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Unravelling the Impact of Grape Washing, SO_2, and Multi-Starter Inoculation in Lab-Scale Vinification Trials of Withered Black Grapes

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Abstract: Wine quality is strongly affected by chemical composition and microbial population of grape must, which, in turn, are influenced by several post-harvest treatments, including grape withering. Different strategies have been suggested to manage the fermenting must microbiota, as it plays a central role in the outcomes of both spontaneous and guided fermentations. This study aimed at evaluating the impact of grape washing, SO_2 addition, and selected starter culture inoculation on population dynamics, fermentation kinetics, and main oenological parameters in lab-scale trials, focusing on withered grapes usually used for Amarone production. Although grape washing treatment was effective in removing heavy metals and undesirable microorganisms from grape berry surface, inoculation of multi-starter cultures impacted more fermentation rates. Further, both grape washing and starter inoculation procedures had a remarkable impact on wine chemical characteristics, while 30 mg/L SO_2 addition did not significantly affect the fermentation process. In summary, the best strategy in terms of limiting off-flavors and potentially reducing the need for SO_2 addition in wine from withered grapes was the use of yeast starters, particularly mixed cultures composed by selected strains of *Metschnikowia* spp. and *Saccharomyces cerevisiae*. Application of a washing step before winemaking showed a potential to improve organoleptic characteristics of wine.

Keywords: withered grapes; grape washing; heavy metals; grape microbiota; mixed starter cultures; fermentations; wine quality; sustainability

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1. Introduction

Chemical and microbiological composition of grape must play a central role in determining final wine characteristics; therefore, good sanitary conditions at harvest and before crushing are fundamental requirements for obtaining optimal results [1]. Post-harvest withering is traditionally carried out in different winemaking regions, inducing deep changes to the composition of grape and must before fermentation [2–5]. In the Valpolicella area (north-east Italy), this traditional practice is applied in the production of renowned dry and sweet wines, such as Amarone and Recioto della Valpolicella, and consists of placing the harvested ripe and healthy grape bunches, mostly from the variety *Vitis vinifera* L.cv Corvina, in large and ventilated rooms called "fruttaio". After usually 2–3 months, the drying process causes concentration of sugars due to water evaporation (approximately 30–40% of the initial weight), and further modifications related to the metabolic activities of berries and their microbial consortia [2,5,6].

The practice of washing pre-crushed grapes has been proposed to improve wine quality, considering that wine grapes are probably the only vegetable raw material for

food use that usually is not washed before being processed [7]. Washing treatment aims to remove the substances that accumulate on grape berry surface and can cause yeast stress during fermentation, such as chemicals, heavy metals, and spoilage microorganisms [7,8]. In fact, some pesticides and fungicides, such as copper, can delay yeast growth and metabolism, therefore leading to stuck or sluggish fermentations [9–11]. To date, some studies have shown that mechanical washing, carried out with a 1% citric acid solution for 3–6 min, reduced heavy metals throughout the winemaking process and positively affected fermentation kinetics of both spontaneous and inoculated (or guided) fermentations [7,8]. However, these studies have focused on Müller-Thurgau fresh grapes, while, to the best of our knowledge, no information is available on washing withered black grapes.

It is well-known that the microbiota associated with grape berry surface plays a fundamental role in the early stages of fermentation [12,13], influencing, positively or negatively, the overall organoleptic characteristics of wine [14–16]. In this perspective, the diversity of the microbial consortium during fermentation is key to generate wine chemical complexity, which is related to the sensory profile [17–19]. Indeed, in recent years, controlled co-cultured fermentations with non-*Saccharomyces* and *Saccharomyces* yeasts have raised interest because co-inoculation is considered a useful tool to take advantage of spontaneous fermentation while avoiding the risk of fermentation problems [19–21].

For both spontaneous and guided fermentations, an adequate microbiological control is aimed at optimizing the fermentation kinetics and obtaining a final product of reliable quality. Removal of wine spoilage organisms is generally achieved with the use of sulphur dioxide (SO_2), which has a strong antimicrobial effect. Combined with its important antioxidant activity, application of SO_2 has become indispensable in the winemaking process. However, in recent years, health issues have been raised regarding SO_2, especially for sensitive individuals. Thus, the wine industry is following the general trend towards a reduction of the amount of SO_2 in food, looking for alternative control strategies to consider consumers' health and process sustainability [21,22]. Indeed, a recent survey of consumers' perceptions, preferences, and willingness to pay for wine showed that wines with no sulphites added were associated with health benefits and were perceived differently from other types of sustainable wines, showing a promising strategy for product differentiation [23].

In this context, we designed an experimental plan to evaluate the impact of grape washing, SO_2 addition, and multi-starter inoculation on population dynamics, fermentation kinetics, and main oenological parameters in lab-scale vinification trials of withered grapes. The washing step of pre-crushed grapes was effective in removing heavy metals and spoilage organisms, indicating the potential of this strategy to have positive impacts on important organoleptic characteristics of wines. Integrated with the inoculation of mixed starter cultures, particularly selected strains of *Metschnikowia* spp. and *Saccharomyces cerevisiae*, it could be a path worth exploring also in the perspective of reducing SO_2 input in winemaking.

2. Materials and Methods

2.1. Withered Grapes

Grapes of the varieties Corvina, Corvinone, and Rondinella, from the Valpolicella classical area, were harvested at ripening during the 2019 vintage. Grapes were partially dried for three months in a traditional "fruttaio", until reaching a sugar content of 31.5 °Brix. Thirty kilograms of withered grapes where transferred to the laboratory and vinified in different conditions, as shown in Figure 1. The experimental design considered a combination of three variables with two options for each: grape washing (washed and non-washed), SO_2 addition (with and without SO_2), and starter inoculation (inoculated and spontaneous).

