WASHING THE GRAPES BEFORE CRUSHING: EFFECTS ON CONTAMINANTS AND FERMENTATION.

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Summary

The effect of grape washing was evaluated at winery scale in two premium white and red vinifications in Trentino (Italy). White and red grapes were washed in winery scale with 1 % citric acid solution, and the compositions of the musts were compared with that of the untreated controls. The metal and pesticide content of musts were well different if grape contamination was detectable. The yeast content of musts was also reduced by the washing procedure. The fermentation rate was measured in musts inoculated with native yeast (spontaneous fermentation) and with two differently prepared starter cultures. In all cases the fermentation rate was higher with washed grapes.

Introduction

Grapes to be used in winemaking are maybe the only raw material that is not washed before processing. This is probably due to avoid risks of sophistication or adulteration of wines with water addition, but in this way everything that is present on grape skins, like chemicals and microorganisms, but also leaves, parts of stems and sometimes of soil, can contaminate the musts. A reduction of these external abiotic contaminants, by washing the grapes before their crushing, could reduce the stress of yeast during fermentation. Moreover some pesticides (i.e. copper) can delay the yeast growth and lead to stuck or sluggish fermentations (Regueiro et al. 1993). The grapes are also a primary source of yeasts and the species that starts the natural fermentation are those predominant on grapes at the time of harvest. Even if Active Dry Yeasts (ADY) are added, the growth of indigenous non-*Saccharomyces* yeasts will also contribute to the overall fermentation (Heard and Fleet, 1985; Fleet et al. 1993; Beltran et al. 2002, Pretorius 2000), usually in a negative way. Thus, with the aim to reduce external contaminants (chemical and microbial) but without removing totally the natural grape microbiota, we checked the effect of a grape washing system (CLU® Grape Cleaning Unit – Tecnicapompe Zanin srl - Fara Vicentino – Italy) that uses a citrate solution to remove contaminants and complex metals. In the present work, we investigated the dynamics of yeast populations and the fermentation kinetics in musts produced with washed grapes, when fermentation was carried on by native yeasts (spontaneous fermentation), or by a Natural Starter Culture (NSC) or Active Dry Yeasts (ADY).

Materials and methods

Washing equipment

Grapes to be washed were put in the 3500 l tank of the CLU® Grape Cleaning Unit (Tecnicapompe Zanin srl - Fara Vicentino – Italy), which contained a washing solution (1% citric acid) in continuous mixing and air bubbling. The average stay of the grapes in the washing tank was 3-6 minutes, then a belt took the grapes that passed under a rinsing solution and an air flow to get dry.

White wines
Fermentation trials were carried out at industrial scale in 1000-L tanks at Pojer e Sandri winery (Faedo, Trento, Italy). Müller-Thurgau grapes (12000 kg) were manually harvested in 250 kg boxes and stored for 2 days at 4°C. Half of them were washed in the CLU® Grape Cleaning Unit (Tecnicapompe Zanin srl Fara Vicentino – Italy) and then crushed as usual, while the other 6000 kg were crushed without any washing. The must was extracted by means of a pressing system operating in positive pressure under nitrogen atmosphere (Mattivi et al., 2005). After crushing, the must was kept for two days at 14 °C for the cold settling. Once racked, the two 6000-L musts were divided and three 10-hL tanks was filled and inoculated. All the wines were fermented under nitrogen atmosphere, thus avoiding any contact with the oxygen.

Red wines
Red grapes (Cabernet Sauvignon – 4000 kg) were harvested and stored for one day at 4°C, then half of them were washed in the same way as Müller-Thurgau grapes, while the other half was the control. Grapes were destemmed and the whole berries were put in 400 kg open tanks (tubs) to ferment. Berries were gradually broken while plunging the cap, twice a day.

Fermentation trials
For each (white and red) vinification six different fermentations were carried: three with musts obtained from washed grapes and three for the control ones. One must was not inoculated, and its Spontaneous Fermentation (SF) was carried on by native yeasts; the second one was inoculated with a commercial Active Dry Yeast (ADY); the strain used in Müller – Thurgau was Fermol Arome Plus (AEB S.p.A. Brescia, Italy), the one used in Cabernet Sauvignon was Premium Rouge (Enologica Vason, Verona Italy). The third lots were inoculated with natural starter cultures (NSC). NSC was prepared in advance by picking and crushing a series of small amounts of grapes, letting them ferment spontaneously in 30-liters tanks and choosing the culture that showed the best kinetics and flavour.

