



ORIGINAL RESEARCH ARTICLE

A comprehensive study on the effect of foliar mineral treatments on grapevine epiphytic microorganisms, flavonoid gene expression, and berry composition

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ABSTRACT

Foliar treatments with mineral-based compounds have aroused interest as a sustainable approach to cope with high-temperature stress in vineyards, improving the colour of grapes and wines without altering the sugar concentration. Since berry-associated microorganisms are crucial for wine production, composition and stability, the present study aims to unravel the effects of kaolin and zeolite treatments on berry quality, flavonoid gene expression and epiphytic microorganisms (total fungi, *Hanseniospora uvarum*, *Metschnikowia pulcherrima*, plant-associated bacteria and lactic acid bacteria). Experiments were performed on 'Sangiovese' during ripening in two growing seasons (2019 and 2020). Both mineral treatments were able to cause the elicitation of several flavonoid-related genes and to increase the anthocyanin concentration in berries without an alteration of the final technological maturity (TSS, TA, pH) of the grapes. Interestingly, neither kaolin nor zeolites negatively affected the analysed microbial population at harvest despite transient alterations observed in the more stressful year (2019). These results support the use of foliar mineral treatments to increase anthocyanin concentration in grape berries and protect them from excessive heat without altering their native microorganisms.

KEYWORDS: climate change, kaolin, lactic acid bacteria, microbiota, *Vitis vinifera*, yeast, zeolite, ICGWS



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INTRODUCTION

Global climatic records have shown a significant intensification of extreme weather events such as heat waves, droughts, and anomalies in both the frequency and intensity of rainfall (Droulia and Charalampopoulos, 2022). In this perspective, viticulture might suffer from warm summers, with several consequences including severe berry damage (Bondada and Keller, 2012). Moreover, temperatures above 35 °C have been demonstrated to impair anthocyanin biosynthesis (Movahed *et al.*, 2016) and thus affect berry colour. Foliar treatments with mineral-based compounds, such as kaolin (a phyllosilicate white mineral) and zeolites (belonging to the mineral group of aluminium silicates), are successful short-term strategies that positively affect grapevine production (Cataldo *et al.*, 2021) by reducing canopy temperature (Dinis *et al.*, 2016; Valentini *et al.*, 2021; Petoumenou, 2023; Teker, 2023) and increasing berry quality (Bernardo *et al.*, 2022; Brillante *et al.*, 2016; Cao *et al.*, 2023). Additionally, the foliar application of kaolin has been proven to increase the total phenols, flavonoids, anthocyanins and vitamins content of berries (Brillante *et al.*, 2016; Dinis *et al.*, 2016) and enhance the photosynthetic activity of vines (Dinis *et al.*, 2018; Frioni *et al.*, 2019; Teker, 2023). Kaolin and zeolites application has also been proven to reduce the appearance of sunburn-induced damages (i.e., necrosis, shrivel and browning) (Petoumenou, 2023) and to control the occurrence of grapevine pests and diseases (Calzarano *et al.*, 2019).

Although zeolite application to the soil has been widely tested (Cataldo *et al.*, 2022, 2024), observing both a positive impact on the physiological profile of vine and on berry skin metabolism (Cataldo *et al.*, 2022), and leading to a more balanced maturation (Cataldo *et al.*, 2024), experiments testing zeolite foliar application are still in their infancy. Recent results highlight a better improvement in wine quality (Valentini *et al.*, 2021) with respect to kaolin. On the contrary, the effects of kaolin use on physiological, productive and qualitative aspects have been extensively discussed (Brillante *et al.*, 2016; Dinis *et al.*, 2016; Dinis *et al.*, 2018; Frioni *et al.*, 2019; Valentini *et al.*, 2021; Teker, 2023). In particular, the application of kaolin reduces the canopy temperature, by modifying its microclimate, to a greater extent than zeolite (Valentini *et al.*, 2021). Whether these applications might act as elicitors of secondary metabolism remains overlooked. Therefore, we decided to assess treatment-induced changes in the expression of flavonoid-related genes in berries treated with kaolin and zeolites.

Microorganisms such as bacteria, fungi and yeast are known to colonise all grapevine organs, affecting vine health, growth, and productivity (Bettenfeld *et al.*, 2022). Berry-associated microbiota may impact berry quality (Sangiorgio *et al.*, 2022a) and aroma (Sangiorgio *et al.*, 2022b), as well as, when transferred to the must, they can influence wine flavour, aroma, quality and stability. Thus, unravelling the effect of factors, such as climate, use of phytosanitary products and agronomic practices (Martins *et al.*, 2014; Sangiorgio *et al.*, 2022c) on

berry-associated microbes is pivotal for successful wine processing. In this view, we quantified the berry population of total fungi and bacteria along the season. Moreover, the effects of the treatments were evaluated on two yeasts commonly associated with grapes (*Metschnikowia pulcherrima* and *Hanseniospora uvarum*), which can impact winemaking (Romano *et al.*, 2022) and lactic acid bacteria, responsible for the malolactic fermentation (Lonvaud-Funel, 1999).

Overall, we aimed to comprehensively study, along two growing seasons, the effects of kaolin and zeolite application on the ‘Sangiovese’ variety, on the evolution of berry composition, flavonoid gene induction and berry microbial population.

