ORIGINAL RESEARCH ARTICLE

Triacontanol (long-chain alcohol) positively enhances the microbial ecology of berry peel in *Vitis vinifera* cv. 'Glera' yet promotes the must total soluble sugars content

Andrea Colautti[1], Fabrizio Golinelli[2], Lucilla Iacumin[1], Diego Tomasi[2], Pierpaolo Cantone[2] and Giovanni Mian[1,2]*

[1] Department of Agricultural, Food, Environmental and Animal Science, University of Udine, via delle Scienze 206, Udine (UD), 33100, Italy
[2] Council for Agricultural Research and Economics-Research Centre for Viticulture and Oenology, Viale 26 Aprile, 31015 Conegliano, Italy

*correspondence:
giovanni.mian@uniud.it

ABSTRACT

Grapevine is one of the most important woody perennial crops in Italy, both in terms of cultivated area and economic income. In recent years, the identification of effective and integrated tools for plant nutrition and manipulation to improve plant health status in agriculture has become increasingly important to lead towards environmentally friendly viticulture. In this sense, plant-associated microbiota of berry carposphere structure and function significantly impact vines performance (must fermentation and final wine profile). For this, a recently developed biostimulant rich in triacontanol (TRIA) obtained from *Fabaceae* tissues has been tested in grapevine cv. Glera. Our study aimed to understand how TRIA application affected the fungal and prokaryotic communities, which is important for fermentation and the final wine olfactory profile. Taxonomical analysis revealed that TRIA application did not lead to significant differences in the number of species among kingdoms, therefore, not reducing the biodiversity of the grapevine carposphere. However, it did modify the ratio of certain species, such as increasing those with biocontrol effects and reducing the number of indigenous yeasts. Finally, the TRIA application resulted in a simple and cost-effective strategy to induce an earlier harvest with higher sugar content, which is important for fermentation. These results suggest the potential of using TRIA to obtain healthier grapes with cleaner sensory profiles during fermentation.

KEYWORDS: biostimulant, bacteria, berry peel, fungi, grapevine, metagenomics, sugars

INTRODUCTION

In recent years, improving plant health using effective and integrated tools for plant nutrition and manipulation is becoming increasingly important in agriculture. The search for applied innovations includes the cultivation of vines, which are high-value woody perennial crops that, given their increased global economic importance and relative expansion of the cultivated area, must aim for increasingly precise and effective practices to preserve the environment. (Viers *et al*., 2013). Several studies on biostimulants are currently being conducted in this regard. Different works have demonstrated their effectiveness as growth promoters and stress relievers, making their application in the field attractive due to the potential reduction in reliance on chemical fertilisers and pesticides and their leaching into groundwater, as well as stress factors such as climate change and extreme weather conditions (Monteiro *et al*., 2022). Furthermore, their foliar application is becoming increasingly appealing due to their ability to prevent plant diseases and improve grapevine berry quality (Jamiołkowska, 2020). However, given the critical importance of microbiota in grapevine plant health as well as the oenological relevance (Bettenfeld *et al*., 2022), it is necessary to investigate the possible selective effect on the microbial ecology that these products can cause (Lau *et al*., 2022). Potential microbiota modifications on grape berries are even more significant when considering the importance of the latter on the final aromas and organoleptic characteristics of the obtained wines, which are also typical of the geographical location, using the so-called French term 'terroir' but influenced by a variety of factors as well (Mezzasalma *et al*., 2017, Carpena *et al*., 2021, Bokulich *et al*., 2016, Liu *et al*., 2020, Tomasi *et al*., 2022). Plants provide a rich and diverse habitat for a diverse range of microorganisms, the majority of which contribute to the growth and health of their plant hosts (Luziatelli *et al*., 2019). Most of these microorganisms can promote plant growth through a variety of mechanisms, including changes in hormonal content, the production of volatile compounds, increased nutrient availability, and improved abiotic stress tolerance (Ruzzi and Aroca, 2015). Plant growth-promoting activity of epiphytic microbes can be affected by environmental conditions, including exposure to biostimulants/fertilisers or their degradation products (Timmusk *et al*., 2017). The surface of the grape berry (carposphere) is inhabited by microbiota of filamentous fungi, yeasts, and bacteria that can affect grape and wine quality (Lleixà *et al*., 2018). The diversity and population sizes of the microbiota are altered when the grape surface is modified (for example, by applying exogenous products). The ecological alteration of the grape affects the vinification process and the final wine quality, typically adding flavours and/or changing their composition (Steel *et al*., 2013). Thus, it is critical to investigate further the microbiota diversity changes in treated grapes and their impact on alcoholic fermentation, as well as how biostimulants application can influence these parameters. Even though the use of biostimulants is becoming more common in sustainable agriculture, little information is available on the effect of these products on the epiphytic bacterial and fungal microbiota. Foliar application of biostimulants can, thus, affect the epiphytic microbiota, promoting the growth of bacteria and fungi that can be beneficial, neutral, or harmful to plants (Colla *et al*., 2017). Plant-associated microbiota structural and functional changes significantly impact the ecosystem, altering antagonistic and synergistic interactions among microorganisms and improving host fitness by increasing plant metabolic capacity, nutrient uptake, and plant response to abiotic and biotic stresses (Valencia *et al*., 2018). Given these considerations, this study aimed to investigate the foliar application of a newly developed biostimulant from a microbiological standpoint. This product derived from *Fabaceae* tissue, whose properties on the ripening dynamic and wine must technological parameters in *Vitis vinifera* were analysed in a previous study (Mian *et al*., 2022a), is high in amino acids, peptides, and various stimulating compounds, as well as a high presence of natural triacontanol (TRIA) $C_{30}H_{62}O$ (> 6 mg kg^{-1}) (Figure 1), developed by the company ILSA, already available on the market (commercial name: ILSAC-ON). This saturated long-chain alcohol, found in epicuticular waxes, is a natural plant growth regulator that has been shown to promote growth in various plants when supplied exogenously. Several studies have shown that it improves plant growth, yield, photosynthesis, protein synthesis, water and nutrient uptake, nitrogen-fixation, enzyme activities, and the contents of free amino acids, higher sugars, soluble protein, and active constituents of essential oil in a variety of crops (Naeem *et al*., 2012).
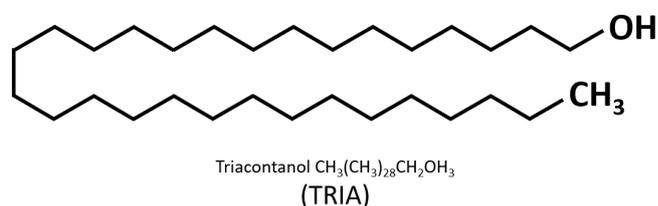

Triacontanol $CH_3(CH_2)_{28}CH_2OH_3$
(TRIA)

FIGURE 1. Structural formula of triacontanol (TRIA)

Following treatment in an open-field environment, DNA was extracted and sequenced. The goal was to understand how TRIA application can affect a single community (bacteria and fungi) in terms of the genus, as well as the implications for fermentation and the final wine olfactory profile since it was not reported before. Grapevines treated with TRIA were compared to untreated grapevines of the cultivar 'Glera' (one of the five most cultivated varieties in the Friuli-Venezia Giulia Region, together with other regions within the Italian sector).