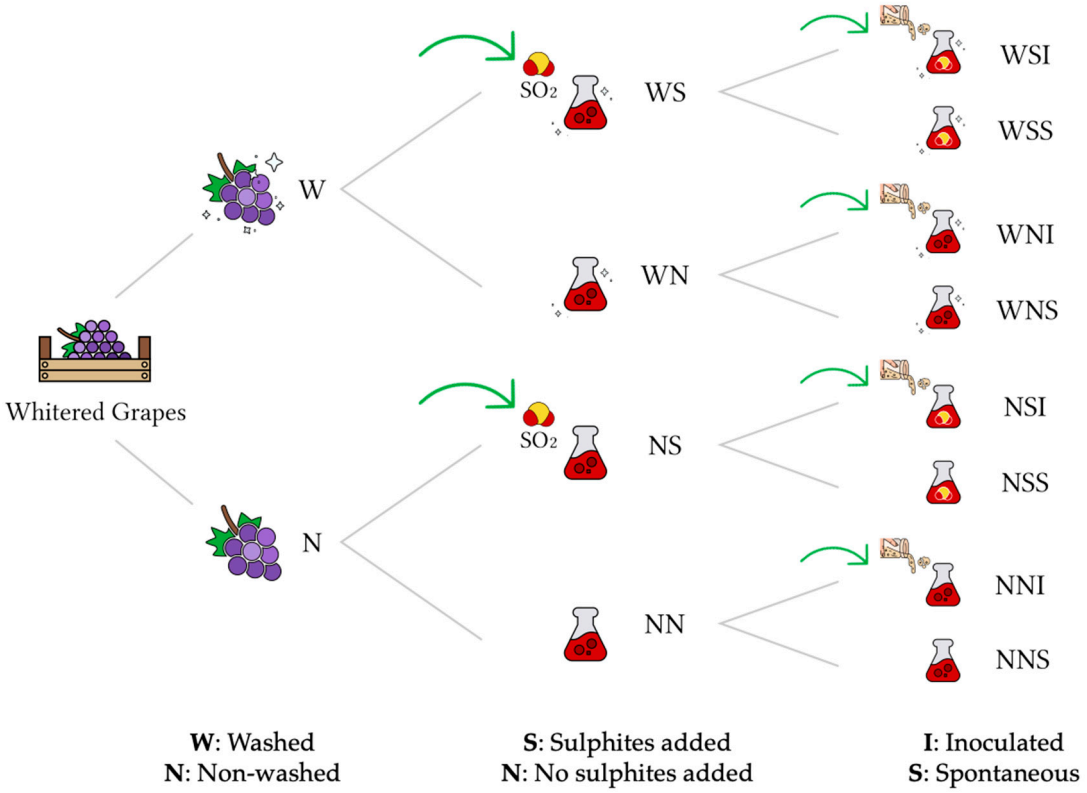


Figure 1. Schematic representation of the experimental plan, with the eight different modalities of winemaking carried out and the acronyms explained.

2.2. Grape Washing

Upon arrival to the laboratory, withered grapes were divided in two batches of 15 kg, and one of them was submitted to a washing step. Treatment was carried out in tanks using a 1% citric acid (Sigma-Aldrich, Milan, Italy) solution prepared in tap water. Grapes were rinsed with washing solution at a volumetric ratio of 1:3. To make washing more effective, compressed air was blown in the tanks during the 10 min treatment (modified from Cavazza et al. [7]). After washing, grapes were left to dry at room temperature for 1 h.

Washing solution samples were collected prior to grape washing and at the end of washing treatment for the microbiological and chemical analysis. For the DNA extraction, these samples were vacuum-filtered through sterile S-Pak Filters® (Sigma-Aldrich, Milan, Italy), which were stored at −20 °C until further analysis.

2.3. Microvinifications

Washed (W) and non-washed (N) withered grapes were manually destemmed and crushed in separate tanks. The obtained grape musts were further split in two new batches, then one of them was added with 30 mg/L SO_2, as potassium metabisulphite ($K_2S_2O_5$, Sigma-Aldrich). Four types of grape must were obtained, namely: washed with SO_2 (WS), washed without SO_2 (WN), non-washed with SO_2 (NS), and non-washed without SO_2 (NN) (Figure 1).

Grape musts (0.8 kg, containing the skins) were transferred in 1 L sterile glass bottles, filling eight bottles for each of the previous four types of must. Finally, four bottles of each must were inoculated (I) with the strain *Metschnikowia* sp. DBT012 (Department of Biotechnology, University of Verona), then sequentially inoculated after 48 h with *S. cerevisiae* EC1118 (Lallemand, Montréal, Canada). *Metschnikowia* sp. DBT012 was previously isolated from grape bunches and thoroughly characterized for its enological potential, also in mixed culture fermentations with *S. cerevisiae* EC1118 [24,25], showing interesting enzymatic activities and high production of aromatic compounds that can positively impact wine

organoleptic profile. Both yeasts, conserved in dried preparations, were rehydrated for 15 min in a volume of 1:10 non-chlorinated water at 37 °C, then acclimatized with a 1:1 dilution in grape must to avoid temperature shock, and inoculated at a concentration of 1×10^6 CFU/mL. The remaining four bottles from each type of must were not inoculated, letting spontaneous (S) fermentation be carried out by native yeasts.

The eight modalities of fermentation were performed in quadruplicates, for a total of 32 bottles. All bottles were kept static in a room with controlled temperature (22 ± 1 °C). Cap management was carried out by punching down twice a day to favor aeration and homogenization, thus improving the fermentation process.

2.4. Fermentation Kinetics and Microbiological Analysis

Fermentations were monitored daily through determination of weight loss due to CO_2 release. When daily weight loss was constant (<0.05 g/L), fermentations were stopped by the addition of 80 mg/L $K_2S_2O_5$, and bottles were tightly closed and kept at 4 °C until further analysis.