MATERIALS AND METHODS

1. Experimental data and environmental data collection

The trial was conducted from 2019 to 2020, at the experimental vineyard of the University of Bologna, Italy (44°32' N–11°22' E), on variety ‘Sangiovese’ (*Vitis vinifera* L.) clone12T nine-years-old vines grafted on SO4 (*V. berlandieri* × *V. riparia*). Vines were trained to the vertical shoot-positioned spur-pruned cordon. The vines were spaced at 2.8 m between rows and 1 m within the North-South oriented rows and six two-bud spurs per vine were left with winter pruning. At flowering, each vine was pruned to uniform for the number of bunches (17 and 14 for 2019 and 2020, respectively). The vineyard was not irrigated and the soil was loamy and deep. It was subjected to one shoot trimming applied at the end of June and to the disease control program according to the Integrated Pest Management (IPM) Guidelines of the Emilia-Romagna Region. In particular, systemic and cytotropic products were applied until the beginning of berry touching (BBCH 77) whereas from BBCH 77 until 10 days before the first foliar mineral application copper and sulphur-based products were applied. In total, 8 applications were performed.

The weather conditions were recorded by a meteorological station located at the experimental vineyard and equipped with a rain gauge, thermocouples and relative humidity sensors (iFarming srl, Ravenna, RA, IT). Winkler index (Winkler, 1974) was calculated for 2019 and 2020 as

$$\sum_{01/04}^{31/10} (T_{mean} - 10).$$

The following treatments were compared in a randomised block design with three replicates of fifteen plants each:

- untreated control vines sprayed with water;
- KAO, kaolin sprayed treatment;
- ZEO, natural Italian zeolite sprayed treatment.

KAO, ZEO and C plants (Table 1) were treated twice, at the beginning and at full veraison, because this is the period when the synthesis of anthocyanins is mostly responsive to external stimuli. Additionally, the trial area (Po Valley, Italy) is in this period characterised by frequent heat waves and lots of rain.

The first application was carried out on July 30 (day of year [DOY] 211) and July 27 (DOY 209) for the years 2019 and 2020, respectively; the second application was carried out on August 7 (DOY 219) and August 5 (DOY 218) for the two years of the trial, respectively. A Knapsack sprayer (Model M3, Cifarelli, Pavia, Italy) was used for careful spraying of suspensions on the whole canopy (clusters and leaves) on both sides for a total of 400 L/ha.

TABLE 1. Details on the KAO, ZEO and C treatments.

Treatment	Active ingredient	Dose	Manufacturer
Kaolin (KAO)	Al ₂ SiO ₅ (100 %)	3 kg hL ⁻¹	Bal.Co S.p.a., Modena, Italy
Zeolite (ZEO)	52 % SiO ₂ , 17 % Al ₂ O ₃ , 12 % H ₂ O*, 6.1 % K ₂ O, 5.7 % CaO, 3.6 % Fe ₂ O ₃ , 2 % MgO, 0.6 % Na ₂ O, 0.5 % TiO ₂ , 0.3 % P ₂ O ₅ , 0.2 % MnO	3 kg hL ⁻¹	ZEOVER, Verdi, Reggio Emilia, Italy
Control (C)	Water	-	-

2. Berry quality and composition

From the first foliar applications until harvest, 4 vines for each treatment were monitored. 50 berries per vine were collected at six time points: immediately before the foliar application, 48 h after the first application, 48 h after the second application, 2 weeks after the second application, and 4 days before harvest and at harvest. Berries were crushed and then sieved for total soluble solids (TSS) and titratable acidity (TA) analysis. TSS concentration was measured by a temperature-compensating CR50 refractometer (Maselli Misure Spa, Parma, Italy). A sample of 5 mL of the same must was diluted seven times with bi-distilled water for titration using a Crison Compact Titrator (Crison, Barcelona, Spain) with 0.25 N NaOH as titrant (Sigma-Aldrich, St. Louis, MO, USA). TA data were expressed as g L⁻¹ of tartaric acid equivalents. From veraison till harvest, for a total of 5-time points (48 h after the first application, 48 h after the second application, 2 weeks after the second application, 4 days before harvest and at harvest), 20-berry sample per vine was collected by cutting through the pedicel with scissors. Berries were peeled and their skins were soaked in 100 mL methanol for 24 h, then extracts were stored at 20 °C. Total anthocyanins were separated by high-performance liquid chromatography (HPLC) using a Waters 1525 instrument equipped with a diode array detector (DAD) and a reversed-phase column (RP18 250_4.6 mm, 5_M) with a precolumn Phenomenex, Castel Maggiore, BO, Italy). Anthocyanins were quantified as mg/g of berry skin at 520 nm using an external calibration curve with malvidin-3-glucoside chloride as the standard (Sigma-Aldrich, St. Louis, MO, USA) according to Mattivi *et al.* (2006).

3. Gene expression

4 vines for each treatment were monitored from which twenty healthy berries were sampled at four sampling dates: 48 hours after the first and second treatment, two weeks after the second treatment and 4 days before harvest. Berries were peeled and their skins were immediately frozen in liquid nitrogen. Berry skins (0.2 g) were pooled together and grounded with a mortar and pestle in liquid nitrogen. RNA extraction was performed on the obtained powder using the Spectrum Plant Total RNA kit (Sigma-Aldrich, St Louis, MO, USA). RNA quality and quantity were determined using a Nanodrop-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). One microgram of extracted RNA was treated with two units of DNase I (Promega, Madison, WI, USA) and then reverse-transcribed using Improm-II Reverse Transcriptase (Promega, USA) according to the manufacturer's instructions. Real-Time quantitative PCR analysis was performed with a dilution of cDNA (1:20), to which a master mix containing SYBR Green (Applied Biosystems, Foster City, CA, USA) and the primers of the genes of interest were added. The PCR reaction was conducted on an ABI PRISM Step One Plus system (Applied Biosystems, Foster City, CA, USA), as reported by Pastore *et al.* (2013). Non-specific PCR products were identified by dissociation curves. Each reaction was performed in three technical replicates, using actin and ubiquitin as housekeeping genes. The expression of all the genes involved in the first and late steps of anthocyanin biosynthesis was assessed, and their primers retrieved from the literature: PAL1 (phenylalanine ammonia-lyase) (Belhadj *et al.*, 2008); DFR (dihydroflavonol reductase), LDOX (leucoanthocyanidin dioxygenase) and UFGT (UDP-glucose: flavonoid 3-O-glucosyl transferase) from (Goto-Yamamoto *et al.*, 2002) and F3H2 from (Jeong *et al.*, 2006). Additionally, the transcription factor MYBA1 was assessed with primers from (Jeong *et al.*, 2006). Amplification efficiency was calculated with the LinRegPCR software and used in the calculation of the MNE (Mean Normalized Expression), as reported in (Simon, 2003). The mean normalized expression (MNE) value was calculated for each sample referring to the actin and ubiquitin expressions according to the Simon equation. Statistical analysis was performed on raw data whereas MNE were presented as root squared for visualisation purposes.