MATERIALS AND METHODS

1. Experimental design

The experiment was carried out in a commercial vineyard of *Vitis vinifera* cultivar 'Glera,' located in the Cormons (Gorizia, Italy) geographical area (45.928155 N, 13.443721 E) during the 2021 vintage. The vines were 21 years old and were trained to a guyot trellis 1.50 m above ground.
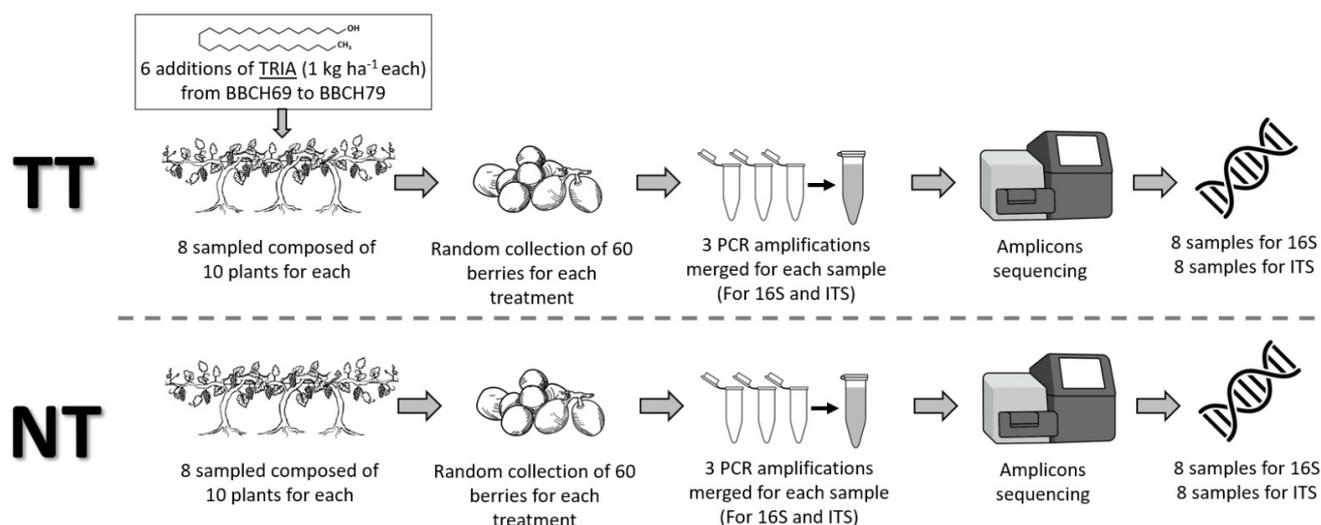
FIGURE 2. Experimental workflow for the two treatments

The intra- and inter-row vine spacing was 0.90 m × 2.30 m south–north, for a density of approximately 4500 plants/ha. The whole following adopted workflow was summarised in Figure 2. Meteorological data is available online (repository: https://www.osmer.fvg.it/archivio.php?ln=).

For the two treatments (TT: treated, NT: non-treated), the randomised experimental design was adopted (de Oliveira Cantao and Mian, 2023) and consisted of 8 replicates made up of 10 plants for each treatment for a total of 160 considered plants, leaving out a row of plants to avoid possible cross-contamination. For both treatments, local standard practices were followed for fertilisation and pest management, applying 150 kg ha^{-1} of a balanced fertiliser (15N, 10P, 20K + Mg + S) and adopting commercial products as generally used in non-integrated management without performing emergency irrigation. On the TT trial plants, a commercial biostimulant whose composition is reported in Table 1 (commercial name ILSAC-ON) was applied six times during the growing season, beginning just after flowering and finishing at the veraison onset (measured on the Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie scale from BBCH 69 to BBCH 79), following manufacturer's instructions (6 kg ha^{-1} per year). No biostimulant applications were performed on the NT plants. The product was applied through an experimental nebuliser specifically built for use.

2. Sampling, DNA extraction and amplification

Berries were sampled right before harvest time (~20 °Brix, i.e., the first decade of September, DOY 249 (Day Of the Year) both for NT and TT and then handled under sterility. For each sample, the peel of 60 berries was put into a 50-mL Falcon tube containing 30 mL of epiphyte removal buffer pH 6.5 (6.75 g of KH_2PO4, 8.75 g of K_2HPO_4, and 1 mL of Triton X-100, to 1 L of deionised water). Samples were then sonicated at 600 Hz with a cycle of 30 s of sonication and 30 s without sonication for 10 minutes at 4 °C. The tubes were centrifuged at 12,000 × g for 10 min at 4 °C to

pellet microorganisms' cells, which were separated from the supernatant and stored at −80 °C. Genomic DNA extraction was carried out using NucleoSpin Plant II (Carlo Erba, Italy), following the manufacturer's instructions. The concentration and quality of the DNA samples were verified using the NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific, Inc., Wilmington, DE, USA) to obtain 25 ng/μL diluted DNA samples. Using Polymerase chain reactions (PCR), the ITS and 16S regions, for fungi and bacteria, respectively, were amplified, where each sample intended for sequencing (8 for the TT and 8 for the NT of 16S, and 8 for the TT and 8 for the NT of ITS for a total of 32 sequenced samples) was obtained from the bulking of 3 amplification products. For bacterial 16S rRNA gene targeting the V3-V4 region amplification, the PCR was performed using the following mixture: 10 μL of 2 × Dr. MAX Master Mix Solution (Doctor Protein Corp., Seoul, Korea), 1 μM of 341F (5'- CCTACGGGNGGCWGCAG 3'), 805R (5'- GACTACHVGGGTATCTAATCC -3') primer set, and 25 ng of the extracted DNA as a template. For eukaryotic fungal ITS region amplification, the same protocol was performed with bacteria using a different primer set (1 μM of ITS3 (5'- GCATCGATGAAGAACGCAGC -3') and ITS4 (5'- TCCTCCGCTTATTGATATGC -3'). PCR amplification was performed with an initial denaturation at 95 °C for 7 min, followed by 30 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 107 °C for 10 min. Amplification was ascertained by running amplicons on electrophoresis gel for 1.5 hours, on TBE (tris-borate EDTA) gel (1.5 % agarose), and subsequently purified using GenElute™ Microbiome DNA Purification Kit (Merck). The amplicons were then sequenced using the Illumina MiSeq platform (Illumina Inc., San Diego, CA) by an external company introducing the Nextera adapters.

3. Bioinformatic analysis

Subsequent bioinformatic analyses were performed using QIIME2 (Bolyen *et al*., 2019) software package (v2021.11). The obtained raw paired-end sequences were filtered,

TABLE 1. Summary of biostimulant composition from the commercial product label

	Parameter	Value	Tolerance
	Total amino acids	5.0 %	-
	Free amino acids	1.5 %	-
	Hydrolysis degree	30.0 %	-
	Triacontanol of natural origin	> 6 mg/kg	-
	Dry matter	30.0–38.0 %	-
	Organic matter	21.0–25.0 %	-
Chemical parameters	Ash	6.0–9.0 %	-
	Chlorides (Cl°)	0.6–0.8 %	-
	Physical parameters		
	Electrical conductivity 1:100 (dS/m)	1.20–1.80	-
	Density (kg/dm3)	1.14	± 0.02
	pH	4.5–5.5	-
	Mw protein component (g/mol)	7096	400

	Parameter	Value	Method of analysis
	Enterobacteriaceae (UFC/g)	< 10	ISO 21528-2 2004
Microbiological analysis	*Salmonella* spp.	Absent in 25 g	UNI EN ISO 6579-1:2017
	Aerobic biodegradability	Biodegradable	OECD 310:2014

trimmed, denoised, and merged using Cutadapt (Martin, 2011) and DADA2 (Callahan *et al.*, 2016), yielding Amplicon Sequence Variants (ASVs). Taxonomy was assigned to ASVs using the sklearn naïve Bayes taxonomy classifier (Bokulich *et al.*, 2018). For training the classifier based on primer sequences used for PCR amplification, SILVA v.138 (Quast *et al.*, 2013) was used for the taxonomic analysis of the 16S reads, while for the analysis of the ITS reads the Unite (Nilsson *et al.*, 2019) v8.3 dynamic 2021-05-10 database with all eukaryotic species was used. Sequences assigned to chloroplasts, mitochondria, with frequency < 2 (singletons), or non-identified were filtered out from the dataset. Subsequent statistical analyses were conducted via R v4.1.2. The rarefaction curves on species richness were calculated using ggrare (ranacapa package (Kandlikar *et al.*, 2018)). After rarefaction on the samples implemented through the phyloseq package (McMurdie & Holmes, 2012), the Alpha-diversity analysis was carried out. Significant effects were tested using a pairwise Kruskal–Wallis H test, with results considered significant when the *p*-value was < 0.05. To graphically represent the microbiological differences between the two treatments, a Principal Coordinates Analysis (PcoA) using Bray–Curtis dissimilarity matrices were constructed using the vegan package (Oksanen *et al.*, 2013) evaluating the presence of a significative effect of the treatment with a PERmutational Multivariate Analysis Of Variance (PERMANOVA) based on 4999 permutations.

