Microbiological Research 232 (2020) 126387

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Ecological impact of a rare sugar on grapevine phyllosphere microbial communities

Michele Perazzolli[a,b,*], Andrea Nesler[b], Oscar Giovannini[b], Livio Antonielli[c], Gerardo Puopolo[a,b], Ilaria Pertot[a,b]

[a] Center Agriculture Food Environment (C3A), University of Trento, via E. Mach 1, 38010 San Michele all'Adige, Italy
[b] Department of Sustainable Agro-ecosystems and Bioresources, Research and Innovation Centre, Fondazione Edmund Mach, Via E. Mach 1, 38010 San Michele all'Adige, Italy
[c] Department of Health and Environment, Bioresources Unit, Austrian Institute of Technology, Konrad-Lorenz-Strasse 24, 3430 Tulln an der Donau, Austria

ARTICLE INFO

Keywords:
Rare sugar
Tagatose
Anti-nutritional effect
Grapevine microbiota
Leaf microbiota engineering

ABSTRACT

Plants host a complex microbiota inside or outside their tissues, and phyllosphere microorganisms can be influenced by environmental, nutritional and agronomic factors. Rare sugars are defined as monosaccharides with limited availability in nature and they are metabolised by only few certain microbial taxa. Among rare sugars, tagatose (TAG) is a low-calories sweetener that stimulates and inhibits beneficial and pathogenic bacteria in the human gut microbiota, respectively. Based on this differential effect on human-associated microorganisms, we investigated the effect of TAG treatments on the grapevine phyllosphere microorganisms to evaluate whether it can engineer the microbiota and modify the ratio between beneficial and pathogenic plant-associated microorganisms. TAG treatments changed the structure of the leaf microbiota and they successfully reduced leaf infections of downy mildew (caused by *Plasmopara viticola*) and powdery mildew (caused by *Erysiphe necator*) under field conditions. TAG increased the relative abundance of indigenous beneficial microorganisms, such as some potential biocontrol agents, which could partially contribute to disease control. The taxonomic composition of fungal and bacterial leaf populations differed according to grapevine locations, therefore TAG effects on the microbial structure were influenced by the composition of the originally residing microbiota. TAG is a promising biopesticide that could shift the balance of pathogenic and beneficial plant-associated microorganisms, suggesting selective nutritional/anti-nutritional properties for some specific taxa. More specifically, TAG displayed possible plant prebiotic effects on the phyllosphere microbiota and this mechanism of action could represent a novel strategy that can be further developed for sustainable plant protection.

1. Introduction

Plants support a complex microbiota on and inside various plant organs (Bulgarelli et al., 2013). The aerial part (phyllosphere) is usually colonised by a variety of bacteria, filamentous fungi and yeasts adapted to this harsh habitat (Lindow and Brandl, 2003; Rastogi et al., 2013). Phyllosphere microorganisms include not only plant pathogens, but also several microorganisms known to exert beneficial effects on the plant (Lindow and Brandl, 2003; Müller and Ruppel, 2014; Vorholt, 2012). In particular, some phyllosphere communities contribute to the plant health through the plant resistance stimulation and the biocontrol activities against phytopathogens (Bulgarelli et al., 2013; Perazzolli et al., 2014; Vorholt, 2012). However, the role and ecology of beneficial microorganisms on the phyllosphere is only partially understood (Müller and Ruppel, 2014). A better knowledge on plant and phyllosphere microbiota interactions may encourage the development of novel approaches for plant protection (Lindow and Brandl, 2003; Rastogi et al., 2013). For example, stimulating natural beneficial communities with the application of selected protein-derived nutritional factors reduced the severity of phytopathogens and may represent a new tool to be integrated into sustainable plant protection strategies (Cappelletti et al., 2016).

The phyllosphere is an open environment and microorganisms may migrate on it from soil, through rain splash, wind or human action in agronomic practices (Martins et al., 2013; Vacher et al., 2016; Zarraonaindia et al., 2015), and from other substrates (other plants, plant residues, *etc.*) through atmosphere dispersion (Fort et al., 2016; Zarraonaindia et al., 2015). The phyllosphere microbiota can be

* Corresponding author at: Center Agriculture Food Environment (C3A), University of Trento, via E. Mach 1, 38010 San Michele all'Adige, Italy.
E-mail address: michele.perazzolli@unitn.it (M. Perazzolli).

https://doi.org/10.1016/j.micres.2019.126387
Received 6 August 2019; Received in revised form 24 November 2019; Accepted 25 November 2019
Available online 26 November 2019
0944-5013/ © 2019 The Authors. Published by Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/BY-NC-ND/4.0/).