Yeast enumeration was performed on samples (10 mL) of washing solution and musts taken at the beginning and throughout fermentation. Samples were serially diluted in 0.9% NaCl solution and spread onto WL Agar (Sigma-Aldrich, Milan, Italy) supplemented with 100 mg/L chloramphenicol and 100 mg/L biphenyl. The plates were incubated at 27 °C for 2–3 days in aerobiosis. Colonies belonging to *S. cerevisiae* and non-*Saccharomyces* yeasts were counted differentially due to clear distinctions of specific morphology on this medium [25].

Colonies putatively belonging to *Metschnikowia* spp. (distinguishable by a characteristic red halo beneath the colonies [25]) were identified on WL Agar plates of fermenting musts, and ten of them were randomly isolated from each plate at 24 and 48 h of fermentation, for a study of implantation. They were isolated, purified, and cultured in YPD broth (10 g/L Yeast extract, 20 g/L bacteriological Peptone, 20 g/L Dextrose) at 27 °C for 48 h. Two milliliters from each culture were centrifuged (14,000 rpm, 5 min) and the cell pellet obtained was frozen at −20 °C for subsequent DNA extraction.

2.5. Chemical Analysis

Samples of grape musts and wines were firstly clarified by centrifugation (5000 rpm, 10 min), then loaded in a Y15 automated enzymatic analyzer (BioSystems, Barcelona, Spain). Residual sugars (glucose/fructose), acetic acid, primary amino nitrogen (PAN), ammonia, free SO_2, total SO_2, and acetaldehyde were measured with dedicated enzymatic kits from the same manufacturer. Yeast Assimilable Nitrogen (YAN) was calculated by the sum of PAN and ammonia.

Copper and iron content in the washing solutions was analyzed by a specialized external laboratory (UIV—Unione Italiana Vini, Verona, Italy) using inductively coupled plasma-mass spectrometry (ICP-MS; Agilent 7700×), following the UNI EN ISO 17294-2:2016 method.

2.6. DNA Isolation

For the isolation of total genomic DNA, the membranes used in the filtration of washing solutions were cut and placed inside the tubes provided in the PowerSoil® DNA isolation kit (Qiagen, Milano, Italy), then DNA extraction followed the manufacturer's protocol. DNA yield and purity were determined by a NanoDrop ND1000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA, USA). DNA concentration was normalized to 8 ng/μL before amplification.

Total genomic DNA from the cell pellets of putative *Metschnikowia* spp. isolates was purified using the Wizard Genomic DNA Purification kit (Promega, Milano, Italy), following the manufacturer's instructions. Quality assessment of DNA was carried out as described above and dilution on DNAse-free water was performed when necessary for the following molecular analysis.

2.7. Molecular Analysis

PCR-DGGE analysis was performed using DNA isolated from washing solutions and from selected strains employed to prepare a reference marker for the presumptive identification of microorganisms. The following strains were considered: *Hanseniaspora uvarum* DESP52, *Botrytis cinerea* B2, *S. cerevisiae* EC1118, *Lachancea thermotolerans* COLC27, *Aureobasidium pullulans* A1, *Torulaspora delbrueckii* PINOTG5, *Starmerella bacillaris* MALV45, *Rhodotorula mucilaginosa* CLINT2, *Pichia membranifaciens* COLBR1, *Penicillium expansum* VSA, *Aspergillus niger* VSE, *Candida diversa* CHIAR8, and *Metschnikowia* sp. DBT012. They belong to the most frequent fungal species associated with grape surface [26], more specifically of withered grapes [2]. All strains are part of the microbial collection of the Department of Biotechnology, University of Verona.

Approximately 250 nucleotides of the 5′-end region of the 26S rRNA gene were amplified by PCR with the universal primers NL-1 containing GC-clamp and LS-2, following the protocol of Cocolin et al. [27].

Separation of GC-clamped amplicons was carried out in a D-Code™ Universal Mutation Detection System (Bio-Rad, Segrate, Italy). PCR products were loaded onto a 30–60% denaturing gel and run for 16 h at 50 V at a constant temperature of 60 °C. The gel was stained with a solution containing EuroSafe colorant Acid Stain (Euroclone, Milano, Italy) to observe the bands by UV transillumination (UVITEC Gel Documentation System, Cleaver Scientific).

Dominance of the strain *Metschnikowia* sp. DBT012 over the native *Metschnikowia* spp. yeasts was assessed using the Repetitive Element Palindromic PCR (REP-PCR) typing method with the primer $(GTG)_5$, following the protocol of Pfliegler et al. [28]. PCR products were examined using 1.5% (*w*/*v*) agarose gel stained with EuroSafe colorant Acid Stain. Visualization and image capturing were made under UV with a ChemiDocXRS+ Imaging System (Bio-Rad, Segrate, Italy).

2.8. Statistical Analysis

Data of fermentation kinetics and analytical determinations were compared by three-way analysis of variance (ANOVA), followed by the post-hoc Tukey's HSD (Honestly Significant Difference) test, with statistical significance threshold set at 95% (p-value < 0.05), and Principal Component Analysis (PCA). Statistical analysis was performed using the package XL-STAT (Addinsoft SARL, Paris, France).

3. Results and Discussion

Microbiological and chemical analyses were conducted on grape musts, washing solutions, and wines to evaluate the impact of grape washing, SO_2 addition, and starter inoculation on the progress and outcomes of the lab-scale winemaking process. These three variables were combined following the experimental design shown in Figure 1.

3.1. Impact of Washing Treatment on Grape Microbiota and Metals Content

Quantitative analyses by plate counts of the grape musts from the different batches showed the minimum microbial load, of 4.56 Log CFU/mL, in the WS sample, obtained from washed grapes added with 30 mg/L SO_2. Similar concentrations were found in the other grape musts: 4.96 Log CFU/mL in NN, 4.98 in WN, and 5.02 in NS.