4. Berry must chemical composition form veraison to harvest time

From veraison onset to harvest time (typically made at around 18–23 °Brix), 160 berries per treatment were sampled weekly and stored at –80 °C for a total of six surveys. At the time of harvest, the total sugars content was measured using a refractometer (Atago PR32) at 23 °C. Total acidity (titratable acidity expressed as g L^{-1} of tartaric acid) and pH were measured using an automatic titrator (Crison Micro TT 2022) by titration with 0.1N NaOH solution.

RESULTS

1. Sequencing features

The bacterial and fungal communities were identified using 16S rRNA and ITS metagenomic sequencing analyses. After filtering low-quality, short, and chimera sequences, 450,132 high-quality reads from 16S rRNA gene sequencing samples and 270,048 high-quality reads from ITS gene sequencing samples were obtained. The 16S rRNA and ITS reads were clustered into 4758 ASVs and 1763 ASVs, respectively. After assessing the sequencing coverage of detected bacterial and fungal ASVs in the samples by rarefaction analysis, the rarefaction curves reached saturation at approximately 8000 and 3800 sequencing depths, respectively, for bacterial and fungal communities, suggesting that the sequencing procedure reached an acceptable proportion of the biological

species richness associated with the berry carposphere of bacterial and fungal communities.

2. ITS sequencing

The analysis of alpha diversity provided a preliminary picture of how the two treatments, TT and NT, influenced fungal ecology. The intragroup difference between TT and NT treatments, based on the evaluation of Species Richness (Figure 3A) and Shannon diversity (thus, also considering the relative abundance of the species) (Figure 3B) was slightly higher for both indicators for the TT treatment. However, statistical analysis revealed no significant differences ($p > 0.05$) between the two treatments, indicating that, despite minor differences, TRIA did not cause appreciable differences in the variability of the fungal population in the plants where it was applied.

Similarly, it was impossible to identify a clear separation of clusters in the microbiota community by comparing the two treatments using beta diversity analysed via Bray–Curtis dissimilarity (Figure 4). In fact, despite a slight separation trend and a high percentage of explained variability, no statistically significant difference was evidenced by the PERMANOVA analysis. As a result, TRIA did not lead to significant differences in fungal biodiversity on the grape bunch.

By analysing the relative percentages of the main genera present (> 0.1 %), the principal differences brought by the treatments to the fungi population were identified. It was possible to observe 15 main genera (Figure 5). *Aureobasidium* and *Alternaria*, which alone for both treatments constituted over 50 % of the genera present (49.43 % and 40.50 % for *Aureobasidium* and 13.04 % and 12.90 % for *Alternaria*, respectively, for TT and NT treatments), were the most present genera. It was also possible to identify important groups of yeasts such as *Saccharomyces* (9.89 % TT,
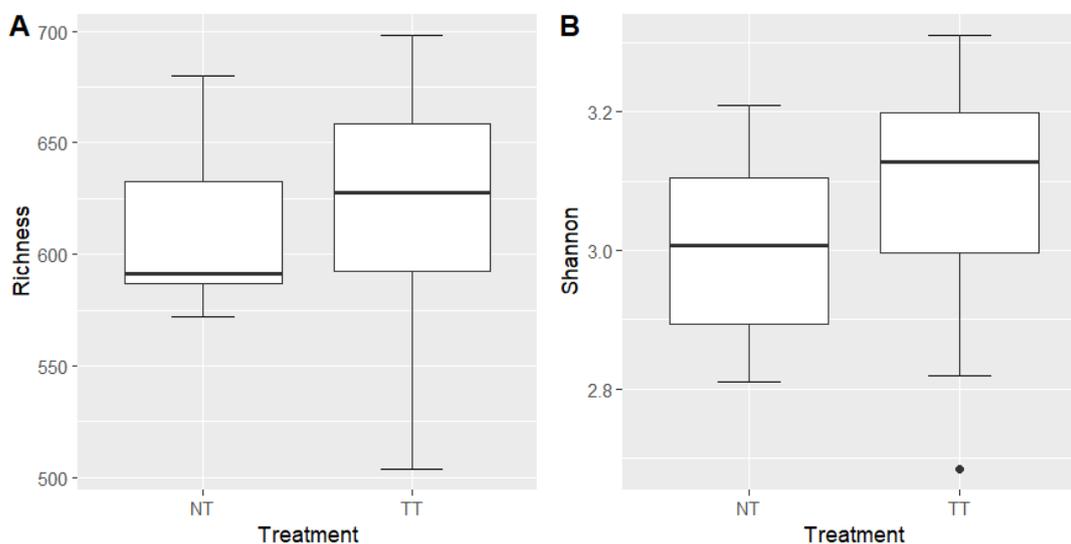

FIGURE 3. Statistical analysis of fungi alpha-diversity. Data are the mean of 8 replicates per treatment. The dots in the figure represent the outliers. Significant effects were tested using a pairwise Kruskal–Wallis H test ($p < 0.05$).
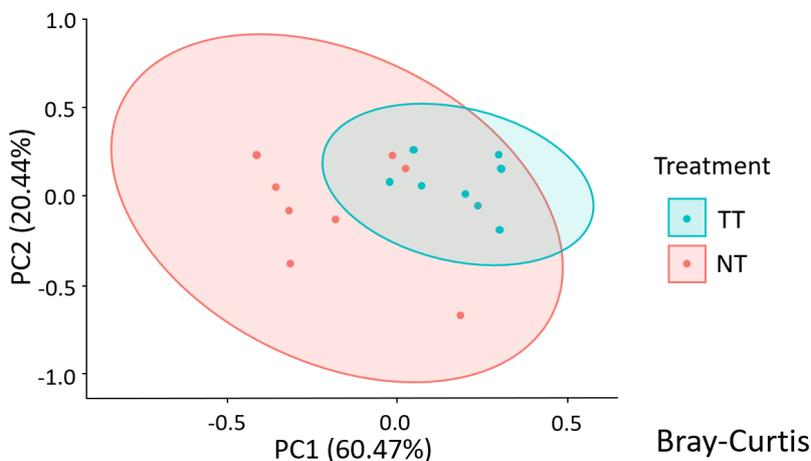