affected by environmental and biotic factors, such as air pollution, leaf age, nitrogen fertilization, plant species, UV radiation and invading microorganisms (Vacher et al., 2016; Vorholt, 2012), as well as the agronomic management (Vitulo et al., 2019). In particular, the microbial structure of grapevine (*Vitis vinifera*) leaf communities changed during the vegetative cycle according to the plant developmental stage and agricultural practices (Fort et al., 2016; Pinto et al., 2014). Phyllosphere communities are resilient to some chemical or biological pesticides (Ottesen et al., 2015; Perazzolli et al., 2014; Sylla et al., 2013a, b) and the abundance of specific taxa on grapevine leaves can be affected by plant treatments (Fort et al., 2016; Perazzolli et al., 2014; Pinto et al., 2014), as in the case of a protein-derived product under greenhouse conditions (Cappelletti et al., 2016). Therefore, it has been suggested that the plant microbiota can be engineered to optimize the plant-associated microbial consortia, but this strategy has not been applied anywhere (Syed Ab Rahman et al., 2018; Toju et al., 2018). In this study, grapevine was used as model system under field conditions, since its phyllosphere microbiota was previously characterized for pathogenic and beneficial interactions (Perazzolli et al., 2014; Pinto et al., 2014) and for impacts on organoleptic properties of grapes and wine (Bokulich et al., 2016; Pinto et al., 2015; Zarraonaindia et al., 2015). Grapevine is susceptible to a large number of devastating diseases (Armijo et al., 2016), such as downy mildew (caused by *Plasmopara viticola*) and powdery mildew (caused by *Erysiphe necator*), that negatively affect grape production (Buonassisi et al., 2017; Gadoury et al., 2012; Gessler et al., 2011).

The term ‘rare sugars’ refers to monosaccharides and their derivatives that rarely exist in nature (Granström et al., 2004) and are metabolised by only certain microbial taxa (Van der Heiden et al., 2013). The ecological role of rare sugars is not yet understood and their promising biological properties are underestimated due to the limited availability of these compounds in nature (Li et al., 2013). However, the implementation of novel enzymatic and microbial processes lowered the cost of rare sugars synthesis (Granström et al., 2004; Izumori, 2006; Oh, 2007) and made scientific studies and industrial applications on these molecules more accessible (Li et al., 2013; Oh, 2007). In particular, tagatose (TAG) is a rare sugar that inhibits the growth of phytopathogenic oomycetes and fungi, such as grapevine and cucumber downy mildew, cucumber and barley powdery mildew, potato and tomato late blight (Ohara et al., 2019). TAG was generally recognised as safe (GRAS) by the food and drug administration (FDA) and it can be used as a natural low-calories sweetener in several countries, United Stated and European Union included (Levin, 2002; Vastenavond et al., 2011), indicating no negative impacts on the human health. TAG showed potential health and prebiotic benefits for humans, including the treatment of type 2 diabetes, hyperglycemia and anemia (Levin, 2002; Oh, 2007). Specifically, TAG increases the proportion of beneficial bacteria (*Enterococcus* spp. and *Lactobacillus* spp.) of the gut microbiota (Hasibul et al., 2018; Vastenavond et al., 2011), inhibits the growth of *Streptococcus mutants* (Hasibul et al., 2018) and is not catabolised by some human pathogenic bacteria, such as *Bacillus cereus, Escherichia coli*, *Listeria monocytogenes*, *Salmonella enterica* Typhimurium and *Staphylococcus aureus* (Bautista et al., 2000; Lobete et al., 2017). Thus, TAG can be catabolised by only certain microbial taxa, such as *Erwinia* spp., *Exiguobacterium* spp., *Lactobacillus* spp. (Hao et al., 1990; Martinussen et al., 2013; Raichand et al., 2012; Wu and Shah, 2017), suggesting some possible nutritional/anti-nutritional effects of this carbohydrate on specific microbial taxa. Building on this potential differential effect on microbial populations, the aim of this study was to investigate whether TAG treatments can shape indigenous leaf microbial populations of grapevine under field conditions acting as nutritional/anti-nutritional factor for the grapevine leaf microbiota.

2. Materials and methods

2.1. Optimization of the tagatose concentration

Two-year-old plants of *V. vinifera* cultivar ‘Pinot noir’ were individually planted in 2.5 l- pots and grown under greenhouse condition for two months as previously described (Perazzolli et al., 2012). To assess the efficacy against downy mildew under greenhouse conditions, healthy plants were sprayed with a water solution of 0.8, 4, 8 and 24 g/l TAG on all leaves using a compressed air hand sprayer (20–30 ml for each plant). As control, plants were left untreated (UNT) or sprayed with copper (2 g/l Copranthol Hi Bio, Syngenta AG, Basel, Switzerland) as reference fungicide against downy mildew. Plants were left to dry for two hours under greenhouse conditions and a *P. viticola* suspension (1×10^5 sporangia/ml) was sprayed on the abaxial surface of all leaves (20–30 ml per plant), according to Perazzolli et al. (2012). Plants were incubated overnight in the dark at 95 ± 4 % relative humidity and kept under greenhouse conditions to allow downy mildew development. Six days after *P. viticola* inoculation, plants were incubated overnight in the dark at 95 ± 4 % relative humidity to allow downy mildew sporulation and the disease severity was assessed visually as the percentage of abaxial leaf area covered by *P. viticola* sporulation, according to the standard guidelines of the European and Mediterranean Plant Protection Organization (EPPO, 2001). To assess the efficacy against powdery mildew under greenhouse conditions, plants naturally infected with *E. necator* (powdery mildew severity 8.5 ± 1.7; mean ± standard error values) were sprayed with a water solution of 0.8, 4, 8 and 24 g/l TAG on all leaves as described above (20–30 ml for each plant). As control, UNT plants and plants sprayed with sulphur (5 g/l Thiamon 80 Plus, Du Pont, Wilmington, DE, USA) were used. Seven days after incubation under greenhouse conditions, powdery mildew severity was assessed visually as the percentage of leaf area covered by *E. necator* sporulation, according to the EPPO standard guidelines (EPPO, 2002). A randomised complete block design with four replicates (plants) was used for each treatment, the downy and powdery mildew experiment was carried out twice.