Yeast isolation
After convenient dilutions in peptone water, each sample was immediately spread onto WL plates (Oxoid, Cambridge, UK) for *Saccharomyces* isolation and Agar lysine medium plates (Oxoid, Cambridge, UK) for *non-Saccharomyces* isolation. Plates were incubated at 25 °C for 4 days for colony development. Plates containing between 30 and 300 colonies were examined. From each plate, colonies with different morphology were counted and, for each morphotype, one or two colonies were streaked for purity determination by repetitive streaking on YPD-agar. Only those colonies that appeared to be *Saccharomyces* because of their colour and morphology (Cavazza et al, 1992; Pallmann et al. 2001) were isolated from WL agar and were assayed to verify whether they belonged to *S. cerevisiae* species by plating them onto Lysine agar medium which is unable to support *S. cerevisiae* growth (Morris and Eddy, 1957; Angel and Siebert 1987).
All yeast isolates were stored at -80°C in 25% glycerol solution.

DNA isolation from yeast cultures
Strains were grown o/n in YPD broth and 2ml were collected and centrifuged at 10000rpm for 5 min. DNA extraction from the cells was performed with a synthetic resin (Instagene Bio-Rad matrix, Richmond, CA, USA) according to the supplier's instructions.

RAPD-PCR assay

Pure *Saccharomyces* cultures among the isolated strains were subjected to Random Amplified Polymorphic DNA (RAPD) for biotype screening, in order to verify the presence of inoculated strain(s) among the strains isolated from must-samples of washed or not washed grapes at different fermentation times.
For RAPD-PCR, XD5 (5′-CTGGCGGCTG-3′) was used as primer. Mixture and run conditions were according to Di Maro et al. (2007). The amplified products (25 µl) were resolved by electrophoresis on 2.5% (w/v) agarose-TAE at 100 V. 1 Kb DNA Ladder (Invitrogen) was used as molecular weight marker.

Analytical methods
Chemical parameters were analysed with official OIV methods and by Near infra-red spectrometry with Winescan equipment (FOSS, Hilleroed, DK).
Traces of residual pesticides, and in particular those applied in the Pojer & Sandry vineyard during 2007 (azoxystrobin, dimetomorf, folpet, iprovalicarb, metalaxil, penconazole, quinoxifen and trifloxystrobin) were analysed solid/liquid extraction with HPLC-DAD, GC-MS, GC-ED detection. Transition metals (Cu, Fe, Mg, Pb, Zn) were analysed by ICP-OES (Larcher and Nicolini, 2001). In the white musts and wines, the hydroxycinnamates were analysed by HPLC, while the reduced and oxidised glutathione by LC-MS (Mattivi et al., 2007).

Results and discussion

Composition of musts and wines
The metal (calcium, magnesium, sodium, zinc and potassium) and pesticide contents were very low in all trials during both white and red vinification. In particular, all the white juices were particularly low in metals, as a consequence of very low spraying in the 2007 vintage. Traces of residual pesticides were found in part of the unwashed samples: iprovalicarb (all 3 musts, average 0,07 mg/L), dimetomorf (2), folpet and trifloxystrobin (1). In the washed samples, the pesticides were either reduced to about the half in the case of iprovalicarb (all 3 musts, average 0,05 mg/L) and dimetomorf (1 sample), or removed.
The winemaking in hyper-reduction, obtained by the use of inert gases such as nitrogen, can be of strong interest for the obtainment of press juices of higher quality, specifically enriched of flavour-active compounds and endogenous antioxidants which are localised in the grape skin. The trial and control wines had similar concentrations of hydroxycinnamates. The contents of reduced glutathione were consistently higher in both musts (+31%) and fermenting must (+35%) and wines (+16%) produced from washed grapes. On the other side, the relatively lower levels of oxidized glutathione were approximately doubled in musts and five times higher in wines from unwashed grape (data not shown). These data suggested that the hyper-reductive winemaking technique assured a lower oxidation level, being in conclusion slightly more efficient, when operating with the washed grape.
In red wines bigger differences were observed (table 1 and 2), probably due to higher pressure of the molds in the vineyard and higher extraction rate since grape skins remained immersed in the fermenting juice for the whole fermentation.
In the vinification procedure adopted for Cabernet Sauvignon grapes, some more juice was extracted every day. This prolonged contact of skin and juice led to a stronger metal extraction, as can be seen in table 1 and 2. While they were low as absolute values, the metal content was significantly higher if grapes were not washed, especially regarding copper, iron, lead and zinc. After 3 days of fermentation, the concentration of copper was reduced from that of the freshly extracted juices, but still in the range of some mg/L in the juices obtained from

unwashed Cabernet grape (tab. 2). These relatively high values could possibly affect the performance of the yeasts in the control fermentations.