These results are in slight contrast with those reported by Cavazza et al. [7], who observed a reduction of the microbial load in the musts of washed Müller-Thurgau grapes, particularly with the removal of apiculate yeasts. However, that study considered fresh white grapes and a more tumultuous process of industrial washing, while the present study investigated black withered grapes which have more wrinkled berries, and a lab-scale washing.

Washing solutions were also microbiologically and chemically analyzed before and after the procedure of grape washing. As for microbiological analysis, a culture-independent approach was applied. The concentration of DNA isolated from the washing solutions before and after grape treatment was determined and considered as an indirect measure

of the removal of microorganisms from the grape surface. The solution before washing had a DNA concentration of 0.08 ± 0.03 ng/mL, while the DNA concentration rose to 3.02 ± 1.31 ng/mL after treatment, as hypothesized. PCR-DGGE was then applied to determine the identity of microorganisms whose DNA was present in washing solutions, with the known limitation that only organisms representing more than 1% in the studied community can be detected with this approach [29]. The saprophytic filamentous fungi *B. cinerea* and *P. expansum* were found in washing solutions after the treatment (Figure 2), as could be expected, since those species have been commonly detected on dried grapes [2,3,30], although their incidence can vary, depending on the drying technique and seasonal conditions [26]. Regarding yeasts, several species, typically associated with grape surface, were detected, such as *H. uvarum* and *P. membranifaciens*. *S. cerevisiae,* which represents a minor population on the surface of heathy grapes [26], was not detected, as also reported by Prakitchaiwattana et al. [31].

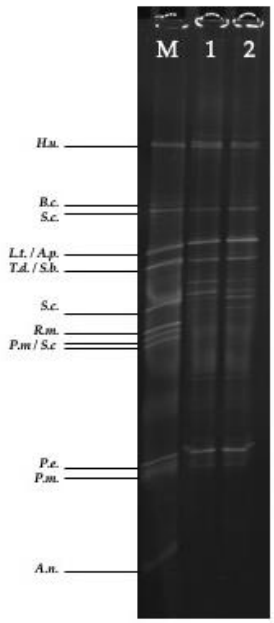


Figure 2. DGGE analysis of amplified 26S rRNA gene (PCR) products obtained from DNA extracted from the grape washing solutions after the treatment (Lane 1 and 2 are replicates). M: molecular marker prepared by loading the DNA from individual species into a single lane, containing: *Hanseniaspora uvarum* DESP52 (*H.u.*); *Botrytis cinerea* B2 (*B.c.)*; *S. cerevisiae* EC1118 (*S.c.*); *Lachancea thermotolerans* COLC27 (*L.t.*); *Aureobasidium pullulans* A1 (*A.p.*); *Torulaspora delbrueckii* PINOTG5 (*T.d.*); *Starmerella bacillaris* MALV45 (*S.b.*); *Rhodotorula mucilaginosa* CLINT2 (*R.m.*); *Pichia membranifaciens* COLBR1 (*P.m.*); *Penicillium expansum* VSA (*P.e.*); *Aspergillus niger* VSE (*A.n.*). Note: it was not possible to unequivocally distinguish between *L. thermotolerans* and *A. pullulans* (*L.t./A.p.*) and between *T. delbrueckii* and *S. bacillaris* (*T.d./S.b.*), due to the overlapping of bands from the reference strains in the marker.

As for chemical composition, an increase of 1.41 ± 0.21 mg/L of copper and 64.93 ± 12.69 µg/L of iron was observed in the washing solution after treatment. That means a removal of metals of 4.23 mg copper and 0.19 mg iron per kg of grapes washed. Metal ions present in wine can come from different sources, such as vineyard soil, agricultural practices (fertilizers and pesticides), dust residues, contamination with winemaking equipment, or through winemaking additions (fining agents, such as copper sulphate) [32–34]. Their concentration in wine, especially copper, is reduced during fermentation, as they are essential trace elements for the growth of *S. cerevisiae* [34–36]. Nevertheless, a high copper concentration (>20 mg/L) in grape must can inhibit the growth of yeasts and slow down fermentation [11,37,38], although this inhibitory concentration can differ as certain strains are able to activate mechanisms for copper resistance and adsorption [35,36,39]. Therefore,

strategies to reduce the heavy metals content in must are important not just to improve the fermentation process, but also to meet the standards related to wine quality.

3.2. Fermentation Performance

Kinetics data of alcoholic fermentations for the different batches are shown in Figure 3, where the same data are alternatively grouped and compared according to one of the three variables considered in this study, namely washing (panels a and b), SO_2 addition (c and d), and starter inoculation (e and f).