2.2. Grapevine treatments under field conditions

A randomised complete block design with four replicates of eight plants for each treatment was used in two locations in San Michele all’Adige (northern Italy), namely, vineyard 1 (‘San Donà’, ‘Pinot gris’ cultivar) and vineyard 2 (‘Facchinelli’, ‘Schiava gentile’ cultivar; Table 1). Grapevine plants were treated with a water solution of 8 g/l TAG or left untreated (UNT). UNT plants were used as control, since water treatment did not cause major changes on the taxonomic structure of bacterial and fungal communities on grapevine leaves (Cappelletti et al., 2016). Treatments were applied weakly before rainfall according to the ‘Meteo 3B’ weather forecast (www.3bmeteo.

Table 1
Characteristics of the two vineyards.

Characteristic [a]	Vineyard 1	Vineyard 2
Coordinates	N 46.184474; e 11.124458	N 46.180685; e 11.125867
Name	San Donà	Facchinelli
Altitude (m a.s.l.)	200	250
Topography	Valley floor	Low hill
Slope (%)	–	3-5
Cultivar	Pinot gris SMA514	Schiava gentile sel. Sebber
Rootstock	SO4	Teleki 5C
Training system	Double ‘pergola trentina’	Simple ‘pergola trentina’
Planting pattern (m)	5.5 × 0.66	3.00 × 0.8
Planting year	2003	1997
Irrigation	Not irrigated	Drip system

[a] Characteristics of the two vineyards analysed in San Michele all’Adige, Trentino (Northern Italy).

com) to predict the probable infections of *E. necator* and *P. viticola* from 18 April to 18 August 2014. A motorised backpack mist blower (450, Solo, Newport News, VA, USA) was used with a spray volume of 500 l/ha. Under field conditions, the downy and powdery mildew severity was assessed visually on 40 leaves randomly chosen on the four central plants of each replicate (plot with eight to nine plants) at the end of the experiment (BBCH 85; 19 August 2014). The disease severity was assessed as a percentage of infected leaf area covered by downy mildew (oil spots, *P. viticola* sporulation or leaf necrosis) or powdery mildew symptoms (*E. necator* sporulation or leaf necrosis), according to the EPPO guidelines (EPPO, 2001, 2002).

2.3. Sample collection and isolation of phyllosphere microorganisms

Phyllosphere microorganisms were collected by leaf washing as described by Perazzolli et al. (2014). Briefly, asymptomatic leaves were randomly collected one day after the last treatment (19 August 2014, BBCH 85 corresponding to the stage ‘softening of berries’, 30 days before harvesting) and three replicates (named from A to C) of 50 leaves were randomly collected from ten plants for each treatment and vineyard. Leaves were washed as described by Cappelletti et al. (2016), to isolate culturable microorganisms and to obtain a bacterial pellet for DNA extraction by centrifuging 900 ml at 4000 × g for 20 min. The TAG quantity in each leaf washing suspension was assessed by ion chromatography (Chemistry Unit at Fondazione Edmund Mach) (Cataldi et al., 2000) and it was converted as TAG residual per unit of fresh leaf weight (mg/g) using a calibration curve of 98.5 % pure TAG (Sigma-Aldrich, St. Louis, MO, USA) dissolved in ultrapure water within a range between 2 and 40 μg/mL. Briefly, samples were diluted 50 fold in ultrapure water, filtered through a 0.45 μm PTFE membrane (Sartorius, Goettingen, Germany) and analysed with an ionic chromatograph ICS 5000 (Dionex-Thermo Scientific, Waltham, MA, USA), equipped with an autosampler, a quaternary gradient pump, a column oven and a pulsed amperometric detector with a gold working electrode and a palladium counter electrode. The separation was obtained by injecting 5 μl of diluted sample onto a CarboPac PA200 3 × 250 mm analytical column (Dionex-Thermo Scientific, Waltham, MA, USA), preceded by a CarboPac PA200 3 × 50 mm guard column (Dionex-Thermo Scientific), with a KOH gradient (from 1 to 100 mM) at 0.4 ml/min flow rate.

Culturable bacteria and fungi were assessed as previously described (Cappelletti et al., 2016) by plating serial dilutions of each leaf-washing suspension on Nutrient Agar supplemented with 100 mg/l cycloheximide and on Potato Dextrose Agar supplemented with 0.25 % lactic acid, respectively. After incubation at 25 °C for 48 h, colony forming units (CFUs) per unit of fresh leaf weight (CFUs/g) were calculated. To test the effect of TAG *in vitro* on culturable microorganisms, leaf-washing suspensions obtained by leaf washing of UNT plants of vineyard 1 and vineyard 2 were incubated overnight in a 50 m L-tube containing 10 ml of distilled water (control) or distilled water amended with 8 g/l TAG under orbital shaking (80 rpm) at 25 °C. Culturable bacteria and fungi were then assessed by plating serial dilutions on the selective media described above and the concentration of bacteria and fungi (CFUs/ml) were determined after incubation at 25 °C for 48 h.