4. Microbial quantification by real-time PCR

Microbial populations were quantified 48 hours after the first and second treatment and 4 days before harvest on the same berry samples used for gene expression analyses. Total DNA was isolated from the same berry skin powder used for gene expression analyses as described by Mercado *et al.* (1999).

Quantitative real-time PCR (qPCR) was carried out in an ABI PRISM Step One Plus system (Applied Biosystems, Foster City, CA, USA), to assess the colonisation levels of the total fungi, *Metschnikowia pulcherrima*, *Hanseniospora uvarum*, typical phyllospheric bacteria and lactic acid bacteria. Real-Time quantitative PCR analysis was performed with 100 ng total DNA of the sample to which a master mix containing SYBR Green (Applied Biosystems, Foster City, CA, USA)

and the primers of the genes of interest were added (Table S1). The thermal cycling conditions were as follows: 95 °C hold for 2 min followed by 40 cycles at 95 °C for 15 s, 58 °C for 30 s, 60 °C for 30 s, and 95 °C for 15 s. Each qPCR assay included duplicates of each sample, water template, and diluted control samples to create a standard curve. Non-specific PCR products were identified by the melting curves. The relative quantification of each microorganism was performed by comparing the Ct of the specific microorganism DNA with the Ct of the DNA of the samples.

5. Statistical analysis

Treatments and seasonal effects on technological ripening, anthocyanin accumulation and microbial colonisation levels were tested both by one-way analysis of variance (ANOVA) and two-way ANOVA, using the basic R studio (Core Team, 2020) function `aov`. Dealing with data expressed as percentages, microbial colonisation levels were arcsine square root transformed before ANOVA. One-way ANOVA was followed by a Tukey's test for pairwise comparison with mean separation by $P < 0.05$. Heatmap and graphs were generated using `ggplot2` (Wickham, 2011) R package.

RESULTS

1. Weather conditions during the vegetative growth and harvest

During both 2019 and 2020, grapevine growing season rainfall, average maximum (Figure 1), minimum and mean temperatures (Figure S1) were recorded. Climatic conditions during the application of kaolin and zeolite were different in the two years (Figure 1, Figure S1). Indeed, in 2019 in between the first and second treatment total rainfall was 6.5 mm, whereas in 2020, it was 68.4 mm. Thus, in 2020, the heavy rainfall immediately after the second application

resulted also in a temperature decline (Figure 1). Winkler index was calculated for 2019 and 2020 and resulted to be substantially different in the two years: 2228 and 2080 Degree Days, respectively.

2. Effect of the treatments on berry composition

In 2019, two weeks after the second spray, we detected a significant increase in TSS concentration in zeolite-treated berries with respect to control berries (Figure 2A). On the other hand, in both years, the kaolin and zeolite treatments did not result in significant changes in the concentration of sugars at harvest. Regarding TA, in 2019, at harvest, we observed a significantly higher level in kaolin-treated berries with respect to zeolite ones (Figure 2C). No effect on pH was recorded (data not shown). Two-way ANOVA revealed a significant influence of the growing season on TSS concentration and TA (Table S2).

Total anthocyanins concentration was analysed from the beginning of veraison to the harvest (Figure 3). Two-way ANOVA revealed both an effect of the treatment and of the year (Table S2). Specifically, despite the trends of anthocyanin increase along the season being similar in the two years, their content was higher in 2020 than in 2019 regardless of the treatment. In both years, the application of kaolin or zeolites increased anthocyanin content from two weeks after the second treatment until harvest. However, those differences showed significance only in 2019 starting from 265 DOY until harvest.

3. Effects of the treatments on anthocyanins gene expression

In 2019 and 2020 gene expression analyses were performed by real-time PCR in berry skin to study the transcription of proteins involved in the early (PAL1, CHS1 and F3H2) and