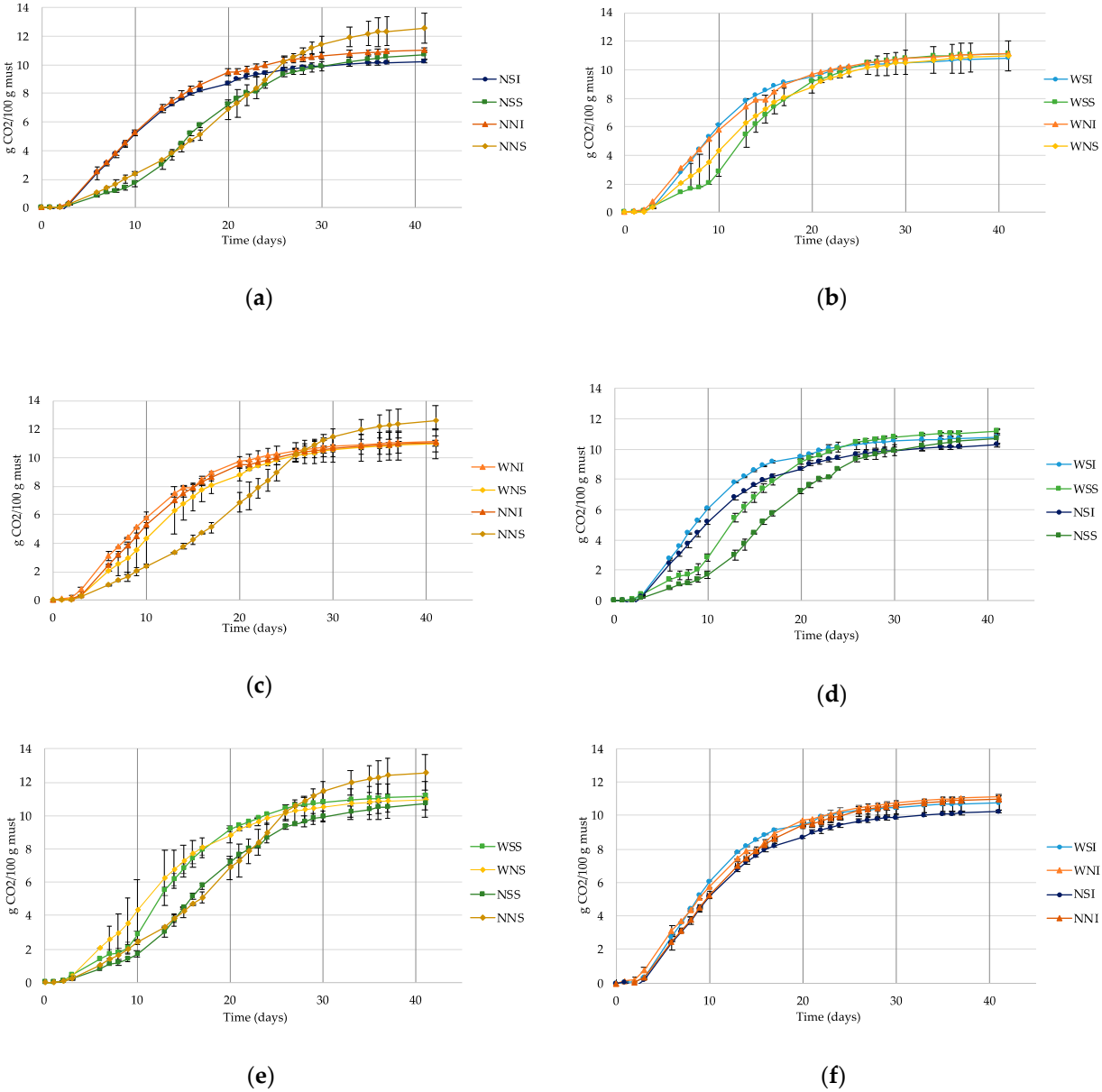


Figure 3. Fermentation kinetics of the eight types of microvinification trials of grape musts grouped according to treatment: washing (top panels, first letter N or W), SO_2 addition (middle panels, second letter N or S), and starter inoculation (bottom panels, third letter S or I). Non-washed (**a**) or washed grapes (**b**), no addition (**c**) or addition of SO_2 (**d**), spontaneous (**e**) or inoculated fermentation (**f**).

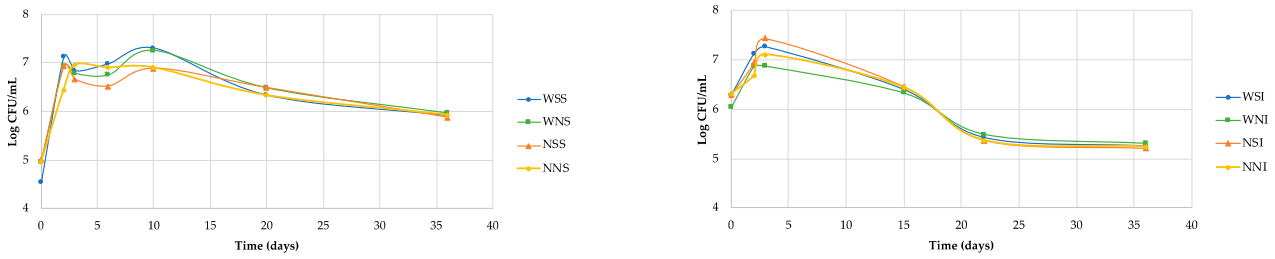
All fermentations showed a continuous consumption of sugars, albeit at different rates. Interestingly, washed grapes showed a faster fermentation rate than non-washed grapes, more evident in the first phases of spontaneous fermentations. This can be related to the

removal of substances that interfere with the yeast performances, including the metals copper and iron, as reported above. Indeed, it is known that pesticides and metals affect growth and activity of yeasts, whether they are native or commercial [7,40]. In addition, the removal of aerobic or low-fermentative yeast species and fungi with oxidative metabolism, as reported above, possibly aids the development of fermentative species and results in a faster fermentation. Nevertheless, in the samples WSS, the decrease in the initial yeast population caused by the washing and SO_2 addition could have also affected fermentative yeasts and be related to the slower fermentation rate compared to WNS in the first 10 days. In general, the SO_2 addition did not cause marked differences in the fermentation kinetics, and as expected, the inoculated trials were faster and more similar among them than the spontaneous ones, during the first half of fermentation, thanks to the fast and reliable growth of the selected starters.

Moreover, it is interesting to note the different behavior of NNS with respect to the other fermentations, since it reached the highest CO_2 production after 30 days, and the fermentative activity continued until the end of the trials. This could be related to the growth of native *S. cerevisiae* yeasts characterized by high alcohol-tolerance and fermentative activity, which were possibly suppressed by the applied interventions in the other trials.