2.4. DNA extraction, amplification and sequencing

DNA was extracted from microbial pellets using the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA). Bacterial and fungal sequences were amplified with primer pairs previously used in grapevine phyllosphere studies (Cappelletti et al., 2016; Pinto et al., 2014) that amplify the V6-V8 region of the 16S rRNA (Baker et al., 2003) and the ITS2 region of the internal transcribed spacer (ITS) fragment (White et al., 1990) respectively (Table S1). Amplification (from 50 ng of extracted DNA), PCR product purification and quantification, library construction and sequencing with a GS FLX + system (Roche, Branford, CT, USA) were carried out as described by Cappelletti et al. (2016) (Sequencing Platform at Fondazione Edmund Mach). Sequences of the 24 samples [two treatments (TAG and UNT), two locations (vineyard 1 and vineyard 2), two amplicons (bacterial 16S and fungal ITS) and three replicates] were obtained and they are available at the Sequence Read Archive of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/sra) under accession number SRP065900 and BioProject number PRJNA301110.

2.5. Bioinformatic analysis and sequence processing

Bacterial and fungal sequences were processed as reported by Cappelletti et al. (2016) with some modifications. Sequence quality check and filtering were carried out with PRINSEQ (Schmieder and Edwards, 2011) and FlowClus (Gaspar and Thomas, 2015), respectively. For quality filtering, reads shorter than 150 bases or longer than 1000 bases were discarded, and homopolymer runs longer than six bases were excluded, as well as ambiguous sequences longer than six bases. A Phred quality score greater than 25 in a sliding window of 50 bases was considered as the minimum average allowed; one barcode correction and two primer mismatches were accepted. Quality filtered reads were processed using V-Xtractor (Hartmann et al., 2010) (http://www.microbiome.ch/software) and ITSx (Bengtsson-Palme et al., 2013) (http://microbiology.se/software/itsx), in order to obtain highly reliable 16S V6-V8 rRNA and ITS2 sequences, respectively. USEARCH v7 (Edgar, 2013) (http://www.drive5.com) was used to de-replicate and sort the extracted regions and chimeras were removed with UCHIME (Edgar et al., 2011) (http://drive5.com/usearch/manual/uchime_algo.html) using the RDPClassifier trainset N.16 database (https://sourceforge.net/projects/rdp-classifier/files/RDP_Classifier_TrainingData/) and the UNITE reference sequences (Kõljalg et al., 2013) for bacterial and fungal sequences, respectively.

Clustering of operational taxonomic units (OTUs) was carried out using the UPARSE algorithm (USEARCH v7 tool) with 97 % of pairwise sequence identity (Edgar, 2013). QIIME (Caporaso et al., 2010) (http://qiime.org) was used for taxonomic assignments of bacterial and fungal OTUs with a naïve Bayesian RDP classifier and a minimum confidence of 0.8 (Wang et al., 2007) respectively against the SILVA 123 database (Quast et al., 2013) (www.arb-silva.de) and UNITE 7.1 database (Kõljalg et al., 2005), which does not include oomycete sequences (https://unite.ut.ee). Taxonomic assignments of fungal OTUs were confirmed by BLAST search against the NCBI nucleotide database (https://www.ncbi.nlm.nih.gov/nucleotide/) and no reads of oomycetes were found, possibly due to the absence of downy mildew sporulation on collected leaves and/or to the washing protocol of leaves that was not able to eradicate *P. viticola* hyphae. After taxonomic classification, OTUs corresponding to chloroplasts and mitochondrial sequences were discarded.

The composition of bacterial and fungal populations on grapevine leaves was visualised using the Krona software (Ondov et al., 2011). A neighbor-joining tree was generated on 16S and ITS sequences of bacterial and fungal OTUs using MEGA 7.0 (Kumar et al., 2016) (www.megasoftware.net) with the Maximum Likelihood method and 999 replicates of bootstrap testing. Phylogenetic trees were visualised using the iTOL software (Letunic and Bork, 2011) (http://itol.embl.de). TAG-affected bacterial and fungal genera comprising species with potential pathogenic and/or beneficial properties (biocontrol) on grapevine plants were identified according to the previous literature.

2.6. Statistical analysis

Statistical analysis of bacterial and fungal data were carried out as reported by Cappelletti et al. (2016), with some modifications. The BIOM table generated by the 16S rRNA gene and ITS analysis was subsampled *via* multiple rarefaction in QIIME (Caporaso et al., 2010) and the Simpson’s diversity index (Simpson, 1949) was calculated to

estimate microbial diversity. For beta-diversity metrics, BIOM tables were obtained by rarefaction at 21,542 and 3831 read depth, for the 16S rRNA and ITS data respectively. A multivariate analysis was performed with an unsupervised Principal Coordinate Analysis (PCoA) followed by its constrained ordination counterpart, *i.e.* a Constrained Analysis of Principal coordinates (CAP) (Anderson and Willis, 2003) applied to Bray-Curtis dissimilarity matrices (Bray and Curtis, 1957) as re-implemented in the vegan R package. To detect significant differences among treatments and locations, a permutation test (anova.cca function, vegan R package) (Legendre and Legendre, 1998) on CAP (capscale function, vegan R package) and a multivariate generalised linear model (multivariate GLM, vegan R package) on the normalised OTU table were applied (manyglm function, vegan R package) on bacterial and fungal data. In both CAP and multivariate GLM, location and treatment were used as fixed categorical predictors, together with their interaction. The test applied on CAP was carried out with the vegan::anova.cca function after 9999 permutations (vegan R package). The applicability of CAP was verified by homogeneity analysis of variances, as implemented by the vegan::betadisper function, followed by permutational test (9999 iterations) for location and treatment factors on bacterial and fungal data (vegan R package). The multivariate GLM was built assuming a negative binomial (log-link) distribution of the data, graphically verified by plotting fitted *vs* residual values. Analysis of deviances was conducted on multivariate GLM (mvabund::anova.-manyglm, vegan R package) using a score test after 9999 permutations with Montecarlo resampling. Ordination analysis was carried out with the phyloseq R package (McMurdie and Holmes, 2013).