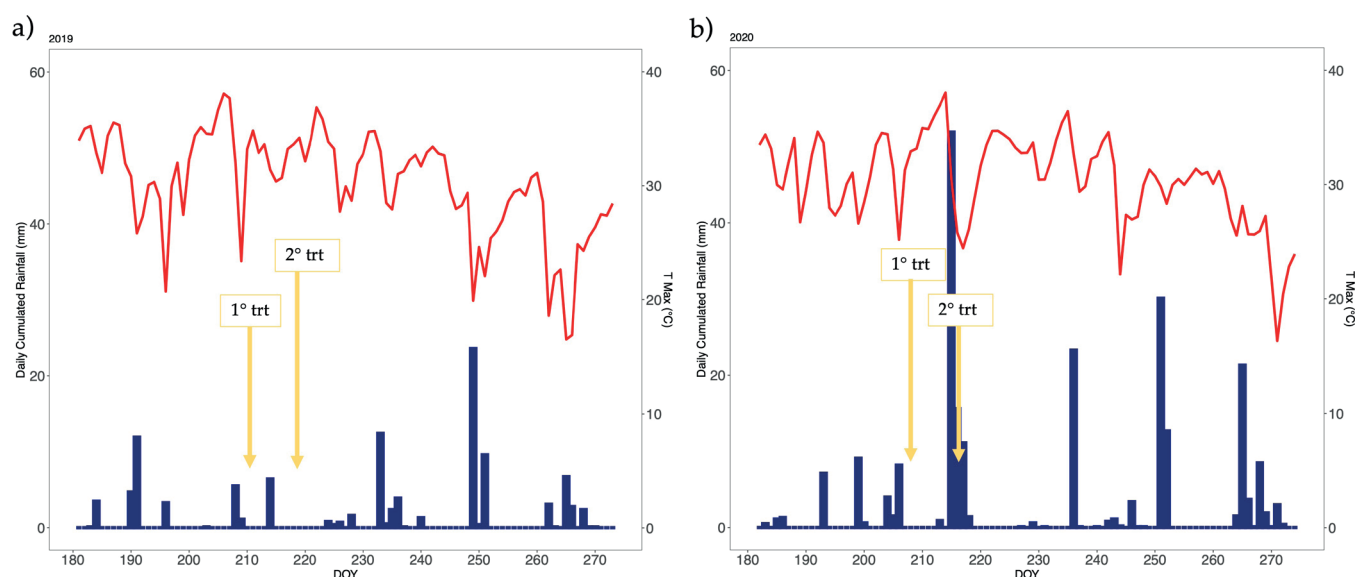


Figure 1. Daily maximum air temperature (°C, red line) and cumulated rainfall (mm, blue bar charts) from berries beginning to touch phase (BBCH 77) to harvest in (a) 2019 and (b) 2020. Kaolin and zeolite applications are indicated as yellow arrows.

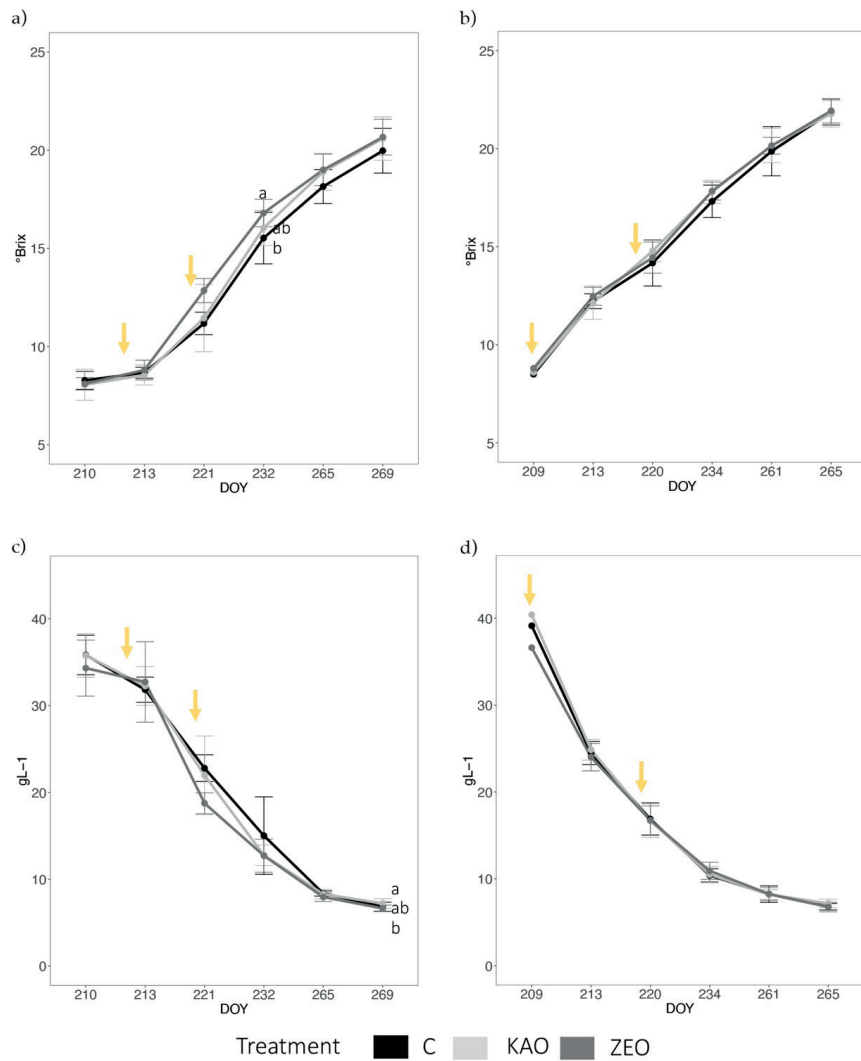


FIGURE 2. Evolution, in control (C), kaolin (KAO) and zeolite treated (ZEO) 'Sangiovese' grape berries, of TSS and TA concentration in 2019 (a and c, respectively) and 2020 (b and d, respectively). Error bars indicate standard deviation (n = 4). Letters indicate significant differences according to ANOVA and post-hoc Tukey test (p < 0.05). Yellow arrows indicate the first and second mineral applications.