	Iron (mg/l)			Lead (µg/l)			Copper (mg/l)			Zinc (mg/l)		
	SF	NSC	ADY	SF	NSC	ADY	SF	NSC	ADY	SF	NSC	ADY
Must	0.48			44			2.66			0.42		
Ferm. 3 day	0.50	0.52	0.39	68	8	24	0.76	1.05	0.54	0.21	0.27	0.38
Wine	1.19	1.21	1.66	<3	<3	<3	0.39	0.42	0.45	0.07	0.07	0.04

Table 1. Iron, Lead, Copper and Zinc content in musts, after three days of fermentation and in Cabernet Sauvignon wines obtained from **washed grapes**. SF = Spontaneous Fermentation; NSC Must inoculated with a Natural Starter Culture; ADY Must inoculated with Active Dry Yeast strain Premium Rouge (Enologica Vason, Verona, Italy)

	Iron (mg/l)			Lead (µg/l)			Copper (mg/l)			Zinc (mg/l)		
	SF	NSC	ADY	SF	NSC	ADY	SF	NSC	ADY	SF	NSC	ADY
Must	0.85			62			10.56			0.88		
Ferm. 3 day	0.88	0.74	0.69	44	118	80	2.05	4.58	4.62	0.57	0.48	0.54
Wine	0.86	1.18	1.35	<3	<3	<3	0.43	0.69	0.58	0.09	0.01	0.09

Table 2. Iron, Lead, Copper and Zinc content in musts, after three days of fermentation and in Cabernet Sauvignon wines without grape washing. SF = Spontaneous Fermentation; NSC Must inoculated with a Natural Starter Culture; ADY Must inoculated with Active Dry Yeast strain Premium Rouge (Enologica Vason, Verona Italy).

Regarding the pesticides, small traces of dimetomorf (0,13 mg/L) and iprovalicarb (0,14 mg/L) were present in the juice from unwashed grape, and their concentration was effectively reduced (respectively 0,09 and 0,07 mg/L) in the juice extracted from the washed grape. As a consequence, both chemicals were below the detection level (< 0,03 mg/L for dimetomorf, 0,01 mg/L for iprovalicarb) in the 3 wines produced from the washed grape, while they were still detectable in the control wines.

Müller-Thurgau

Yeast growth was monitored from must settling to the end of wine fermentation, and the results are shown in figure 1, 2 and 3. Yeast initial content was clearly lower in must from washed grapes. During the first two days of cold settling (from day -2 to day 0, common to all trials) Immediately after crushing grapes, yeast counts were quite high, probably due to the fact that after harvesting, grapes were stored at 4 °C for two days before crushing. After one day, yeast growth was observed: *Saccharomyces* yeasts appeared in one day, and stayed constant until the racking of clear juice, while non-*Saccharomyces* ones showed a slight decline after a growth the first day.

Müller-Thurgau grapes: Spontaneous fermentation

After cold settling, each of the two musts from washed grapes and from the control ones was divided in three 20-hl amounts and put in fermentation tanks, to let them ferment. The total yeast number was still higher in must from grapes not treated with the washing equipment, as can be seen in fig.1. Apiculate yeasts dominated the must microbiota: where the grapes had not been washed their number was more than 1.5×10^5 cfu/ml, in clean must their number was 3.7×10^4.

During the fermentation, the non-*Saccharomyces* yeasts grew for one day, to decrease and disappear after nine days. Their number was initially one-log higher in the must from

untreated grapes. In the next days the difference declined until the sixth day, when their number was the same in the two musts, and two days later they disappeared in both musts.