Bacterial and fungal alpha-diversity was analysed in R by fitting richness (OTU counts) and diversity (Simpson's index) values in a linear model where location and treatment were considered as factors. After graphical verification of fitted *versus* residual values, differences between locations and between treatments were assessed by analysis of variance (ANOVA). Data on disease severity and relative abundances were processed using Statistica 13.3 software (TIBCO Software Inc., Palo Alto, CA, USA). After validating data for normal distribution (K–S test, $P > 0.05$) and variance homogeny (Cochran's test, $P > 0.05$), ANOVA was carried out for each taxon. Tukey's test or Student's *t*-test was used to detect significant differences among treatments ($P \leq 0.05$) in pairwise and multiple comparison, respectively. When ANOVA prerequisites were not met, disease severity and relative abundance values were transformed by square root and CFU counts were transformed by Log_{10}, in order to satisfy ANOVA assumptions without affecting the statistical results (Kim et al., 2018).

3. Results

3.1. Tagatose treatments controlled downy and powdery mildew symptoms and affected culturable phyllosphere microorganisms under field conditions

TAG treatments reduced downy mildew and powdery severity on grapevine leaves under greenhouse conditions, the lowest concentration that maximise the disease reduction was 8 g/l (Fig. S1) and it was used in the following experiments. Under field conditions, downy mildew severity on grapevine leaves (vineyard 1, UNT: 46.0 ± 6.5 %; vineyard 2, UNT: 42.0 ± 5.1 %) was reduced by 8 g/l TAG treatments in the two vineyards characterised by different cultivars and agricultural characteristics (vineyard 1, TAG-treated: 33.6 ± 2.7 %; vineyard 2, TAG-treated: 21.2 ± 3.2 %; Student's *t*-test, $P \leq 0.05$). Powdery mildew severity was reduced by TAG treatments in vineyard 2 (UNT: 21.4 ± 6.8 %; TAG-treated: 1.2 ± 0.2 %; Student's *t*-test, $P \leq 0.05$), while no powdery mildew symptoms were detected on UNT and TAG-treated plants in vineyard 1. TAG treatments did not affect the number of culturable epiphytic bacteria and fungi per leaf unit under field conditions (Fig. 1A), although TAG residues were present in the leaf washing suspension of TAG-treated leaves (Table 2). However, when culturable phyllosphere microorganisms of UNT plants were incubated

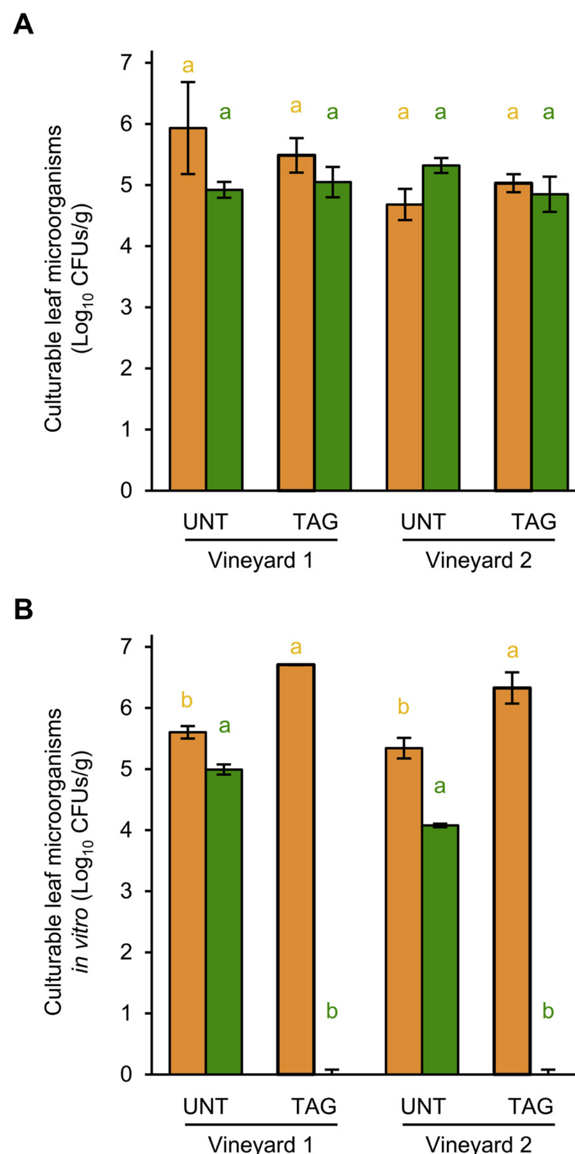

Fig. 1. Tagatose effect on culturable microorganisms of the grapevine phyllosphere. Plants were left untreated (UNT) or treated with tagatose (TAG) in vineyard 1 and vineyard 2 and colony forming units (CFUs) of phyllosphere bacteria (orange) and fungi (green) per unit of leaf fresh weight (CFUs/g) were assessed by plating method (A). Phyllosphere bacteria (orange) and fungi (green) collected form UNT plants were incubated with water (UNT) or TAG overnight *in vitro* and CFUs were assessed by dilution plating method (B). Mean Log_{10}-transformed values and standard errors from three replicates are presented for each sample. Bacterial and fungal data were analysed separately, different coloured letters indicate significant differences among treatments and vineyards according to Tukey's test ($P \leq 0.05$) with the 'a' letter assigned to the condition with the highest mean CFU/g value.

with TAG *in vitro*, the number of bacteria and fungi was respectively increased and impaired (Fig. 1B).