3.3. Population Dynamics

Since starter addition had the highest impact on the fermentation kinetics, data on yeast population throughout the fermentation process were compared highlighting the inoculation strategy. After crushing, as reported above, the total yeast population in grape must was around 1×10^5 CFU/mL, with a slightly lower value for WSS (Figure 4a). The addition of *Metschnikowia* sp. DBT012 in the inoculated trials raised this concentration to about 1×10^6 CFU/mL (Figure 4b).



(**a**) (**b**)

Figure 4. Total yeast population during spontaneous (**a**) and inoculated (**b**) microvinification trials of Figure 2 (WSS and WSI), washed grapes without SO_2 (WNS and WNI), non-washed grapes with SO_2 (NSS and NSI), non-washed grapes without SO_2 (NNS and NNI).

In all fermentations, spontaneous or guided, an increase in the yeast concentration was observed in the first 48 h, then raised again in the trials inoculated with *S. cerevisiae* EC1118. Interestingly, a small decrease was observed in the spontaneous fermentations from the second to the third day, but then increased to the highest levels at day 10, greater in the musts of washed grapes compared to the non-washed ones. In both spontaneous and inoculated fermentations, it was observed that the counts after the first quarter of the fermentation onwards started to drop and became similar in all trials (washed and non-washed grapes, with and without SO_2). Nevertheless, this decrease observed until the end of fermentations was more marked in the inoculated trials, resulting in higher cell concentrations at the end of spontaneous fermentations.

In the inoculated trials, total yeast population was differentially counted as *Metschnikowia* sp. DBT012, *S. cerevisiae*, and other non-*Saccharomyces* yeasts (Figure 5). Molecular genotyping by REP-PCR performed on putative *Metschnikowia* spp. isolates (data not shown) showed

that strain DBT012 predominated over the native *Metschnikowia* spp. On the first day after inoculation of DBT012, 20–30% of the yeast population detected in the fermenting musts belonged to this strain, with a higher implantation in the must of washed grapes with SO_2 (WSI). This result is in accordance with the previous observation that grape washing and SO_2 addition removed part of the native population, favoring the inoculated starter DBT012. Even after the inoculation of *S. cerevisiae* EC1118, 48 h after the beginning of fermentations, the population of non-*Saccharomyces* yeasts, including native strains and the inoculated DBT012, was still dominating the fermentation yeastome until the 6th day, except for the trial WSI.

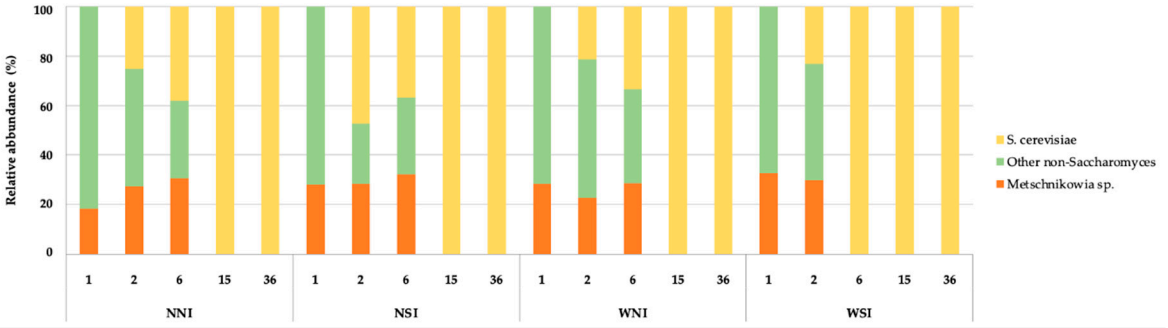


Figure 5. Yeast population dynamics in the fermentations inoculated (I) with *Metschnikowia* sp. DBT012 and sequentially with *S. cerevisiae* EC1118 after 48 h, in four different grape musts. Non-washed grapes without SO_2 (NNI), non-washed grapes with SO_2 (NSI), washed grapes without SO_2 (WNI), washed grapes with SO_2 (WSI).

The analysis after 15 and 36 days (Figure 5) revealed the dominance of *S. cerevisiae*. Indeed, it has been reported that non-*Saccharomyces* species cannot withstand the competition with the wine yeast *S. cerevisiae*, which is more tolerant to the ethanol and CO_2 produced during fermentation [19,25,41].

3.4. Chemical Analysis

The chemical composition of the wines obtained is reported in Table 1. In all wines, fermentations did not reach the completion and they were arrested with more than 30 g/L of residual sugars, without significant differences between samples. As observed in fermentation kinetics (Figure 3), although the fermentations of washed and/or inoculated grape musts were significantly faster in the first half of fermentation, at the end they all reached around the same plateau of CO_2 released from sugar consumption. Considering the high sugar concentration of these grape musts obtained from withered grapes (300.20 $\pm$ 0.14 g/L in non-washed grapes must and 291.73 $\pm$ 6.71 g/L in washed grapes must), it is possible that the combination of high alcohol with still high sugar content towards the end of fermentation caused a stress that impaired yeast activity and led to the fermentation arrest.