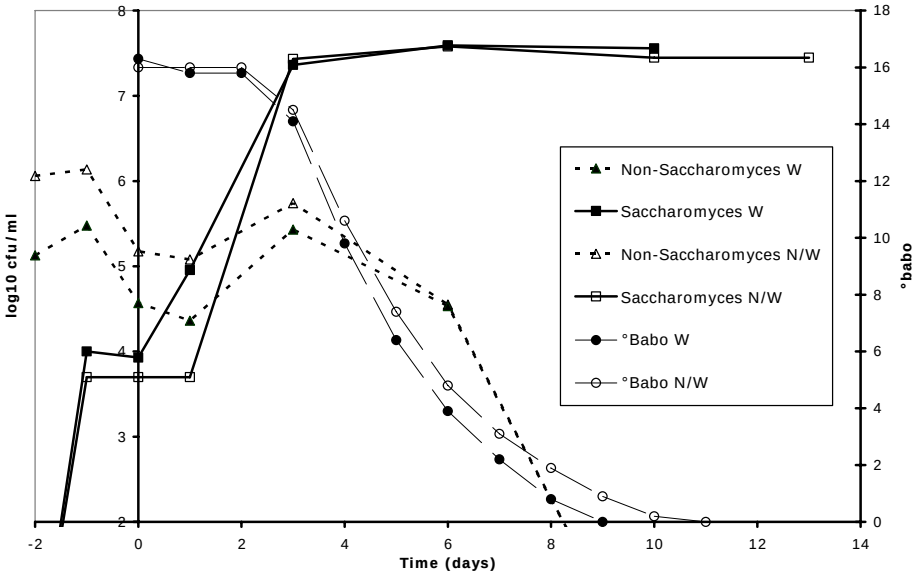

Fig. 1. Yeasts growth and sugar consumption during the **spontaneous fermentation** of Müller-Thurgau must from grapes treated (W) or not (N/W) with washing equipment. Must cold settling is shown as negative period on the left of x-axis.

Saccharomyces yeasts grew better in the clean must: after the settling their number was low, but slightly higher in the clean must, maybe indicating a more favourable environment. The bigger difference in the clean and "dirty" musts was observed after 24 hours, when *Saccharomyces* yeasts number was more than one-log higher in the clean must, and then any difference disappeared. Their better growth in the must from washed grapes could explain the faster fermentation of this one, which took 9 days to come to the end, compared to that of the other must, which took 11 days.

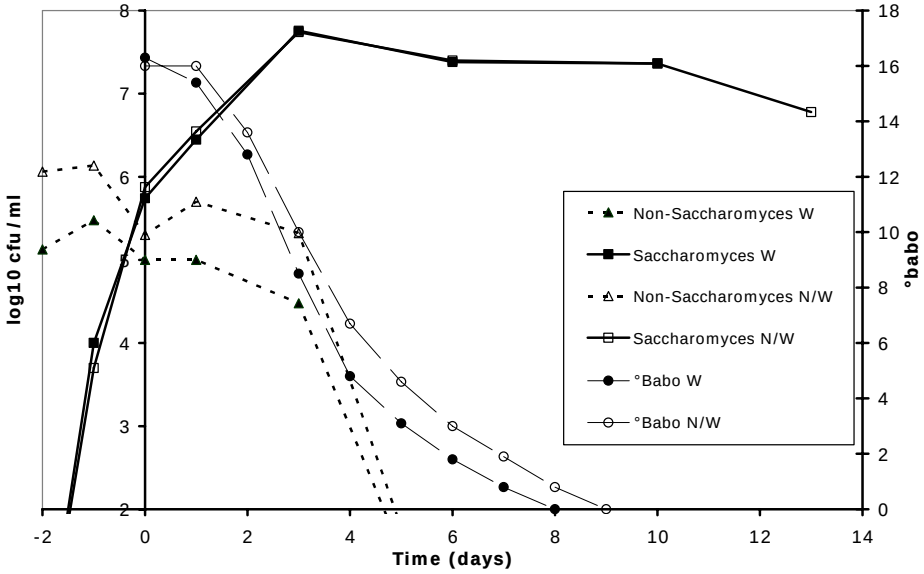

Fig. 2. Yeasts growth and sugar consumption during the fermentation of Müller-Thurgau must from grapes treated (W) or not (N/W) with washing equipment, inoculated with an **autochthonous Natural Starter Culture**. Must cold settling is shown as negative period on the left of x-axis.