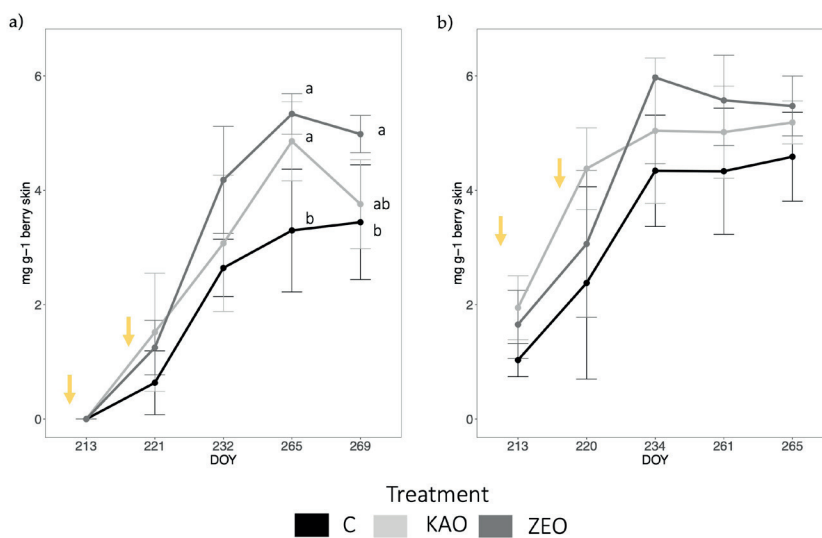


FIGURE 3. Evolution of total anthocyanin concentration in control (C), kaolin (KAO) and zeolite treated (ZEO) 'Sangiovese' grape berries in (a) 2019 and (b) 2020 growing seasons. Error bars indicate standard deviation (n = 4). Letters indicate significant differences according to ANOVA and post-hoc Tukey test (p < 0.05). Yellow arrows indicate the first and second mineral applications.

late (DFR, LDOX, UFGT and MYBA1) steps of anthocyanin biosynthesis. Additionally, a gene involved in the transport of anthocyanins to vacuole (GST4), and a gene involved in the flavonol biosynthesis (FLS4) were analysed (Figure 4). A relation was found between the effectiveness of the treatments on anthocyanin accumulation and the gene expression analyses, as differences between C, KAO and ZEO berries in terms of gene expression were detected. However, the results of gene expression analysis in the differently treated berries differed in the two growing seasons, 2019 and 2020. Regarding genes involved in the early steps of flavonoid biosynthesis, in 2019 PAL1 was significantly overexpressed in ZEO both at 48 h from the first (213 DOY) and second treatments (221 DOY). A similar result, although not statistically different, can be observed in ZEO in 2020 48 h from the first treatment (213 DOY). In 2019, CHS1, 48 h from the second treatment (221 DOY), was significantly overexpressed both in KAO and ZEO. In 2020, significant differences were found for CHS1 only before harvest (261 DOY) where KAO was downregulated in comparison to C. An overexpression of the F3H2 gene was observed in ZEO in 2019 48 h after the second application (221 DOY) whereas in 2020 KAO led to an overexpression before harvest. Concerning the genes involved in the late steps of anthocyanin formation, in 2019, 48 h after the first treatment, the DFR of ZEO-treated berries was overexpressed, whereas in 2020, a downregulation in KAO and ZEO was observed. In 2020, downregulation occurred in ZEO also at 48h after the second treatment. LDOX gene expression was not influenced by treatments in 2019, while both KAO and ZEO-treated berries were downregulated 48 h after the second treatment and overexpressed before harvest, with respect to the control. ZEO treatment led to an overexpression of the

UFGT gene 48 h after the second treatment in 2019 whereas in 2020 also KAO led to an overexpression, but it emerged 48 h from the first treatment. The transcription factor MYBA1 was overexpressed in ZEO-treated berries 48 h after the first and second treatment in 2019, whereas in 2020, a significant induction was observed in ZEO only before harvest.

No clear effect of the treatment was observed for the expression of GST4, which was stimulated in ZEO-treated berries only in 2020 (48 h after the second treatment and 4 days before harvest).

4. Effects of the treatments on the microbial population of berry surfaces

The microbial population of the surfaces of differently treated grapevine berries was analysed in 2019 and 2020 48 h after the first and second treatment and 4 days before harvest. Total fungi, *Metschnikowia pulcherrima*, *Hanseniospora uvarum* (Figure 5), phyllospheric bacteria and lactic acid bacteria (Figure 6) were quantified by real-time PCR. For the total epiphytic fungal populations, we observed, in 2020, an increase in population at 4 days before harvest with respect to the second sampling date (48 h after the second spray) (Figure 5A), whereas this increment was negligible in the first year of the experiment. In 2019, 48 h after the second treatment, the population of total fungi was greater on the surface of ZEO-treated berries than on the surface of the control berries. In both 2019 and 2020, the population of *H. uvarum* was highest at the first sampling date (48 h after the first application) and became later barely detectable (Figure 5B). Furthermore, we found that in 2019, after the second foliar application, kaolin-treated berries showed a higher quantity of *M. pulcherrima* (Figure 5C).