A high residual YAN concentration was found at the end of fermentations; thus, the depletion of nutrients was probably not determinant for causing the fermentation arrests. An initial concentration of 140 mg/L YAN is widely considered as the minimum required for yeasts to complete alcoholic fermentation. These nutrients are mainly consumed in the first days of fermentation and depleted, commonly leading to nitrogen limitation in winemaking and strongly affecting fermentation kinetics and wine characteristics. The YAN content can be very variable in different grape musts, depending mostly on grape variety, geographical origin, and ripening [42,43]. In the present study, a presumably higher consumption of YAN was noticed in spontaneous fermentations (final content 70.41 $\pm$ 6.75 mg/L) compared to guided fermentations (82.03 $\pm$ 5.51 mg/L), as the initial YAN was considered the same in all trials. In particular, the highest YAN consumption was observed in the sample WNS and the lowest in NNI. Lower final YAN values in spontaneous fermentations could be related to higher nitrogen requirements by the native yeasts in comparison with the selected starters inoculated. As demonstrated by several authors [44–47], in spontaneous fermentations, competition mechanisms for the consumption of nutrients could be triggered between the yeast species

present. On the contrary, in guided fermentations, the starters could activate mechanisms to inhibit other species with consequent lower consumption of nutrients. Depletion of nutrients could be negative in slowing down yeast activity and possibly leading to stuck or sluggish fermentation, but, on the other hand, it could be viewed as positive if the fermentation is successfully finished and little nutrition is available for the development of contaminating microorganisms [25,48,49]. No significant differences were observed in YAN consumption due to grape washing or SO_2 addition.

Table 1. Chemical analysis of wines obtained with spontaneous (S) and inoculated (I) microvinification trials of four grape musts. Washed grapes with SO_2 (WSS and WSI), washed grapes without SO_2 (WNS and WNI), non-washed grapes with SO_2 (NSS and NSI), non-washed grapes without SO_2 (NNS and NNI). Data are represented as the mean ± standard deviation of two replicates.

Samples	Residual Sugar (g/L)	CO_2 Middle (g/L) †	YAN (mg/L) ‡	Free SO_2 (mg/L)	Total SO_2 (mg/L)	Acetaldehyde (mg/L)	Acetic Acid (g/L)
WSI	44.36 ± 2.49 ^{a}	94.74 ± 0.27 ^{a}	83.20 ± 5.80 ^{a}	8.85 ± 1.20 ^{a}	66.00 ± 2.83 ^{a}	25.65 ± 0.49 ^{ab}	0.74 ± 0.13 ^{b}
WSS	34.09 ± 4.38 ^{a}	92.00 ± 0.96 ^{a}	76.05 ± 10.68 ^{ab}	7.65 ± 0.64 ^{a}	49.00 ± 2.83 ^{ab}	27.00 ± 1.41 ^{a}	1.33 ± 0.04 ^{a}
WNI	53.02 ± 8.83 ^{a}	97.07 ± 4.44 ^{a}	81.70 ± 1.27 ^{a}	9.50 ± 2.69 ^{a}	62.00 ± 8.49 ^{ab}	25.16 ± 0.54 ^{ab}	0.73 ± 0.03 ^{b}
WNS	32.62 ± 6.57 ^{a}	88.02 ± 1.05 ^{a}	62.70 ± 3.82 ^{b}	7.70 ± 1.27 ^{a}	64.50 ± 2.12 ^{ab}	28.51 ± 2.21 ^{a}	1.09 ± 0.12 ^{a}
NSI	38.02 ± 8.46 ^{a}	87.11 ± 1.93 ^{a}	75.35 ± 1.06 ^{ab}	11.30 ± 0.00 ^{a}	50.00 ± 8.49 ^{ab}	26.50 ± 0.71 ^{a}	0.78 ± 0.04 ^{b}
NSS	34.00 ± 20.40 ^{a}	72.20 ± 1.33 ^{b}	71.00 ± 0.99 ^{ab}	6.95 ± 0.49 ^{a}	46.50 ± 3.54 ^{b}	24.50 ± 0.71 ^{ab}	1.16 ± 0.01 ^{a}
NNI	42.91 ± 6.69 ^{a}	94.78 ± 2.27 ^{a}	87.85 ± 4.03 ^{a}	11.00 ± 0.14 ^{a}	50.50 ± 2.12 ^{ab}	21.50 ± 0.71 ^{b}	0.82 ± 0.01 ^{b}
NNS	49.36 ± 2.72 ^{a}	68.69 ± 7.19 ^{b}	71.90 ± 1.41 ^{ab}	6.90 ± 1.27 ^{a}	46.50 ± 2.12 ^{b}	24.00 ± 1.41 ^{ab}	1.21 ± 0.02 ^{a}

† CO_2 middle = amount of CO_2 released after 20 days of fermentation. ‡ YAN = yeast assimilable nitrogen. Different letters in the same column indicate a significant difference in HSD Tukey's test ($p < 0.05$).

Also, grape washing and SO_2 addition did not cause any significant differences in the final concentrations of free SO_2 in wine. Nevertheless, the three-way ANOVA showed that it was significantly higher when comparing the wines obtained from guided fermentations (10.16 ± 1.56 mg/L) with the wines from spontaneous (7.30 ± 0.85 mg/L). The amount of total SO_2 was also higher in guided compared with spontaneous fermentations, while acetaldehyde concentration was lower due to starter inoculation, although the differences were not statistically significant. For both total SO_2 and acetaldehyde, the only significant difference was related to grape washing, which resulted in greater values in the fermentations of washed grapes compared to non-washed (60.38 ± 8.05 mg/L versus 48.38 ± 4.17 mg/L SO_2 respectively, 26.58 ± 1.73 mg/L versus 24.13 ± 2.03 mg/L acetaldehyde, respectively).

The results highlight the complex mechanisms behind the interactions between these compounds, but some general trends were observed, such as the lower acetaldehyde content resulting in higher free SO_2, when comparing wines with similar total SO_2. Indeed, acetaldehyde is a very active carbonyl compound that may derive from yeast metabolism and can bind to free SO_2, hindering the effectiveness of SO_2 as a preservative [50–52]. Its final levels may depend on the yeast strain, but also on fermentation conditions [21,53–55]. In particular, it is known that the acetaldehyde production is higher in the presence of SO_2 [54,56]. Acetaldehyde has a perception threshold between 100 and 125 mg/L in wine, with levels above that potentially contributing with negative descriptors such as bruised apple and nutty [57]. In the present study, the maximum concentration observed was 28.51 ± 2.21 mg/L in WNS, therefore considerably below the threshold.