3.2. Tagatose changed bacterial populations and increased the relative abundance of putative beneficial bacteria on grapevine leaves

The composition of bacterial communities was analysed on TAG-treated and UNT leaves in the two vineyards. After filtering out low-quality and short sequences, a total of 378,083 bacterial reads were obtained, with more than 21,542 of quality filtered reads for each replicate (Table S2), and rarefaction curves reached the saturation (Fig. S2A). Bacterial richness and diversity were affected by the grapevine

Table 2
Tagatose residual on grapevine leaves.

Vineyard	Treatment [a]	Tagatose residual (mg/g) [b]	
Vineyard 1	UNT	< 0.05	b
	TAG	1.07 ± 0.03	a
Vineyard 2	UNT	< 0.05	b
	TAG	0.92 ± 0.11	a

[a] Plants were left untreated (UNT) or treated with tagatose (TAG) in vineyard 1 and vineyard 2.

[b] Tagatose residual was assessed on supernatants of leaf washing suspension and expressed per unit of leaf fresh weight (mg/g). Different letters indicate significant differences across treatments and vineyards according to Tukey's test ($P \leq 0.05$) with the 'a' letter assigned to the condition with the highest mean tagatose value.

location (F = 5.98, P = 0.04 and F = 5.98, P = 0.04), respectively) and treatment (F = 7.16, P = 0.03 and F = 7.29, P = 0.03, respectively), according to ANOVA test (Fig. S3A and S3B). Almost the totality of bacterial reads was assigned to taxa at the taxonomic level of family and genus (99.2 and 91.4 %, respectively) and 315 OTUs were identified in total (Table S3). Gammaproteobacteria was the most abundant phylum of phyllosphere bacteria (relative abundance of 97.1 ± 1.69 %); *Orbaceae*, *Enterobacteriaceae* and *Pseudomonadaceae* were the most abundant bacterial families (Figs. S4A and S5A).

Global effects on bacterial phyllosphere populations were examined using an unsupervised approach offered by PCoA and its constrained counterpart represented by CAP analysis. The first CAP axis discriminated samples according to the grapevine location (75.6 % of eigenvalue variance), the second axis highlighted differences between TAG-treated and UNT plants (20.0 %, Fig. 2A) and PCoA analysis corroborated these results (Fig. S6A), with significant differences among locations (pseudo-F = 14.55, P = 0.001) and treatments (pseudo-F = 4.05, P = 0.025) according to the permutation test (Table S4). A multivariate GLM analysis confirmed the significant effect of the location (score = 401.20, P = 0.001) and treatment (score = 356.40, P = 0.001) on beta-diversity of phyllosphere bacteria and highlighted significant changes (P = 0.001) on TAG-treated plants as compared with UNT plants in both locations (Table S4).

The relative abundance of bacterial phyla was generally consistent in the two vineyards, except for Betaproteobacteria (Fig. S5A), but the relative abundance of bacterial families varied according to grapevine locations pooling data of UNT and TAG-treated samples of each vineyard (Fig. 3A, Table S5). In particular, the relative abundance of *Bifidobacteriaceae*, *Leuconostocaceae*, *Neisseriaceae* and *Orbaceae* was greater in vineyard 2 than in vineyard 1, while that of *Micrococcaceae*, *Enterobacteriaceae*, *Pseudomonadaceae* and *Xanthomonadaceae* showed an opposite profile (Fig. 3A, Table S5). Considering UNT leaves, vineyard 2 showed greater relative abundance of the dominant bacterial genera (relative abundance > 2 %) *Gilliamella*, *Frischella* and *Snodgrassella*, while vineyard 1 showed greater relative abundance of *Pseudomonas*, *Pantoea* and *Stenotrophomonas* (Fig. 4A). However, the relative abundance of dominant genera *Acinetobacter*, *Enterobacter*, *Lactococcus*, *Orbus* and *Variovorax* was consistent among treatments and locations and they represent the conserved core microbiota of the grapevine phyllosphere.

The relative abundance of dominant genera was affected by TAG treatments, which increased the relative abundance of the *Pseudomonas* genus in both vineyards and reduced that of *Frischella* and *Snodgrassella* in vineyard 2 (Fig. 4A). The pairwise comparison between TAG-treated and UNT leaves within each vineyard confirmed profiles of *Pseudomonas*, *Frischella* and *Snodgrassella* and revealed a TAG-dependent increase in relative abundance of the rare genera (relative abundance ≤ 2 %) *Chroococcidiopsis*, *Exiguobacterium*, *Leifsonia*, *Methylobacterium*, *Pelomonas* and *Rhodobium* in vineyard 1 (Table S6). Moreover, TAG treatments increased and decrease the relative abundance of *Erwinia*