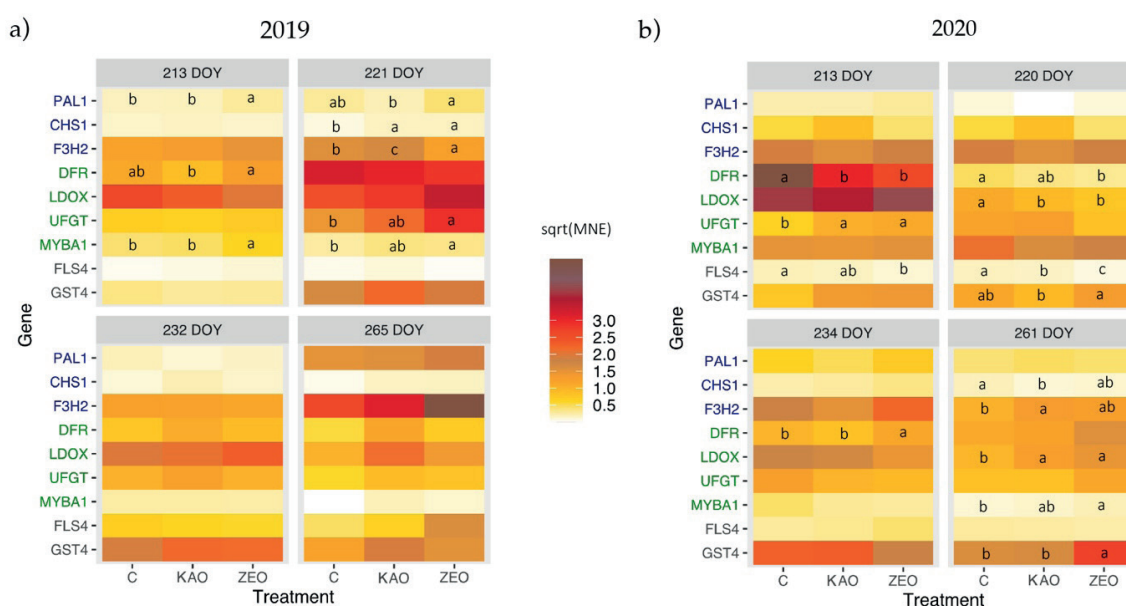


FIGURE 4. Heatmap showing anthocyanins gene expression (expressed as squared MNE) results for differently treated berries (C, KAO and ZEO) at different sampling time points in (a) 2019 and (b) 2020. Letters indicate significant differences according to ANOVA and post-hoc Tukey test ($p < 0.05$). Genes involved in the early and late steps of anthocyanin biosynthesis are blue and green coloured, respectively. Genes involved in the transport of anthocyanins to the vacuole and in the flavonol biosynthesis are grey-coloured.

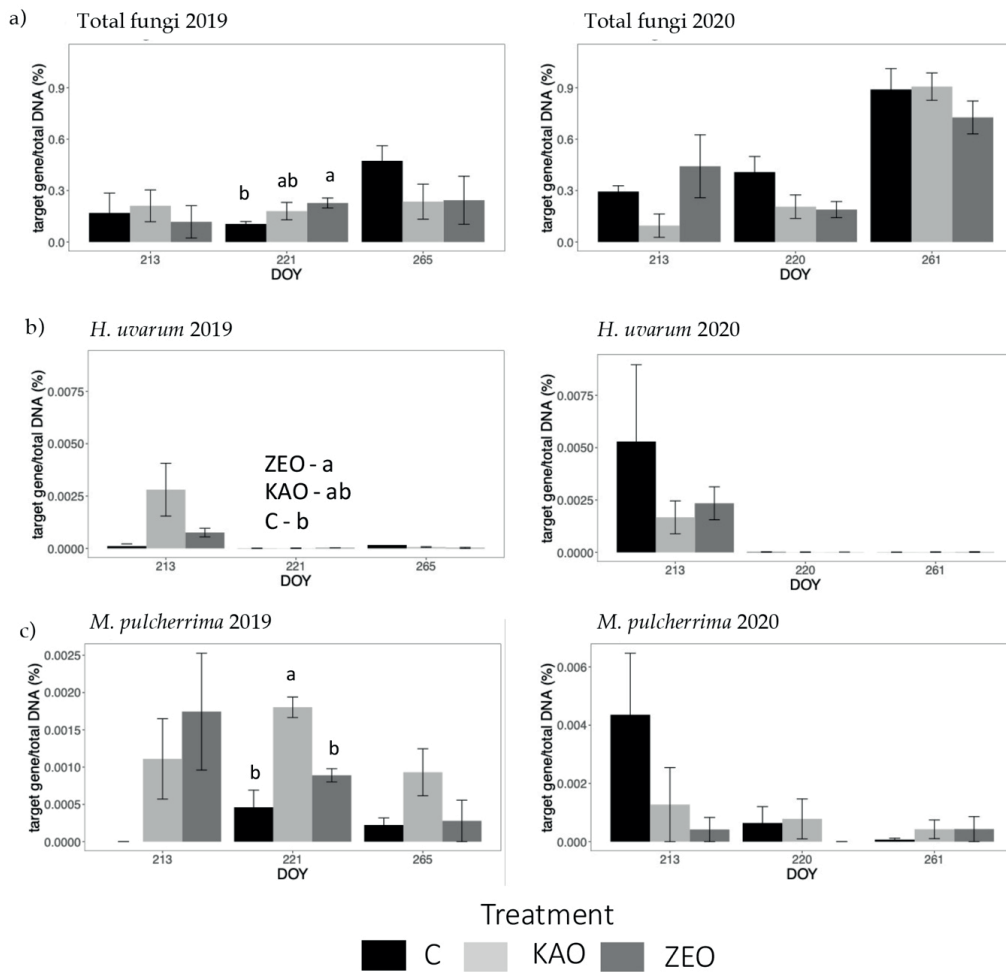


FIGURE 5. Barplots showing 2019 and 2020 (a) total fungi, (b) *H. uvarum*, (c) *M. pulcherrima* colonising berry surfaces during the growing season. Different letters indicate significant differences between treatments ($p \leq 0.05$) for each microorganism, according to Tukey's post-hoc test.