FIGURE 4. Beta diversity of samples plotted with R software regarding treatments in berry carposphere calculated using Bray–Curtis dissimilarity. The percentage presence on the quadrant shows the explained variability.
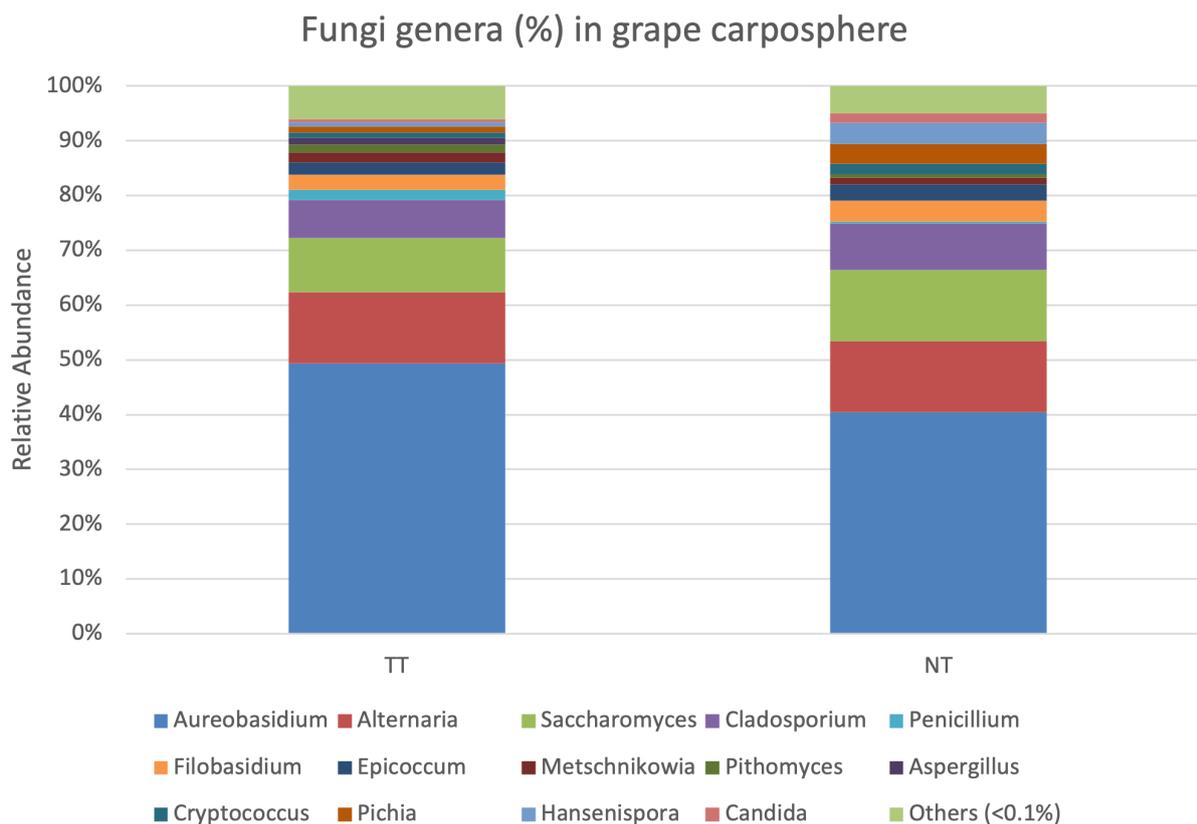
FIGURE 5. Fungi found in berry carposphere. Data are the percentage (%) of ASVs number for each microorganism based on the total identified ASVs. *p*-value and standard deviation are available in Table S1.
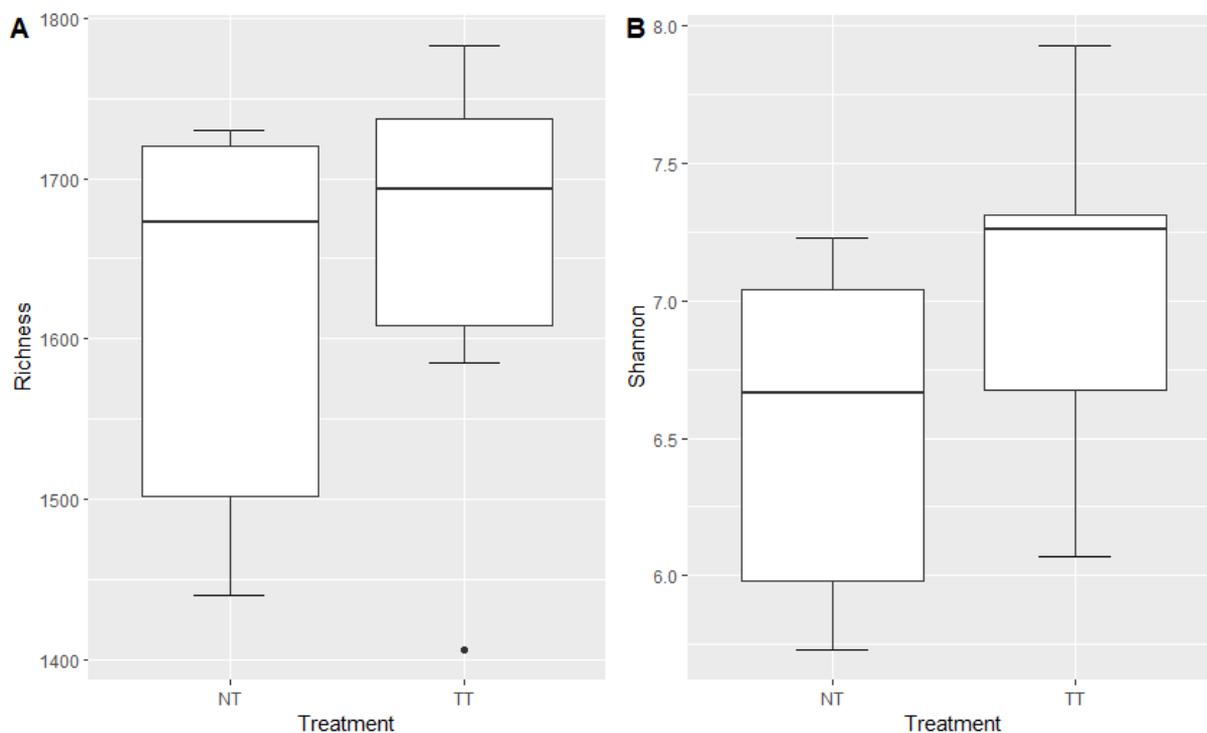

FIGURE 6. Statistical analysis of bacterial and fungi alpha-diversity. Data are the mean of 8 replicates per treatment. The dots in the figure represent the outliers. Significant effects were tested using a pairwise Kruskal–Wallis H test ($p < 0.05$).

13.05 % NT), *Metschnikowia* (1.88 % TT, 1.16 % NT), *Pichia* (1.03 % TT, 3.57 % NT), *Hanseniaspora* (0.80 % TT, 3.88 % NT) and *Candida* (0.57 % TT, 1.73 % NT), along with other ascomycetes such as *Cladosporium* (6.96 % TT, 8.47 % NT), *Penicillium* (1.84 % TT; 0.26 % NT), *Filobasidium* (2.74 % TT, 3.87 % NT), *Epicoccum* (2.12 % TT, 3.03 % NT), *Pithomyces* (1.46 % TT, 0.69 % NT), *Aspergillus* (1.15 % TT, 0.001 % NT), and *Cryptococcus* (1.05 % TT, 1.90 % NT). Analysing the results obtained, it was possible to identify significant differences for the genera *Penicillium* (p < 0.05), *Pithomyces* (p < 0.05), *Aspergillus* (p < 0.001), *Pichia* (p < 0.05), *Hanseniaspora* (p < 0.001), and *Candida* (p < 0.001) (Table S2). Of these, *Penicillium*, *Pithomyces*, and *Aspergillus* presented higher percentages for the TT

samples, while *Pichia*, *Hanseniaspora* and *Candida* were present in higher percentages in the NT samples.

2.1. 16S RNA gene sequencing

Alpha diversity analysis of bacteria provided an outcome similar to the fungi kingdom: the intragroup difference between TT and NT treatments based on the evaluation of Species Richness (Figure 6A) and Shannon diversity (Figure 6B) was only slightly higher for both indicators for the TT treatment. However, statistical analysis revealed no significant differences (p > 0.05) between the two treatments, indicating that, despite minor differences, TRIA did not cause appreciable variations in the variability of plants where it was applied.
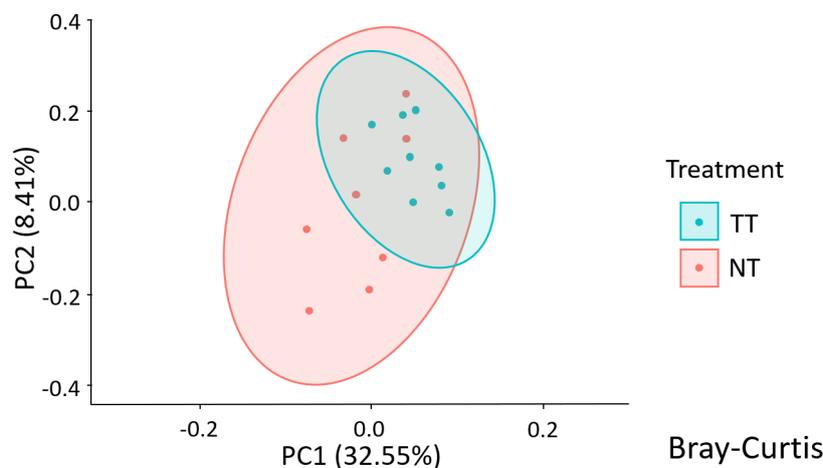

FIGURE 7. Beta diversity of samples plotted with R software regarding treatments in berry carposphere. The percentage presence on the quadrant shows the explained variability.

Even comparing the beta diversity values (Figure 7), no significant differences were highlighted between the two groups from the PERMANOVA, indicating that the application of TRIA did not induce effects on biodiversity compared to untreated samples.

