (Approx 3700 CFU/mL vs 160 CFU/mL of control Grillo wines at the end of AF). Lactic bacteria were at similar levels at the end of the MLF of all Nero d'Avola wines
The chemical analysis of the wines revealed that a markedly reduced concentration of acetic aldehyde was present in no-SO_2 Grillo wines, compared to their controls. This however was not the case for the Nero d'Avola wines, were similar amounts were found between control and no-SO_2 wines. Finally, free and total SO_2 was completely absent in no-SO_2 red wines while a residual amount was present in no SO_2 white wines (table 1; average values from 4 separate trials; standard deviations indicated in parentheses; NdA, Nero d'Avola).

Table 1

Parameter	Control Grillo	No-SO_2 Grillo	Control NdA	No-SO_2 NdA
Acetic Aldehyde	25.90 (1.10)	6.04 (2.24)	1.31 (0.25)	1.255 (0.19)
Free SO_2	6.88 (0.76)	0.00 (0.00)	22.39 (0.51)	0.00 (0.00)
Total SO_2	43.95 (2.88)	2.56 (0.02)	35.82 (0.52)	0.00 (0.00)

To understand the sensory consequences of our fermentation protocol, we subjected our wines to "duo-trio" and preference tests. The first test revealed that while no differences existed between replicates, assessors could however distinguish between control and no-SO_2 Grillo wines.No difference was noted between Nero d'Avola control and no-SO_2 wines. In preference tests, no predilection toward one or the other product was expressed. As these results refer to pre-bottling white wines and post-bottling red wines, they will need further confirmation. Chemical, microbiological and sensory analyses will be performed in the incoming months, to monitor the evolution of the wines. Although preliminary, these results are nonetheless encouraging, as they show that our fermentation protocol allowed us to produce wines which were as appealing as those made in the traditional way.

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BIBLIOGRAPHY

Bemrah, N., Vin, K., Sirot, V., Aguilar, F., Ladrat, A.-C., Ducasse, C., Gey, J.-L., Rétho, C., Nougadere, A., Leblanc, J.-C., 2012. Assessment of dietary exposure to annatto (E160b), nitrites (E249-250), sulphites (E220-228) and tartaric acid (E334) in the French population: the second French total diet study. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 29, 875–885.

Cavazza, A., Grando, M.S., Zini, C., 1992. Rilevazione della flora microbica in mosti e vini. Vignevini 9, 17–20.

Cavazza, A., Poznanski, E., 1998. Le analisi microbiologiche nel laboratorio enologico. Vignevino 25, 42–53.

Giraud, E., Ivanova, A., Gordon, C.S., Whelan, J., Considine, M.J., 2012. Sulphur dioxide evokes a large scale reprogramming of the grape berry transcriptome associated with oxidative signalling and biotic defence responses. Plant Cell Environ. 35, 405–417.

Di Maio, S., Polizzotto, G., Di Gangi, E., Foresta, G., Genna, G., Verzera, A., Scacco, A., Amore, G., Oliva, D., 2012. Biodiversity of Indigenous *Saccharomyces* Populations from Old Wineries of South-Eastern Sicily (Italy): Preservation and Economic Potential. PLoS ONE 7, e30428.

ISO 5495, 2005. Sensory analysis - Methodology - Paired comparison test.

Mischek, D., Krapfenbauer-Cermak, C., 2012. Exposure assessment of food preservatives (sulphites, benzoic and sorbic acid) in Austria. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 29, 371–382.

OIV, 2006. Compendium of International Methods of Analysis OIV.

Pallmann, C.L., Brown, J.A., Olineka, T.L., Cocolin, L., Mills, D.A., Bisson, L.F., 2001. Use of WL Medium to profile native flora fermentations. American Journal of Enology and Viticulture 52, 198–203.

Pocock, K.F., Alexander, G.M., Hayasaka, Y., Jones, P.R., Waters, E.J., 2007. Sulfate--a candidate for the missing essential factor that is required for the formation of protein haze in white wine. J. Agric. Food Chem. 55, 1799–1807.

Ribéreau-Gayon, P., Dubourdieu, D., Doneche, B., Lonvaud, A., 1998. Traité d'oenologie. 1. Microbiologie du vin. Vinifications. Dunod, Paris.

Romancino, D.P., Di Maio, S., Muriella, R., Oliva, D., 2008. Analysis of non-Saccharomyces yeast populations isolated from grape musts from Sicily (Italy). Journal of Applied Microbiology 105, 2248–54.

Squadrito, M., Corona, O., Ansaldi, G., Di Stefano, R., 2007. Relazioni fra i percorsi biosintetici degli HTCAs, dei flavonoli e degli antociani nell'acino d' uva. Riv. Vitc. Enol. 60, 59–70.

UNI EN ISO 10399, 2004. Sencory analysis- Methodology- Duo-trio test.
Vally, H., Thompson, P.J., 2003. Allergic and asthmatic reactions to alcoholic drinks. Addict Biol 8, 3–11.

Vandevijvere, S., Temme, E., Andjelkovic, M., De Wil, M., Vinkx, C., Goeyens, L., Van Loco, J., 2010. Estimate of intake of sulfites in the Belgian adult population. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 27, 1072–1083.