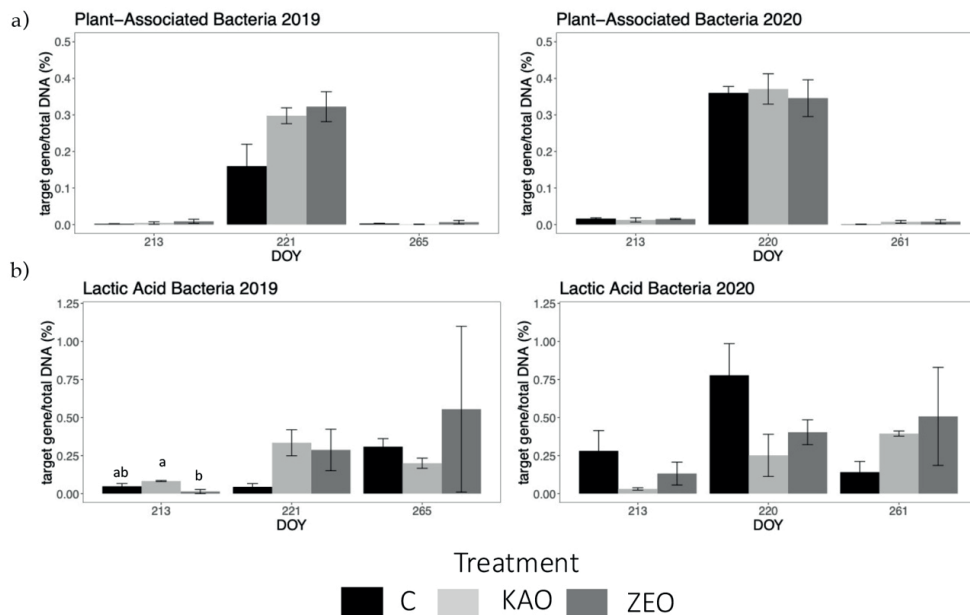


FIGURE 6. Barplots for 2019 and 2020: (a) phyllospheric bacteria, (b) lactic acid bacteria colonising berry surfaces during the growing season. Different letters indicate significant differences between treatments ($p \leq 0.05$) for each microorganism, according to Tukey's post-hoc test.

DNA amplification by primers 335f and 769r allowed the quantification of bacteria typically associated with plants (e.g., *Chryseobacterium* spp., *Stenotrophomonas rhizophila*, *Sphingomonas* spp., *Pantoea vagans*, *Rhizobium* spp. and *Ruminococcus albus*) (Dorn-In *et al.*, 2015). Both in 2019 and 2020, we observed an increase in bacterial populations at the second sampling date (48 h after the second application) with respect to the first and third time points (Figure 6A, Table S2). No treatment effect was detected in the epiphytic bacterial populations. Regarding lactic acid bacteria (LAB), we observed a significant difference between treatments only in 2019 at the first sampling date, being kaolin-treated berries populated by higher LAB levels with respect to zeolite ones (Figure 6B).

DISCUSSION

1. Effects of the treatments on berry composition and gene expression of anthocyanins

Climate change-induced heat waves might cause a decoupling between technological and phenolic maturity (Sadras and Monzon, 2006; Toda and Balda, 2015; Allegro *et al.*, 2020). Thus, the foliar application of mineral compounds could mitigate this divergence by reducing berry temperature (Dinis *et al.*, 2016; Valentini *et al.*, 2021; Petoumenou, 2023; Teker, 2023). In agreement with previous studies (Petoumenou, 2023; Teker, 2023), looking at the soluble solid content evolution, we did not observe an effect of the treatments, with the exclusion of the significant increase registered in 2019 two weeks after the second treatment with ZEO. Higher temperatures boost the activity of malate enzymes, leading to a reduction of the malic acid content of berries (Sweetman *et al.*, 2014). Kaolin has been demonstrated to have a longer cooling effect on berries (Valentini *et al.*, 2021). Thus, the longer cooling effect of kaolin may explain the higher acidity found in kaolin-treated berries with respect to control and zeolite.

Synthesis and accumulation of anthocyanins are influenced, in a cultivar-dependent manner, by the complex interaction of genes with climatic conditions, cultural practices and cultivation sites (Arapitsas *et al.*, 2012; Dal Santo *et al.*, 2018). In particular, in ‘Sangiovese’, anthocyanin accumulation has been demonstrated to be highly dependent on temperature, with prolonged temperatures above 35 °C significantly reducing their synthesis (Pastore *et al.*, 2017). The year 2019 was warmer and drier in respect to 2020. Indeed, although anthocyanin accumulation in the differently treated berries (C, KAO, ZEO) occurred similarly in the two years (being KAO and ZEO characterised by a higher anthocyanins concentration with respect to C), an effect of the season was detected, being 2020 characterised by a general higher anthocyanin accumulation. A boost of anthocyanins content of berries following foliar kaolin application was observed also in cv Touriga Nacional (Dinis *et al.*, 2016; Conde *et al.*, 2016), Merlot (Song *et al.*, 2012) and in Cabernet Sauvignon (Brillante *et al.*, 2016). In this work, we observed a lower anthocyanins concentration in KAO

with respect to ZEO, which might be partly attributed to the reduced photosynthetic activity of KAO-treated vines. Indeed, in Valentini *et al.*, 2021, we observed a reduction of the photosynthetic activity right after kaolin application.