Regarding acetic acid, inoculation of starter cultures had an evident impact on the production of this compound, since a higher concentration was observed in spontaneous fermentations (1.20 ± 0.11 g/L) with respect to guided fermentations (0.77 ± 0.07 g/L). As concentrations above the threshold of 0.80 g/L can compromise the quality of wine [15,17], in our trials, inoculation played a central role in preventing possible faults associated with volatile acidity. Other authors already suggested the use of mixed culture fermentations to reduce acetic acid concentration in wines, in particular *Metschnikowia* spp. strains [58–60]. Nevertheless, neither grape washing nor SO_2 addition showed an impact on acetic acid production.

To better visualize the similarity between the replicates and the separation of the winemaking strategies, a PCA was carried out using chemical data of wines obtained for each fermentation (Figure 6). The first two components explained 67.74% of the variability, with PC1 accounting for 43.19% and PC2 for a further 24.55%.

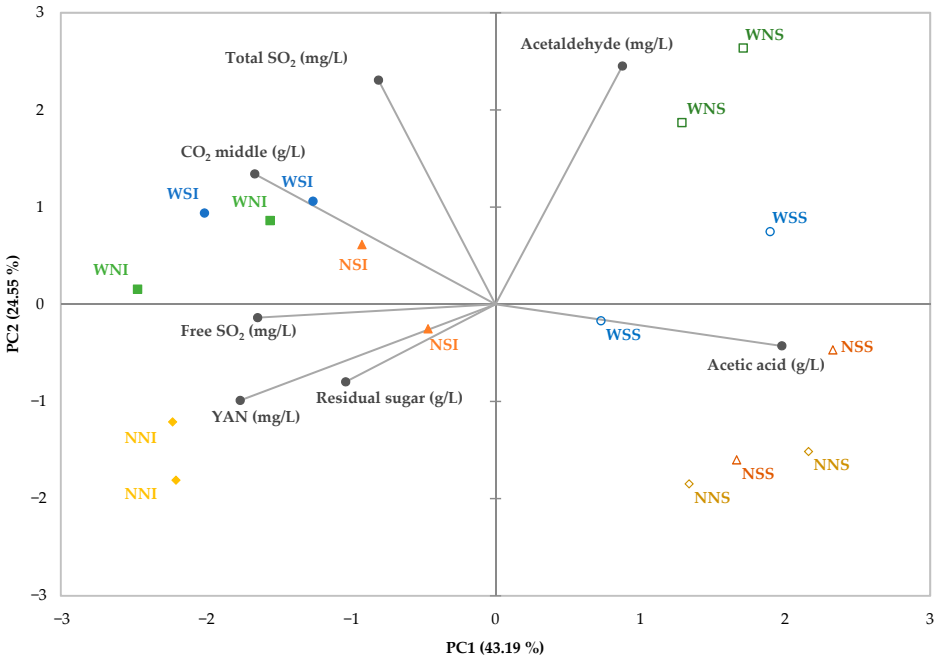


Figure 6. Bi-plot of the Principal Component Analysis (PCA) with the main enological parameters of wines obtained with spontaneous (S) and inoculated (I) microvinification trials of four grape musts. Washed grapes with SO_2 (WSS and WSI), washed grapes without SO_2 (WNS and WNI), non-washed grapes with SO_2 (NSS and NSI), non-washed grapes without SO_2 (NNS and NNI).

A good reproducibility of the replicates was observed, and some particular effects caused by the different treatments, as discussed above, were highlighted. The first component (PC1) discriminated the wines obtained from spontaneous fermentations from the ones obtained from inoculated ones, mainly based on the different content of acetic acid, YAN, and free SO_2 in the final wines and the CO_2 release by the middle of fermentation.

On the other hand, the second component (PC2) separated the wines fermented from washed grapes and the ones obtained from non-washed grapes, with differences particularly related to acetaldehyde and total SO_2.

In summary, the combined effect of grape washing and starter inoculation had a remarkable effect on the wine characteristics, while SO_2 addition did not significantly influence the chemical composition of wines. It seems that the best strategy in terms of reducing off-flavors (volatile acidity) and increasing the availability of free SO_2, thus reducing the need of SO_2 addition, is the inoculation of selected starter cultures, including or not the grape washing step before winemaking. Particularly, the multi-starter fermentation with selected strains of *Metschnikowia* spp. and *S. cerevisiae* used in the present study confirmed the previously demonstrated advantages compared to the traditional single inoculation of *S. cerevisiae* [25].

4. Conclusions

Overall results indicated that grape washing treatment applied in this investigation was effective in removing heavy metals, especially copper, and in reducing the concentration of the native grape berry microbiota, especially oxidative fungi and aerobic or low fermentative yeasts, with consequent positive impacts on the fermentation process. In both spontaneous and guided fermentation trials, a noticeable increase in fermentation activity

was observed during the first phases of the process in washed grape musts, although, as expected, yeast starter cultures' inoculation was associated with the highest fermentation rates. Grape washing and starter inoculation had different impacts on the final wine chemical composition, sometimes with synergistic effects. While washing caused a general increase of total SO_2 and acetaldehyde levels, starter inoculation augmented free SO_2 and reduced acetic acid production and YAN consumption. Addition of 30 mg/L SO_2 to grape musts, on the other hand, did not cause significant differences neither in the progress of fermentation nor in the main oenological parameters assessed at the end of fermentations.

The removal of heavy metals and spoilage organisms accomplished with the grape washing step indicate the potential of this strategy to have positive impacts on important organoleptic characteristics of wines, and, combined with the inoculation of mixed starter cultures, also in the perspective of reducing SO_2 input in winemaking. These features could be assessed in future studies, such as the wine aromatic profile and the oxidative phenomena that occur in the post-fermentative phases.

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