Müller-Thurgau grapes: NSC fermentation

Kinetic data of the musts from washed and untreated grapes, inoculated with this NSC, are shown in fig. 2. The counts during the settling periods are also reported, even if they were the same as in fig 1, because the two musts were divided in three parts after racking. At time 0, NSC was added at a 5.5 x10^5 cfu/ml concentration in washed grape must, and 7.5 x10^5 cfu/ml in the non-treated must. In this must *Saccharomyces* yeasts were the dominant group at the moment of inoculation.

Non-*Saccharomyces* yeasts grew for some days, always in a higher number in must from non treated grapes, and after five days they dropped to an undetectable level.

Saccharomyces yeast showed similar growth in the two musts: the differences in the counts were negligible, but, once more, sugar consumption was clearly faster in the clean must, in which the fermentation took one day less.

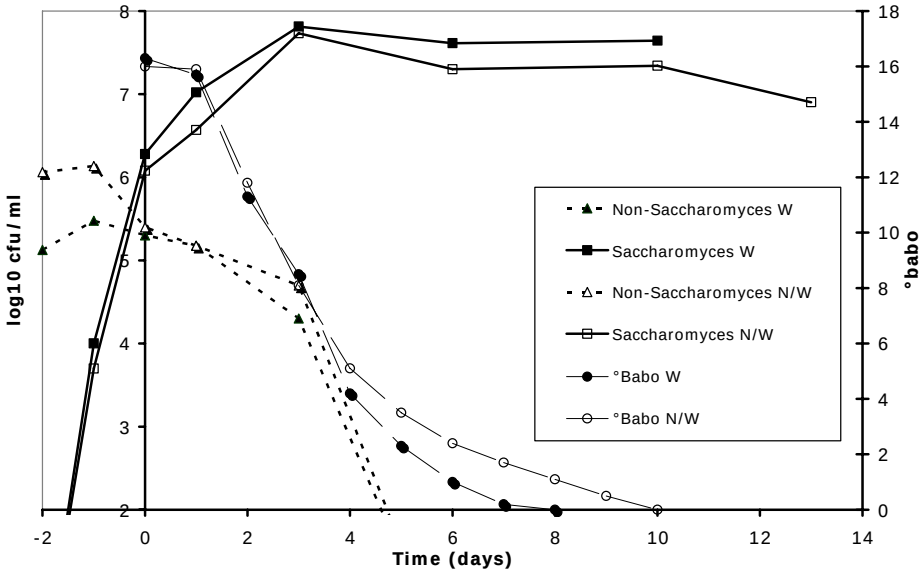

Fig. 3. Yeasts growth and sugar consumption during the fermentation of Müller-Thurgau must from grapes treated (W) or not (N/W) with washing equipment, inoculated with **Active Dry Yeast**. Must cold settling is shown as negative period on the left of x-axis.

Müller-Thurgau grapes: ADY fermentation.

Kinetic data of the musts from washed and untreated grapes, inoculated with ADY, are shown in fig. 3. The counts during the settling periods are also reported, even if they were the same as in fig 1 and 2, because the two musts were divided in three parts after racking. Non-*Saccharomyces* yeasts were detectable for three days; their initial number was slightly higher in musts from untreated grapes, but in both musts they declined from the first day. *Saccharomyces* yeast number was always higher in the clean must. Interestingly, in this case, sugar consumption rate was the same in the two musts in the first three days, and then a difference appeared. Again, in the must from washed grapes the fermentation was completed two days before than in the control one.

Cabernet Sauvignon

As cold (4°C) grapes were processed, and they were only destemmed, the washing procedure had an important side-effect: washed grapes, when put in fermentation open tanks were at 14°C, while the control ones, were at 8-9°C. Yeast growth, monitored in the must before inoculum (M) to the end of wine fermentation (24 days), was affected by this phenomenon, as can be seen in the results shown in figure 4, 5 and 6. As at the moment of the inoculation

the red must there was only a mall amount of juice at the bottom of the open tanks containing mostly whole berries, and juice was extracted in the plunging of the cap, it was difficult to have reliable counts at t0. In fig 4, 5 and 6 t0 counts indicates the yeast counts in must before its separation and inoculum in three thanks.
The total yeast number was higher in must from washed grapes. Apiculate yeasts dominated the must microbiota both in must from washed grapes and in the control ones; their number was 2,5 x 10^4 and 5.0 x 10^3 cfu/ml in clean and "dirty" and must respectively.