Analysing the differences in the proportions of bacterial genera present in berry carposphere, it was possible to identify 18 genera above 0.1 % (Figure 8 and Table S3). Most of the bacteria identified were Gram-negative (91.75 % for TT and 88.92 % for NT), in particular, belonging to the genera *Undibacterium* (33.16 % TT, 10.01 % NT), *Pedobacterium* (20.78 % TT, 13.16 % NT), *Burkholderia* (18.18 % TT, 6.86 % NT), *Massilia* (12.04 % TT, 10.11 % NT), *Sphingomonas* (4.06 % TT, 4.71 % NT), *Pantoea* (1.67 % TT, 4.94 % NT), *Mesorhizobium* (0.71 % TT, 1.89 % NT), *Hymenobacter* (0.61 % TT, 0.90 % NT), *Methylobacterium* (0.20 % TT, 0.50 % NT), *Gluconobacter* (0.12 % TT, 15.47 % NT), *Pseudomonas* (0.12 % TT, 0.25 % NT) and *Flexibacter* (0.10 % TT, 0.13 % NT). Of these, a significantly higher presence was identified in the TT treatment for *Pedobacter*, *Burkholderia*, and *Massilia*, while the genera *Pantoea*, *Mesorhizobium*, *Brevibacterium*, *Methylobacterium*, *Gluconobacter*, *Pseudomonas* and *Flexibacter* were found

to be present in significantly higher percentages in the NT control. Gram positives represented by *Rhodococcus* (3.96 % TT, 3.60 % NT), *Paenibacillus* (2.00 % TT, 4.00 % NT), *Brevibacterium* (0.49 % TT, 1.74 % NT), *Staphylococcus* (0.07 % TT, 0.16 % NT), *Bacillus* (0.07 % TT, 0.63 % NT) and *Lactobacillus* (0.04 % TT, 0.12 % NT) instead constituted the minority part of the bacterial genera found. For these, only the genera *Paenibacillus*, *Staphylococcus* and *Lactobacillus* were detected at higher percentages in the NT control.

2.2. Chemical analysis

As regards the chemical characteristics of the must evaluated throughout the veraison-ripening stage, it was possible to observe a significant increase in the sugar content in the grapes obtained by applying TRIA. In fact, while for the values of total acidity and pH, there were no deviations between TT and NT during the whole monitoring, instead as regards the value of °Brix, a significant difference was observed (p-value < 0.05) with a higher concentration of 2.14 °Brix in favour of the TT treatment (20.53° for TT, 18.39° for NT) (Figure 9). Thus, there was a delay in NT that was no longer recovered since the technological sugar ripening of TT for this wine was brought forward, being putative harvested 4 days earlier than NT.
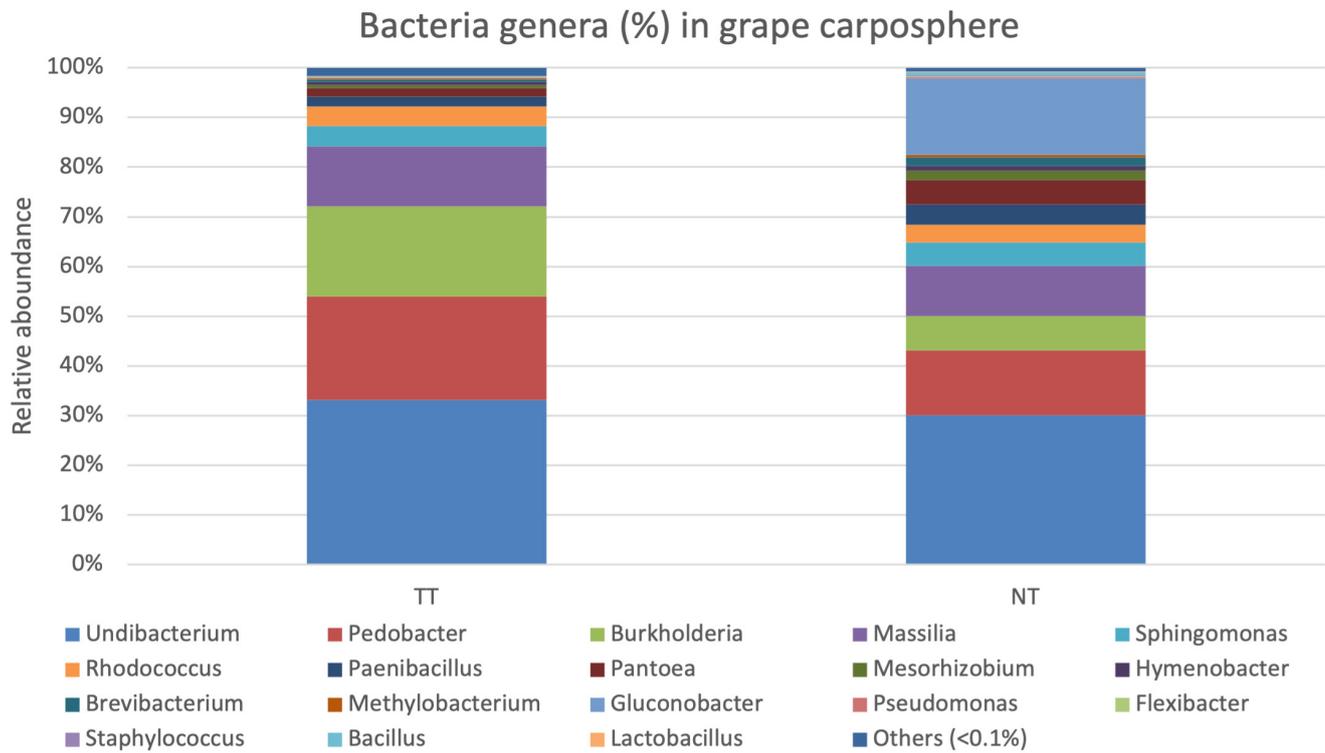
Bacteria genera (%) in grape carposphere

FIGURE 8. Bacteria found in the berry carposphere. Data are the percentage (%) of ASVs number for each microorganism based on the total identified ASVs. *p*-value and standard deviation are available in Table S2.
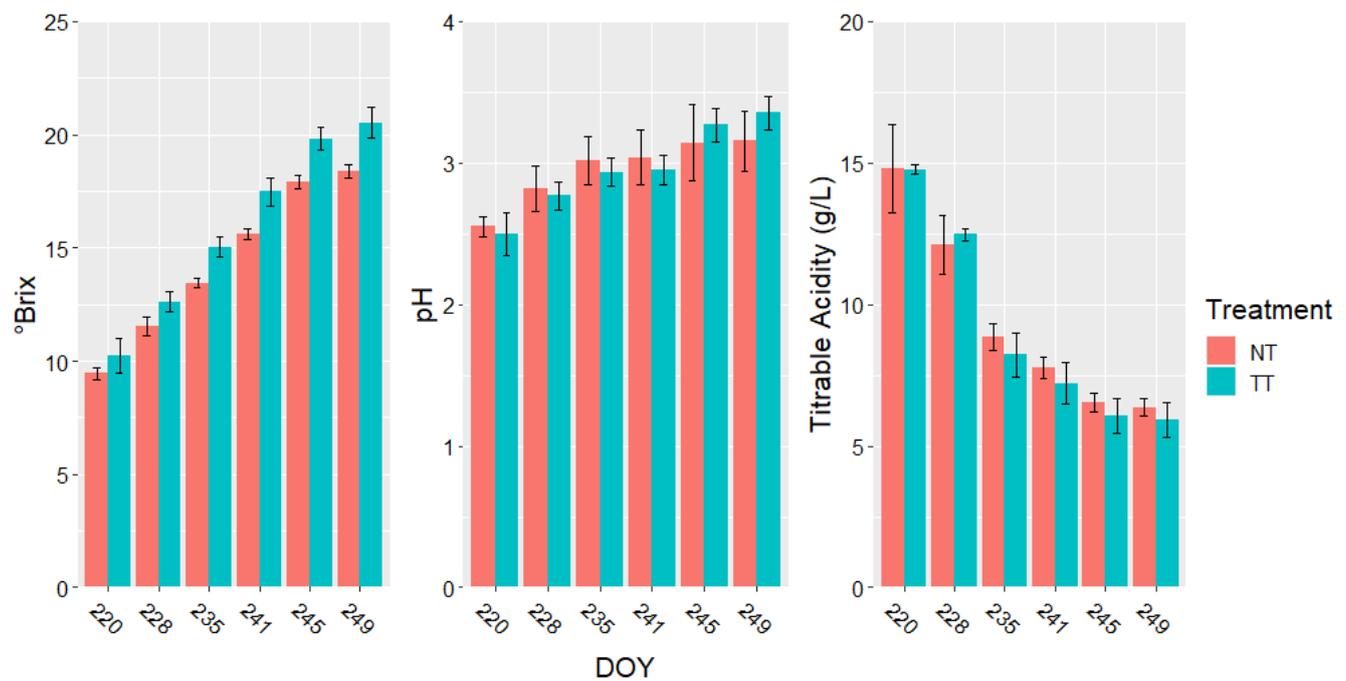

FIGURE 9. Data of soluble solids (°Brix), pH and titratable acidity, of TT and NT musts recorded in the ripening stage. Days are expressed as Day Of Year (DOY).