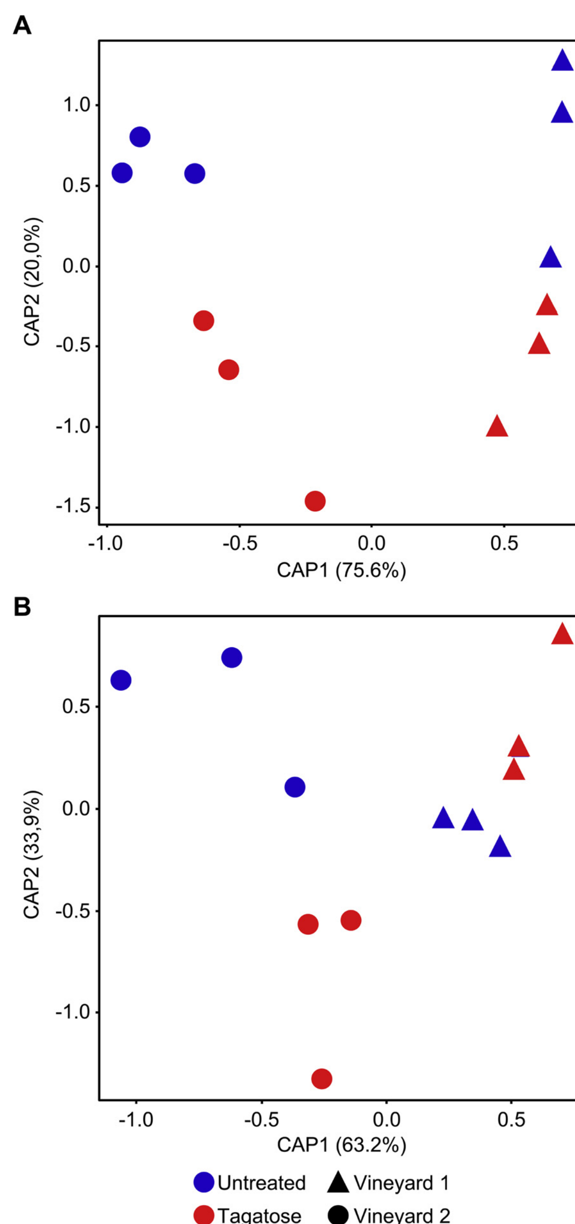

Fig. 2. Tagatose effects on the grapevine phyllosphere microbiota. Constrained analysis of principal coordinates (CAP) was obtained with the Vegan R package using a Bray-Curtis dissimilarity matrix on bacterial (A) and fungal data (B) of untreated plants (blue) and plants treated with tagatose (red) in vineyard 1 (triangles) and vineyard 2 (circles) in triplicate. Significant differences were detected between treatments and vineyards according to the permutation test and multivariate generalised linear model analysis (Table S4).

and *Fructobacillus* in vineyard 2, respectively (Table S6). TAG-affected bacterial genera were classified as potential phytopathogens or potential biocontrol agents of grapevine according to the previous literature and the relative abundance of a genus (*Exiguobacterium*) comprising potential biocontrol species was increased by TAG (Fig. 5A and Table S6). *Pseudomonas* comprised species with both pathogenic and beneficial activities on grapevine and potential properties cannot easily be assigned at the level of genus without a further taxonomic characterisations.

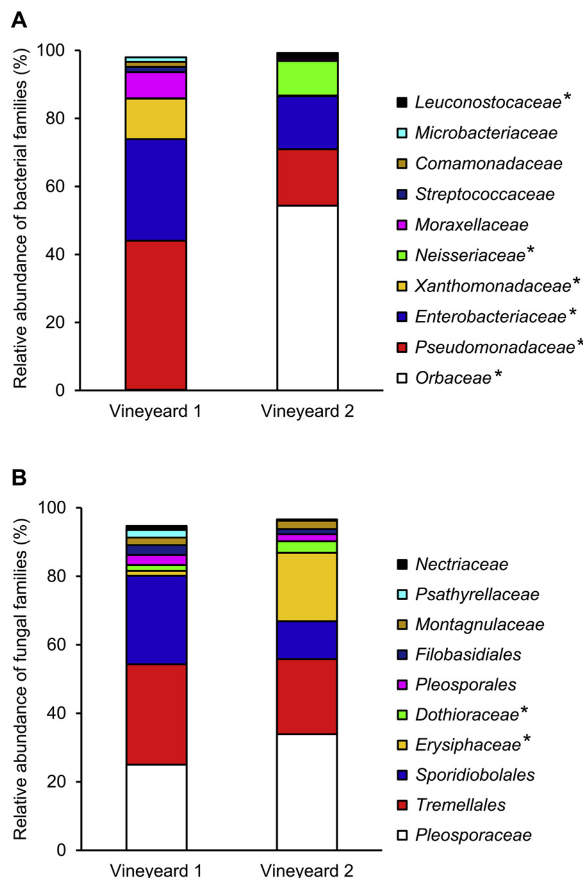

Fig. 3. Taxonomic composition of bacterial and fungal families in the two vineyards. Mean values of relative abundance (percentage) of dominant (relative abundance > 0.2 and 1 %, respectively) bacterial (A) and fungal (B) families was determined for vineyard 1 and vineyard 2 pooling the data of untreated plants (UNT) and plants treated with tagatose (TAG). For each taxon indicated with the colour specified in the legend, asterisks indicate significant differences between vineyards according to Student *T*-test ($P \leq 0.05$). Asterisks were omitted for taxa with no significant differences. The *Filobasidiales*, Pleosporales, Sporidiobolales and Tremellales orders include fungal operational taxonomic units with incomplete taxonomic annotation. The full list of bacterial and fungal families with significant changes in the relative abundance between vineyard 1 and vineyard 2 is reported in Table S5.