Cabernet Sauvignon: Spontaneous fermentation

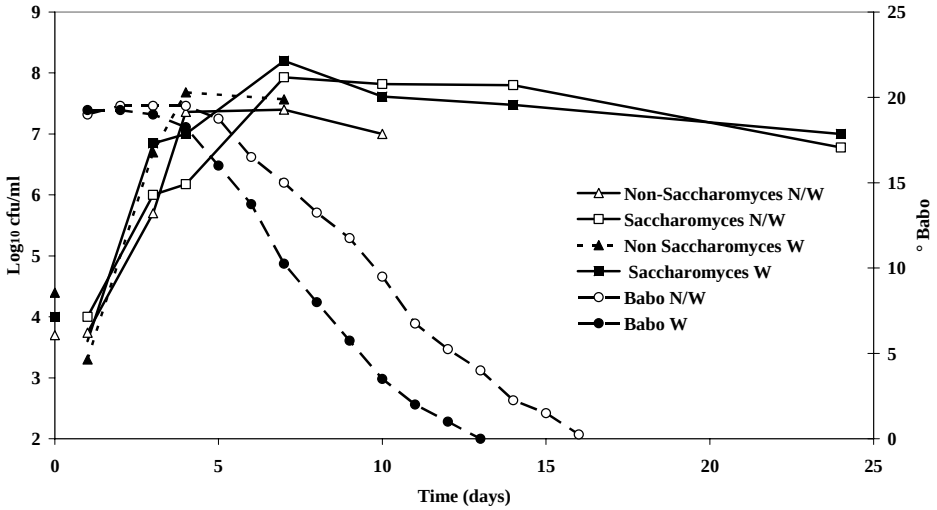

Fig. 4. Yeasts growth and sugar consumption during the spontaneous fermentation of Cabernet-Sauvignon must from grapes treated (W) or not (N/W) with washing equipment.

During the fermentation, the non-*Saccharomyces* yeasts grew for four days, and then they declined and were no more detectable after ten days in washed must and fourteen days in the control must. After the washing procedure, *Saccharomyces* yeasts number was low, but slightly higher in the clean must. The bigger difference between clean and "dirty" musts was observed after three and four days, when *Saccharomyces* yeasts number was more than one-log higher in the clean must. After day 7, their counts were higher in must from control grapes, probably because the sugar content of must from washed grapes was lower and fermentation was close to the end (fig. 4). Their better growth in the must from washed grapes, and the higher initial temperature, could explain the faster fermentation, which took 13 days to come to the end, compared to that of the control must, which took 16 days.

Cabernet Sauvignon: NSC fermentation

Kinetic data of the musts from washed and unwashed grapes, inoculated with NSC, are shown in fig. 5. Non-*Saccharomyces* yeasts were no more detectable after 24 hours fermentation.
Saccharomyces yeasts showed similar growth in the two musts: the differences in the counts were negligible; in both cases they reached the higher concentration at the fourth day when they were about 7,3 - 7,4 log cfu/ml in both washed and control musts.
Once more, sugar consumption was clearly faster in the clean must, in which the fermentation took two days less.

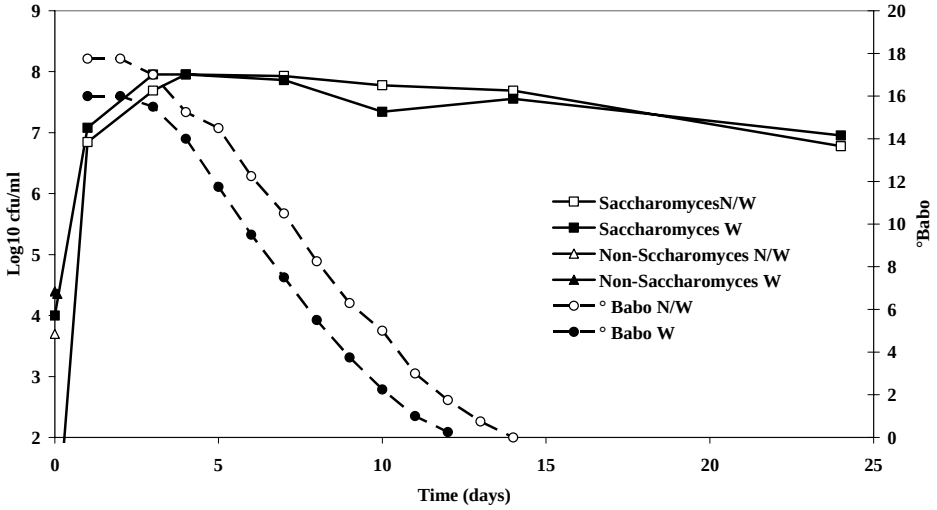

Fig. 5. Yeasts growth and sugar consumption during the fermentation of Cabernet-Sauvignon must from grapes treated (W) or not (N/W) with washing equipment, inoculated with an **autochthonous Natural Starter Culture**.