DISCUSSION

Grapevine is one of the most important woody perennial crops in Italy, both economically and in terms of cultivated area (Tomasi *et al.*, 2020). Given the high extension of this crop with its relative environmental impact (Schaller, 1991, Serpa *et al.*, 2017, Lamastra *et al.*, 2016), an increasingly important challenge for the future is the management of vineyards with a sustainable approach. For this purpose, biostimulants offer interesting properties for grapevine growers and winemakers (Mian *et al.*, 2022b), which, however, should maintain a rich and diversified microbial population in the vineyards to give typicality to the product obtained during fermentation (i.e., *Terroir*) (Miliordos *et al.*, 2022, Samuels *et al.*, 2022, Droby and Wisniewski, 2018, Padmaperuma *et al.*, 2019). With regards to the use of TRIA, this was the first study concerning its effect on the microbiota of the grapevine carposphere.

Alcohols generally exert an antimicrobial effect on both kingdoms (fungi and bacteria).

However, analysing the alfa and beta diversity of the two kingdoms, TRIA did not have a negative effect in terms of the biodiversity of the microbial population, albeit with changes in the abundance of some species (Belda *et al.*, 2017).

As for the bacteria, it was, in fact, possible to observe a significantly higher relative percentage of the most abundant genera in the TT treatment, specifically for the *Pedobacter* and *Burkholderia genera*. *Pedobacter* spp. possess genes coding for lanthipeptides, which have antifungal activities (Caetano *et al.*, 2020), whilst *Burkholderia* spp. isolates have been exploited for the biological control of plant pathogens and growth promotion (Coenye and Vandamme, 2003). Similar functions were also observed for *Paenibacillus* spp. (McSpadden Gardener, 2004), however, in this case, present in higher percentages in the NT treatment. Other genera that were observed in lower percentages in treatment TT than in control NT were *Pantoea*, *Mesorhizobium*, *Brevibacterium*, *Methylobacterium*, *Gluconobacter*, *Pseudomonas*, and *Lactobacillus*. *Brevibacterium* is an important genus known as a biocontroller of anthracnose of grapes (Arfaoui *et al.*, 2019). *Methylobacterium* spp., found at almost a two-fold higher percentage in the NT trial, were suggested to stimulate plant development through phytohormone production (Zarraonaindia *et al.*, 2015). Of note is the drastic reduction of the genus *Gluconobacter* present to a percentage of 0.13 % for the TT treatment compared to a percentage equal to 15.47 % in the NT control. This genus exerts a slight effect on plant health status, as it was found able to reduce the mycelia growth of *Botrytis cinerea*, *Aspergillus* spp., *Penicillium* expansum and Rhizopus stolonifera (Delgado *et al.*, 2021). On the other hand, *Gluconobacter* is reported to produce a great number of Volatile Organic Compounds (VOCs), which might influence the sensory profile of the resulting wines. Indeed, *Staphylococcus* spp. strongly correlates to plant/grape health status due to the production of biogenic amines, which are undesirable compounds in all foods and beverages because they induce food-borne intoxications when consumed at high concentrations (Benavent-Gil *et al.*, 2016), and in this sense, TRIA application reduced its presence. Lastly, the *Lactobacillus* genus was found to be present in greater quantities in NT. This bacterial group is of significant oenological interest as during winemaking, it can contribute to the aromatic and organoleptic profile of the final product (Virdis *et al.*, 2021), as well as altering the sensory profile.

Considering the fungi kingdom, also in this case, significant differences were found at the genera level. The *Penicillium* genus was identified in higher percentages in the TT than in the NT trial. This microorganism influences berry health, as many species belonging to this genus can lead to the onset of rot (Jahani *et al.*, 2020). Similarly, the *Aspergillus* genus, known as a pathogen (Kasfi *et al.*, 2018), was also found to be present at higher concentrations in the TT samples. However, a greater presence was also found for the genus *Pithomyces* in TT. This is an important genus since it has biocontrol capabilities against *Botrytis cinerea* (grey mould) on grapes (Dodd and Stewart, 2003), which can negatively influence both fermentation and sensory profile. It is also possible to observe how three important groups of yeasts such as *Pichia* spp., *Hanseniaspora* spp. and *Candida* spp., were negatively influenced. These results may be due to the inhibitory effect of alcohols on yeasts (Iacumin *et al.*, 2022). Yeasts of the genus *Pichia* and *Candida* were reported to have biocontrol capacity and able to contrast grey mould (Fiori *et al.*, 2008, Saligkarias *et al.*, 2002); therefore, their decrease could be considered negative, while the reduction of *Hanseniaspora* spp. in some cases could be considered advantageous, as strains of these non-*Saccharomyces* yeasts could lead to undesired flavours during fermentation (Jolly *et al.*, 2014).

Regarding must technological parameters, we can state how the biostimulant application positively affected the sugar content, as already reported (Sharma *et al.*, 2018), yet has led to the possibility of earlier harvesting. A harvest brought forward must be taken as a general advantage for reaching the end of the ripening process. In fact, bringing forward the whole technological maturity can result in a lower risk of phytoiatric issues (Iltis *et al.*, 2020), and vine growers can also be present on the market earlier than any other competitors.

CONCLUSIONS

The exogenous application of TRIA resulted in a simple and cost-effective strategy to get a trend towards the different abundance of some bacteria and fungi genera while also leading to an earlier harvest with higher sugar content. Additionally, our taxonomical analysis showed that the application of TRIA reduced the amount of indigenous *Saccharomyces* yeast. This is of great importance since it is possible to avoid natural fermentation, whereas Prosecco wine is of enormous interest as it is crafted/fermented using specific strains. TRIA promoted *Pithomyces* spp., important as a bio controller of grey mould, giving a great chance to get fewer undesirable flavours.

ACKNOWLEDGEMENTS

REFERENCES

Arfaoui, M., Vallance, J., Bruez, E., Rezgui, A., Melki, I., Chebil, S., Sadfi-Zouaoui, N. & Rey, P. (2019). Isolation, identification and in vitro characterization of grapevine rhizobacteria to control ochratoxigenic *Aspergillus* spp. on grapes. *Biological Control*, *129*(November 2018), 201–211. https://doi.org/10.1016/j.biocontrol.2018.10.019

Belda, I., Ruiz, J., Esteban-Fernández, A., Navascués, E., Marquina, D., Santos, A. & Moreno-Arribas, M. V. (2017). Microbial contribution to Wine aroma and its intended use for Wine quality improvement. *Molecules*, *22*(2), 1–29. https://doi.org/10.3390/molecules22020189

Benavent-Gil, Y., Berbegal, C., Lucio, O., Pardo, I. & Ferrer, S. (2016). A new fear in wine: Isolation of Staphylococcus epidermidis histamine producer. *Food Control*, *62*, 142–149. https://doi.org/10.1016/j.foodcont.2015.10.026

Bettenfeld, P., Cadena i Canals, J., Jacquens, L., Fernandez, O., Fontaine, F., van Schaik, E., Courty, P. E. & Trouvelot, S. (2022). The microbiota of the grapevine holobiont: A key component of plant health. *Journal of Advanced Research*, *40*, 1–15. https://doi.org/10.1016/j.jare.2021.12.008