Besides increasing the ability of berries to minimise potentially damaging radiation and lowering grape temperatures (Dinis *et al.*, 2016; Valentini *et al.*, 2021; Petoumenou, 2023; Teker, 2023), kaolin and zeolite were hypothesised to elicit the anthocyanins biosynthetic pathway. In this work, the expression of genes related to flavonoid biosynthesis was analysed in differently treated berries. The application of both kaolin and zeolite enhanced the expression of anthocyanins-related genes in at least one sampling date. However, climatic factors such as rainfall seem to play a role in determining product effectiveness and thus the timing of activation of genes. Indeed, besides being a cooler year, 2020 was characterised by a heavy rainfall event that occurred right after the application of the second treatment, which might have provoked partial leaching of the product and a subsequent alteration of its efficacy with respect to the previous year. However, a third application to supply for the product leaching was avoided to maintain the same number of treatments and timing as for 2019. In ZEO-treated berries, an elicitation of transcription was detected for the PAL1 gene both after the first and second treatment in 2019 and, although not statistically different, also in 2020 after the first treatment. These results shed light on the possible mechanisms underlying ZEO effectiveness in increasing anthocyanin concentration. Thus, zeolite treatment activated the anthocyanins gene expression more often than kaolin suggesting a different mechanism of action between the two minerals. Our findings are strengthened by results obtained on table grape where natural zeolite was able to significantly enhance different antioxidants, total antioxidant activity and phenolics, flavonoids and carotenoids contents (Huwei *et al.*, 2021).

An increase in the abundance of genes related to flavonoid biosynthetic and transport pathways (phenylalanine ammonia lyase, chalcone synthase, flavonol synthase, UDP-glucose:flavonoid 3-Oglucosyltransferase, glutathione S-transferase, tonoplast anthocyanin transporters) in black cv. ‘Touriga nacional’ vines treated with kaolin was observed (Conde *et al.*, 2016; Dinis *et al.*, 2018). However, gene activation following mineral treatments greatly depends on the grapevine cultivar (Bernardo *et al.*, 2022). In our work, although we observed that kaolin application affected the regulation of flavonoid-related genes, its effects on anthocyanin accumulation were mostly linked to the improvement of canopy microclimatic conditions, lowering berry temperature (Valentini *et al.*, 2021; Valentini *et al.*, 2022).

2. Effect of the treatment on microbial colonisation of berries

Grapevine fungal microbiome changes according to plant development (Liu and Howell, 2021) and environmental variables (Barata *et al.*, 2012). In samples collected in 2020, we found an increase in fungal populations close to

harvest. This agrees with other works (Čadež *et al.*, 2010; Combina *et al.*, 2005) that found that colder harvests with higher rainfall lead to higher yeast counts on berry surfaces, which might be attributed to the preference of some fungal strains for humid and mild-cold conditions.

Sugar accumulation and exudation from berries favour the multiplication of epiphytic fungi, especially close to harvest. In 2019, sugar levels of zeolite-treated berries were higher with respect to C and KAO despite not in a statistical significant manner at 48 h after the second mineral application (DOY 221), which could explain the higher fungal levels found, at that sampling, in zeolite-treated berries. *H. uvarum* and *M. pulcherrima* are two of the most abundant species of yeasts, present on grape surfaces, which have an impact also on winemaking. Their abundance may vary depending on plant location and environmental conditions (Brilli *et al.*, 2014; Gómez-Albarrán *et al.*, 2021). In our work, both species showed a reduction over time despite the treatment applied, except for *M. pulcherrima* levels that were higher than the control upon kaolin treatment in 2019. Several consequences of kaolin spray, such as berry and leaf temperature modification (Janick, 2010; Valentini *et al.*, 2021), transpiration levels modification (Janick, 2010), and anthocyanins accumulation (Calzarano *et al.*, 2019; Valentini *et al.*, 2022) might be responsible for *M. pulcherrima* altered levels. On the grape surface, *Aureobasidium pullulans* is one of the most represented yeast-like fungi (Gómez-Albarrán *et al.*, 2021). This aspect may explain why a reduction in some yeast species was observed while total fungi remained stable or increased over time.

Interestingly, bacterial population levels were higher close to veraison in both years analysed. In grapevine, an influence of the developmental stage on berry bacterial microbiome composition has been observed (Ding *et al.*, 2021). Veraison is one of the most pivotal stages for grape metabolism and growth, being the moment when anthocyanin production and accumulation, pectin and cellulose degradation, acidity reduction, sugar accumulation, and berry softening occur. Thus, all these processes might play a role in the changes observed in the epiphytic bacterial levels, by creating a more favourable environment for microbial colonisation (Renouf *et al.*, 2005).

Lactic acid bacteria are mainly responsible for malolactic fermentation, an important secondary fermentation that occurs in many wines, generally about 2–3 weeks after completion of the alcoholic fermentation (Lonvaud-Funel, 1999). In our work, no effect of the treatment was detected on lactic acid bacteria, except for in 2019, where a slight increase in population was observed at the first sampling date on kaolin-treated berries.

Altogether, despite a general increasing effect of the treatments on microbial populations 48 h after the second treatment in 2019, treated grapes did not show significant differences in microbial abundance at harvest.

CONCLUSION

In this work, we aimed to unravel the effects of kaolin and zeolite treatments on berry quality, flavonoid gene expression and epiphytic microorganisms. Gene expression analyses revealed a higher elicitation of anthocyanins-related genes in zeolite-treated plants compared to kaolin, strengthening the hypothesis that while kaolin effects are mostly linked to improvement of grape microclimatic conditions, zeolite might act as elicitor of flavonoid-biosynthesis-related genes. This question, however, demands further study and analysis of RNA sequencing could reveal major insights into the mechanisms of action of foliar mineral treatments. Although fungal and bacterial levels fluctuated over the growing season, being seasonality and rainfall events key factors for determining the grape-associated microbial populations, treated grapes did not show significant differences in microbial abundance, and to some extent composition, at harvest. These results support the use of foliar mineral treatments to ameliorate anthocyanins concentration in grapes berries, protecting them from excessive heat without altering their native microbiota.

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