Cabernet Sauvignon: ADY fermentation

Kinetic data of the musts inoculated with ADY are shown in fig. 6. In both musts Non-*Saccharomyces* yeasts were no more detectable after the first day. *Saccharomyces* yeast number was higher in the clean must until the seventh day of fermentation. They reached the higher concentration (8,0-8,1 log cfu/ml) after four and seven days of fermentation in washed and control must, respectively. This could be explained by the faster fermentation rate in the clean must, in which the fermentation was completed three day before than in the control one.

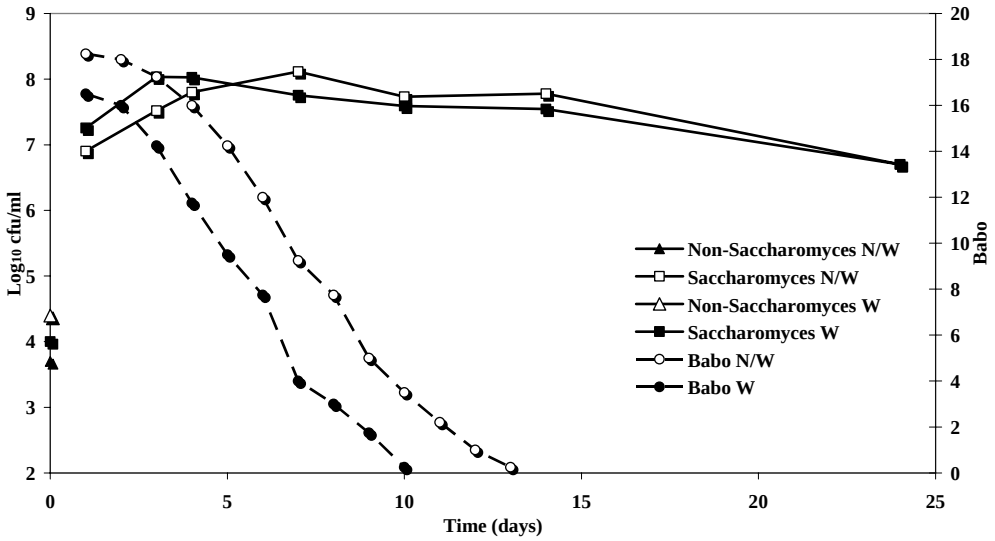

Fig. 6. Yeasts growth and sugar consumption during the fermentation of Cabernet-Sauvignon must from grapes treated (W) or not (N/W) with washing equipment, inoculated with **Active Dry Yeast**.

RAPD-PCR assay

RAPD-PCR assay was used to track the *Saccharomyces* strains inoculated after ADY or NSC addition, and in spontaneous fermentations to monitor biodiversity and population changes among *Saccharomyces* strains during fermentation. During all the fermentations, 67 non-Saccharomyces and 171 *Saccharomyces* colonies were isolated and characterized, after 1, 3, 4, 7, 14 and 24 days.

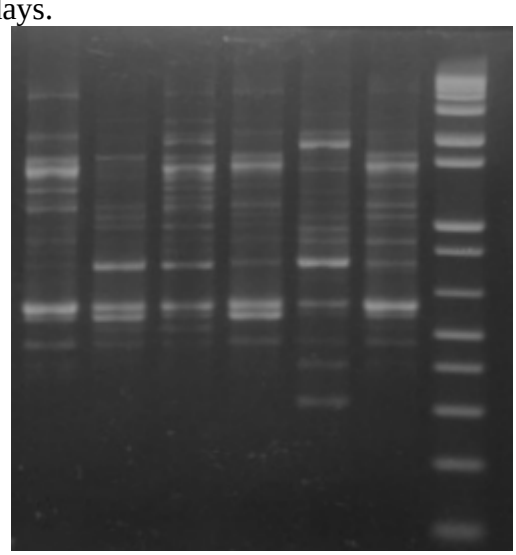

Fig. 7: Comparison of the electrophoresis gels profiles obtained from six isolates after amplification with XD5 primer. Lane 1 R7 isolate; 2: R11 isolate; 3: R12 isolate; 4: R9 isolate; 5: R15 isolate; 6: R3 isolate and 7: molecular weight

Spontaneous fermentation.