Bokulich, N. A., Collins, T., Masarweh, C., Allen, G., Heymann, H., Ebeler, S. E. & Mills, D. A. (2016). Fermentation Behavior Suggest Microbial Contribution to Regional. *MBio*, *7*(3), 1–12. https://doi.org/10.1128/mBio.00631-16.Editor

Bokulich, N. A., Dillon, M. R., Zhang, Y., Rideout, J. R., Bolyen, E., Li, H., Albert, P. S. & Caporaso, J. G. (2018). q2-longitudinal: Longitudinal and Paired-Sample Analyses of Microbiome Data. *MSystems*, *3*(6). https://doi.org/10.1128/msystems.00219-18

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, *37*(8), 852–857. https://doi.org/10.1038/s41587-019-0209-9

de Oliveira Cantao, F. R., & Mian, G. Spinosad Application Prevents Damage by Agriotes spp. Larvae (Wireworms) and Protects Maize (Zea mays) Yield in Northeast Italy. *Plant Protection* 7.1 (2023): 101-108. DOI: 10.33804/pp.007.01.4549

Caetano, T., van der Donk, W. & Mendo, S. (2020). Bacteroidetes can be a rich source of novel lanthipeptides: The case study of Pedobacter lusitanus. *Microbiological Research*, *235*(November 2019), 126441. https://doi.org/10.1016/j.micres.2020.126441

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A. & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Carpena, M., Fraga-Corral, M., Otero, P., Nogueira, R. A., Garcia-Oliveira, P., Prieto, M. A. & Simal-Gandara, J. (2021). Secondary aroma: Influence of wine microorganisms in their aroma profile. *Foods*, *10*(1), 1–26. https://doi.org/10.3390/foods10010051

Coenye, T. & Vandamme, P. (2003). Diversity and significance of Burkholderia species occupying diverse ecological niches. *Environmental Microbiology*, *5*(9), 719–729. https://doi.org/10.1046/j.1462-2920.2003.00471.x

Colla, G., Hoagland, L., Ruzzi, M., Cardarelli, M., Bonini, P., Canaguier, R. & Rouphael, Y. (2017). Biostimulant action of protein hydrolysates: Unraveling their effects on plant physiology and microbiome. *Frontiers in Plant Science*, *8*(December), 1–14. https://doi.org/10.3389/fpls.2017.02202

Delgado, N., Olivera, M., Cádiz, F., Bravo, G., Montenegro, I., Madrid, A., Fuentealba, C., Pedreschi, R., Salgado, E. & Besoain, X. (2021). Volatile organic compounds (Vocs) produced by gluconobacter cerinus and hanseniaspora osmophila displaying control effect against table grape-rot pathogens. *Antibiotics*, *10*(6). https://doi.org/10.3390/antibiotics10060663

Dodd, S. L. & Stewart, A. (2003). RAPD-PCR and UP-PCR techniques distinguish a Pithomyces chartarum isolate with biocontrol capabilities against Botrytis cinerea on grape (*Vitis vinifera*). *New Zealand Journal of Crop and Horticultural Science*, *31*(1), 55–64. https://doi.org/10.1080/01140671.2003.9514235

Droby, S. & Wisniewski, M. (2018). The fruit microbiome: A new frontier for postharvest biocontrol and postharvest biology. *Postharvest Biology and Technology*, *140*(January), 107–112. https://doi.org/10.1016/j.postharvbio.2018.03.004

Fiori, S., Fadda, A., Giobbe, S., Berardi, E. & Migheli, Q. (2008). Pichia angusta is an effective biocontrol yeast against postharvest decay of apple fruit caused by Botrytis cinerea and Monilia fructicola. *FEMS Yeast Research*, *8*(6), 961–963. https://doi.org/10.1111/j.1567-1364.2008.00424.x

Iacumin, L., Colautti, A. & Comi, G. (2022). Zygosaccharomyces rouxii is the predominant species responsible for the spoilage of the mix base for ice cream and ethanol is the best inhibitor tested. *Food Microbiology*, *102*(October 2021), 103929. https://doi.org/10.1016/j.fm.2021.103929

Iltis, C., Moreau, J., Pecharová, K., Thiéry, D. & Louâpre, P. (2020). Reproductive performance of the European grapevine moth Lobesia botrana (Tortricidae) is adversely affected by warming scenario. *Journal of Pest Science*, *93*(2), 679–689. https://doi.org/10.1007/s10340-020-01201-1

Jahani, M., Beheshti, M., Aminifard, M. H. & Hosseini, A. (2020). Effects of Essential Oils to Control Penicillium Sp. In In Vitro and in In Vivo on Grapevine (*Vitis Vinifera* L.) Fruit. *International Journal of Fruit Science*, *20*(S2), 812–826. https://doi.org/10.1080/15538362.2020.1769526

Jamiołkowska, A. (2020). Natural compounds as elicitors of plant resistance against diseases and new biocontrol strategies. *Agronomy*, *10*(2). https://doi.org/10.3390/agronomy10020173

Jolly, N. P., Varela, C. & Pretorius, I. S. (2014). Not your ordinary yeast: Non-Saccharomyces yeasts in wine production uncovered. *FEMS Yeast Research*, *14*(2), 215–237. https://doi.org/10.1111/1567-1364.12111

Kandlikar, G. S., Gold, Z. J., Cowen, M. C., Meyer, R. S., Freise, A. C., Kraft, N. J. B., Moberg-Parker, J., Sprague, J., Kushner, D. J. & Curd, E. E. (2018). Ranacapa: An R package and shiny web app to explore environmental DNA data with exploratory statistics and interactive visualizations [version 1; referees: 1 approved, 2 approved with reservations]. *F1000Research*, *7*, 1–19. https://doi.org/10.12688/f1000research.16680.1

Kasfi, K., Taheri, P., Jafarpour, B. & Tarighi, S. (2018). Characterization of antagonistic microorganisms against *Aspergillus* spp. from grapevine leaf and berry surfaces. *Journal of Plant Pathology*, *100*(2), 179–190. https://doi.org/10.1007/s42161-018-0042-x

Lamastra, L., Balderacchi, M., Di Guardo, A., Monchiero, M. & Trevisan, M. (2016). A novel fuzzy expert system to

assess the sustainability of the viticulture at the wine-estate scale. *Science of the Total Environment*, *572*, 724–733. https://doi.org/10.1016/j.scitotenv.2016.07.043

Lau, S.-E., Teo, W. F. A., Teoh, E. Y. & Tan, B. C. (2022). Microbiome engineering and plant biostimulants for sustainable crop improvement and mitigation of biotic and abiotic stresses. *Discover Food*, *2*(1). https://doi.org/10.1007/s44187-022-00009-5

Liu, D., Chen, Q., Zhang, P., Chen, D. & Howell, K. S. (2020). The Fungal Microbiome Is an Important Component of Vineyard Ecosystems and Correlates with Regional Distinctiveness of Wine. *MSphere*, *5*(4). https://doi.org/10.1128/msphere.00534-20

Lleixà, J., Kioroglou, D., Mas, A. & Portillo, M. del C. (2018). Microbiome dynamics during spontaneous fermentations of sound grapes in comparison with sour rot and Botrytis infected grapes. *International Journal of Food Microbiology*, *281*(May), 36–46. https://doi.org/10.1016/j.ijfoodmicro.2018.05.016

Luziatelli, F., Ficca, A. G., Colla, G., Švecová, E. B. & Ruzzi, M. (2019). Foliar application of vegetal-derived bioactive compounds stimulates the growth of beneficial bacteria and enhances microbiome biodiversity in lettuce. *Frontiers in Plant Science*, *10*(February), 1–16. https://doi.org/10.3389/fpls.2019.00060

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.Journal*, *17*(1), 10. https://doi.org/10.14806/ej.17.1.200

McMurdie, P. J. & Holmes, S. (2012). Phyloseq: A bioconductor package for handling and analysis of high-throughput phylogenetic sequence data. *Pacific Symposium on Biocomputing*, 235–246. https://doi.org/10.1142/9789814366496_0023

McSpadden Gardener, B. B. (2004). Ecology of Bacillus and Paenibacillus spp. in agricultural systems. *Phytopathology*, *94*(11), 1252–1258. https://doi.org/10.1094/PHYTO.2004.94.11.1252

Mezzasalma, V., Sandionigi, A., Bruni, I., Bruno, A., Lovicu, G., Casiraghi, M. & Labra, M. (2017). Grape microbiome as a reliable and persistent signature of field origin and environmental conditions in Cannonau wine production. *PLoS ONE*, *12*(9), 1–20. https://doi.org/10.1371/journal.pone.0184615

Mian, G., Cantone, P. & Golinelli, F. (2022a). First evidence of the effect of a new biostimulant made by Fabaceae tissue on ripening dynamics and must technological main parameters in *Vitis vinifera* 'Ribolla Gialla.' *Acta Horticulturae*, *1333*, 317–322. https://doi.org/10.17660/ActaHortic.2022.1333.41

Mian, G., Belfiore, N., Musetti, R., Tomasi, D., Cantone, P., Lovat, L., Lupinelli, S., Iacumin, L., Celotti, E. & Golinelli, F. (2022b). Effect of a triacontanol-rich biostimulant on the ripening dynamic and wine must technological parameters in *Vitis vinifera* cv. 'Ribolla Gialla.' *Plant Physiology and Biochemistry*, *188*(August), 60–69. https://doi.org/10.1016/j.plaphy.2022.07.032

Miliordos, D.-E., Tsiknia, M., Kontoudakis, N., Zaharias, N. & Kotseridis, Y. (2022). A holistic approach to using biostimulants on the red grapevine variety, Mouhtaro - from grape to wine. *IVES Technical Reviews, Vine and Wine*, *7*(2016JULY), 1–2. https://doi.org/10.20870/IVES-TR.2022.5504

Monteiro, E., Gonçalves, B., Cortez, I. & Castro, I. (2022). The Role of Biostimulants as Alleviators of Biotic and Abiotic Stresses in Grapevine: A Review. *Plants*, *11*(3), 1–18. https://doi.org/10.3390/plants11030396

Naeem, M., Khan, A., Masroor, M. & Moinuddin. (2012). Triacontanol: A potent plant growth regulator in agriculture. *Journal of Plant Interactions*, *7*(2), 129–142. https://doi.org/10.1080/17429145.2011.619281

Nilsson, R. H., Larsson, K. H., Taylor, A. F. S., Bengtsson-Palme, J., Jeppesen, T. S., Schigel, D., Kennedy, P., Picard, K.,

Glöckner, F. O., Tedersoo, L., Saar, I., Kõljalg, U. & Abarenkov, K. (2019). The UNITE database for molecular identification of fungi: Handling dark taxa and parallel taxonomic classifications. *Nucleic Acids Research*, *47*(D1), D259–D264. https://doi.org/10.1093/nar/gky1022

Oksanen, A. J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., Mcglinn, D., Minchin, P. R., Hara, R. B. O., Simpson, G. L., Solymos, P., Stevens, M. H. H. & Szoecs, E. (2013). *Package ' vegan .' December 2016*, 0–291.

Padmaperuma, G., Butler, T. O., Shuhaili, F. A. B. A., Almalki, W. J. & Vaidyanathan, S. (2019). Microbial consortia: Concept and application in fruit crop management. In *Fruit Crops: Diagnosis and Management of Nutrient Constraints*. Elsevier Inc. https://doi.org/10.1016/B978-0-12-818732-6.00025-3

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J. & Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*, *41*(D1), 590–596. https://doi.org/10.1093/nar/gks1219

Ruzzi, M. & Aroca, R. (2015). Plant growth-promoting rhizobacteria act as biostimulants in horticulture. *Scientia Horticulturae*, *196*, 124–134. https://doi.org/10.1016/j.scienta.2015.08.042

Saligkarias, I. D., Gravanis, F. T. & Epton, H. A. S. (2002). Biological control of Botrytis cinerea on tomato plants by the use of epiphytic yeasts Candida guilliermondii strains 101 and US 7 and Candida oleophila strain I-182: II. A study on mode of action. *Biological Control*, *25*(2), 151–161. https://doi.org/10.1016/S1049-9644(02)00052-X

Samuels, L. J., Setati, M. E. & Blancquaert, E. H. (2022). Towards a Better Understanding of the Potential Benefits of Seaweed Based Biostimulants in *Vitis vinifera* L. Cultivars. *Plants*, *11*(3). https://doi.org/10.3390/plants11030348

Schaller, K. (1991). Ground Water Pollution by Nitrate in Viticultural Areas. *International Symposium on Nitrogen in Grapes and Wine*, *29*, 12–22.

Serpa, D., Nunes, J. P., Keizer, J. J. & Abrantes, N. (2017). Impacts of climate and land use changes on the water quality of a small Mediterranean catchment with intensive viticulture. *Environmental Pollution*, *224*, 454–465. https://doi.org/10.1016/j.envpol.2017.02.026

Sharma, R., Sindhu, S. & Sindhu, S. S. (2018). Suppression of Alternaria blight disease and plant growth promotion of mustard (Brassica juncea L.) by antagonistic rhizosphere bacteria. *Applied Soil Ecology*, *129*(May), 145–150. https://doi.org/10.1016/j.apsoil.2018.05.013

Steel, C. C., Blackman, J. W. & Schmidtke, L. M. (2013). Grapevine bunch rots: Impacts on wine composition, quality, and potential procedures for the removal of wine faults. *Journal of Agricultural and Food Chemistry*, *61*(22), 5189–5206. https://doi.org/10.1021/jf400641r

Timmusk, S., Behers, L., Muthoni, J., Muraya, A. & Aronsson, A. C. (2017). Perspectives and challenges of microbial application for crop improvement. *Frontiers in Plant Science*, *8*(February), 1–10. https://doi.org/10.3389/fpls.2017.00049

Tomasi, D., Gaiotti, F., Petoumenou, D., Lovat, L., Belfiore, N., Boscaro, D. & Mian, G. (2020). Winter pruning: Effect on root density, root distribution and root/canopy ratio in vitis vinifera cv. Pinot Gris. *Agronomy*, *10*(10). https://doi.org/10.3390/agronomy10101509

Tomasi, D., Marcuzzo, P., Nardi, T., Lonardi, A., Lovat, L., Flamini, R. & Mian, G. (2022). Influence of Soil Chemical Features on Aromatic Profile of V. vinifera cv. Corvina Grapes and Wines: A Study-Case in Valpolicella Area (Italy) in a Calcareous

and Non-Calcareous Soil. *Agriculture (Switzerland)*, *12*(12). https://doi.org/10.3390/agriculture12121980

Valencia, E., Gross, N., Quero, J. L., Carmona, C. P., Ochoa, V., Gozalo, B., Delgado-Baquerizo, M., Dumack, K., Hamonts, K., Singh, B. K., Bonkowski, M. & Maestre, F. T. (2018). Cascading effects from plants to soil microorganisms explain how plant species richness and simulated climate change affect soil multifunctionality. *Global Change Biology*, *24*(12), 5642–5654. https://doi.org/10.1111/gcb.14440

Viers, J. H., Williams, J. N., Nicholas, K. A., Barbosa, O., Kotzé, I., Spence, L., Webb, L. B., Merenlender, A. & Reynolds, M. (2013).

Vinecology: Pairing wine with nature. *Conservation Letters*, *6*(5), 287–299. https://doi.org/10.1111/conl.12011

Virdis, C., Sumby, K., Bartowsky, E. & Jiranek, V. (2021). Lactic Acid Bacteria in Wine: Technological Advances and Evaluation of Their Functional Role. *Frontiers in Microbiology*, *11*(January), 1–16. https://doi.org/10.3389/fmicb.2020.612118

Zarraonaindia, I., Owens, S. M., Weisenhorn, P., West, K., Hampton-Marcell, J., Lax, S., Bokulich, N. A., Mills, D. A., Martin, G., Taghavi, S., van der Lelie, D. & Gilbert, J. A. (2015). The soil microbiome influences grapevine-associated microbiota. *MBio*, *6*(2). https://doi.org/10.1128/mBio.02527-14