69 *Saccharomyces* colonies were isolated during the fermentation: 31 from the must coming from washed grapes and 38 from the control one. No cluster was found with more than four isolates (considering similarity value of about 80%). Among them, 15 and 21 different profiles were found, indicating that none of the strain clearly dominated the fermentation, neither in the clean must, nor in the control one. In both cases a mixed population carried on the fermentation. Six strains were found both in the clean must and in the control ones.

NSC fermentations

The microbial composition of the NSC used to inoculate the musts was studied. Five different RAPD-patterns were found in it (Fig.7): R7, R9, R10, R11, R15.

The NSC was added to the clean and to the control must.

Before the inoculation of the must, wild *Saccharomyces* was present in the clean must, in very low concentration. The two isolated colonies had the same morphology, whose profile was signed as R3 strain (fig. 7).

R3 and R9 (one of the five NSC strains) showed a clustering similarity value of 93%, so they can be considered as the same isolate (further analysis with different primer are necessary).

69 *Saccharomyces* colonies were isolated from **NSC fermentations;** 31 from washed grapes and 38 from the control ones. 39 out of their 69 RAPD-pattern clustered with NSC isolates (with a similarity value of at least 80% using Pearson correlation). In particular, the amplification patterns R7, R9, R10, R11 and R15 were usually all predominant within four days, then only R9 profile was further found in clean and control must, and was predominant in this latter one. If confirmed with different primers, it is noteworthy that one strain, maybe winery-resident, was found in must, in NSC and in the two fermentations.

ADY fermentations

56 *Saccharomyces* colonies were isolated from **ADY fermentations;** 30 from must produced with washed grapes and 26 from the control one.

Two *S. cerevisiae* amplification patterns were observed to dominate both fermentations: 14 isolates clustered with ADY profile and 22 with the R9 profile. Their distribution did not varied among clean and control must, isolates but varied among fermentation times: at early fermentation stages; 12 out of 26 isolated colonies, showed an electrophoresis pattern clustering with R9 profile, and no isolate clustered with ADY profile. Only after 7 days, the dominating yeasts clustered with ADY strain used for the inoculum and R9 profile disappeared.

Conclusions

A new equipment for washing the grapes before crushing was tested during 2007 vintage in a winery-scale vinification of ten tons of premium quality white and red grapes.

The must produced from un-washed grape had initial yeast content clearly higher, mostly non-*Saccharomyces*. This observation can be easily explained by the washing process. *Saccharomyces* yeast were presumably washed away like all other yeasts, but in all cases, whether they were native or inoculated, autochthonous or of industrial origin, they often grew faster and in all cases fermented more efficiently with higher sugar consumption rate.

Grape washing did not affect the metal composition of premium quality, almost “metal free” Müller-Thurgau musts and wines. It was effective in reducing or removing traces of pesticides and in preserving the wines from oxidation, with respect to control wines.

In red, Cabernet Sauvignon wines, the metal content (copper, iron, zinc, and lead) were well lower if grapes were washed before destemming. The copper levels in must were effectively reduced below those considered toxic for the microbiota, confirming the effectiveness of the citric acid for copper removal. The small traces of residual pesticides that were present in the control musts and wines were greatly reduced in musts and lowered down to undetectable levels in the wines produced from washed grape.

Fermentation kinetics in red wines showed bigger differences than in white wines, not only depending on the longer contact of “dirty” skins with the juice, but also because the grapes, that were stored at 4° C for two days, were heated by the water in the washing equipment. The practice of refrigerate the picked grapes for 24-48 hors is unusual, but the heating side-effect of the washing procedure could be of some interest in cold climates, especially for late-ripening varieties which are often collected with low temperatures.

Regarding wild *Saccharomyces* yeast populations, no clear behaviour was observed. One strain, probably the most resistant to stress conditions, were found in many fermentations, differently inoculated, and may be a winery-resident strain.

In conclusion, these preliminary experiments confirmed the feasibility and enological interest of the introduction of a washing step in both the white and red the winemaking processes.

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