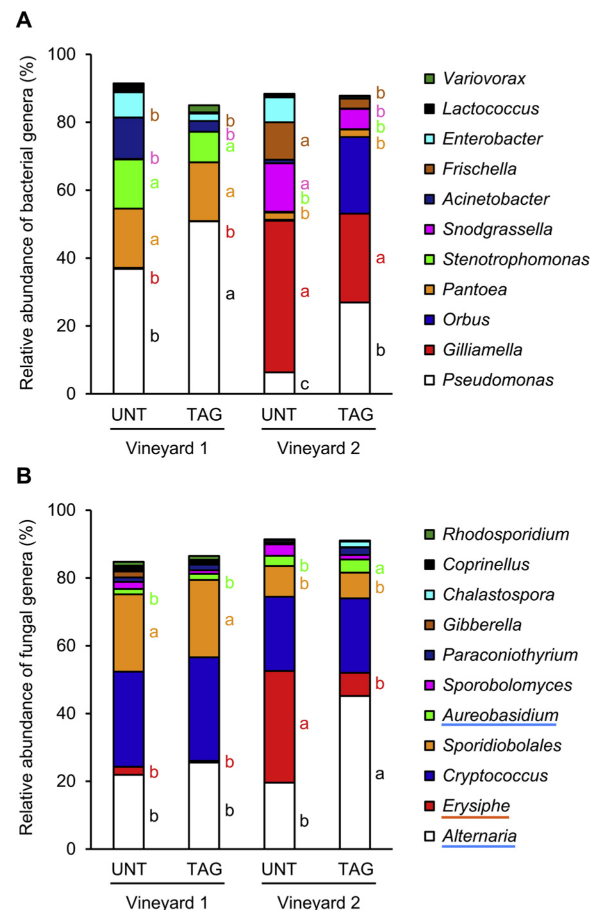

Fig. 4. Tagatose effects on relative abundance of dominant bacterial and fungal genera. Relative abundance (percentage) of dominant (relative abundance > 2 and 1 %, respectively) bacterial (A) and fungal genera (B) was determined for leaves of untreated plants (UNT) and plants treated with tagatose (TAG) in vineyard 1 and vineyard 2. Mean values of three replicates are presented for each sample. For each taxon indicated with the colour specified in the legend, different letters indicate significant differences among treatments and vineyards according to Tukey's test ($P \leq 0.05$). Letters were omitted for taxa with no significant differences among treatments and vineyards (*i.e.Acinetobacter*, *Enterobacter*, *Lactococcus*, *Orbus* and *Variovorax* bacterial genera, *Cryptococcus*, *Gibberella*, *Paraconiothyrium* and *Rhodosporidium* fungal genera). The Sporidiobolales order includes operational taxonomic units with incomplete taxonomic annotation. Dominant genera that comprise potential biocontrol agents [*Alternaria alternata* (Musetti et al., 2006), *Aureobasidium pullulans* (Harm et al., 2011)] and phytopathogens (*Erysiphe necator*) of grapevine are underlined by blue and orange lines, respectively.

3.3. Tagatose changed fungal populations, reduced the relative abundance of fungal pathogens and increased that of putative beneficial fungi on grapevine leaves

The composition of fungal communities was analysed on TAG-treated and UNT leaves in the two vineyards. A total of 61,559 quality filtered reads were obtained for fungal data (Table S2), and rarefaction curves reached the saturation for each replicate (Fig. S2B). Fungal richness was not affected by the grapevine location (F = 2.15, P = 0.18) and treatment (F = 0.0004, P = 0.99), fungal diversity was affected by the grapevine treatment (F = 7.99, P = 0.02), but not by the location (F = 0.60, P = 0.46), according to ANOVA test (Fig. S3A and S3B). The majority of fungal reads were assigned to taxa at family and genus level (79.4 and 77.9 %, respectively) and 458 OTUs were identified in total (Table S7). In particular, Ascomycota (relative abundance of 50.6 ± 4.9 %) and Basidiomycota phyla (relative abundance of 49.3 ± 4.8 %) dominated on grapevine leaves (Fig. S4B).

The CAP analysis on fungal data discriminated samples according to the grapevine locations in the first axis (63.2 %) and according to the grapevine treatments in the second axis (33.9 %, Fig. 2B) and PCoA analysis corroborated these results (Fig. S6B). Significant differences among locations and treatments were confirmed by the permutation test (pseudo-F = 7.81, P = 0.001 and pseudo-F = 3.08, P = 0.026, respectively) and the multivariate GLM analysis (score = 569.60, P = 0.001 and score = 431.50, P = 0.001, respectively). The former test also highlighted significant effects of the TAG treatments in vineyard 2 (P = 0.001) and vineyard 1 (P = 0.012; Table S4). Specifically, the relative abundance of fungal phyla differed according to the grapevine location and it was not affected by TAG treatments; Ascomycota and Basidiomycota were dominant phyla in vineyard 2 and vineyard 1, respectively (Fig. S5B). Therefore, the distribution of fungal families varied according to the grapevine location pooling data of UNT and TAG-treated samples of each vineyard (Fig. 3B, Table S5). The relative abundance of *Dothioraceae*, *Erysiphaceae* and *Mytilinidiaceae* was greater in vineyard 2 than in vineyard 1, while that of *Amphisphaeriaceae*, *Agaricaceae*, *Botryobasidiaceae*, *Cystofilobasidiaceae*, *Hydnodontaceae*, *Sarcosomataceae* and *Xylariaceae* showed the opposite profile. However, the dominant fungal genera (relative abundance > 2%) *Chalastospora*,

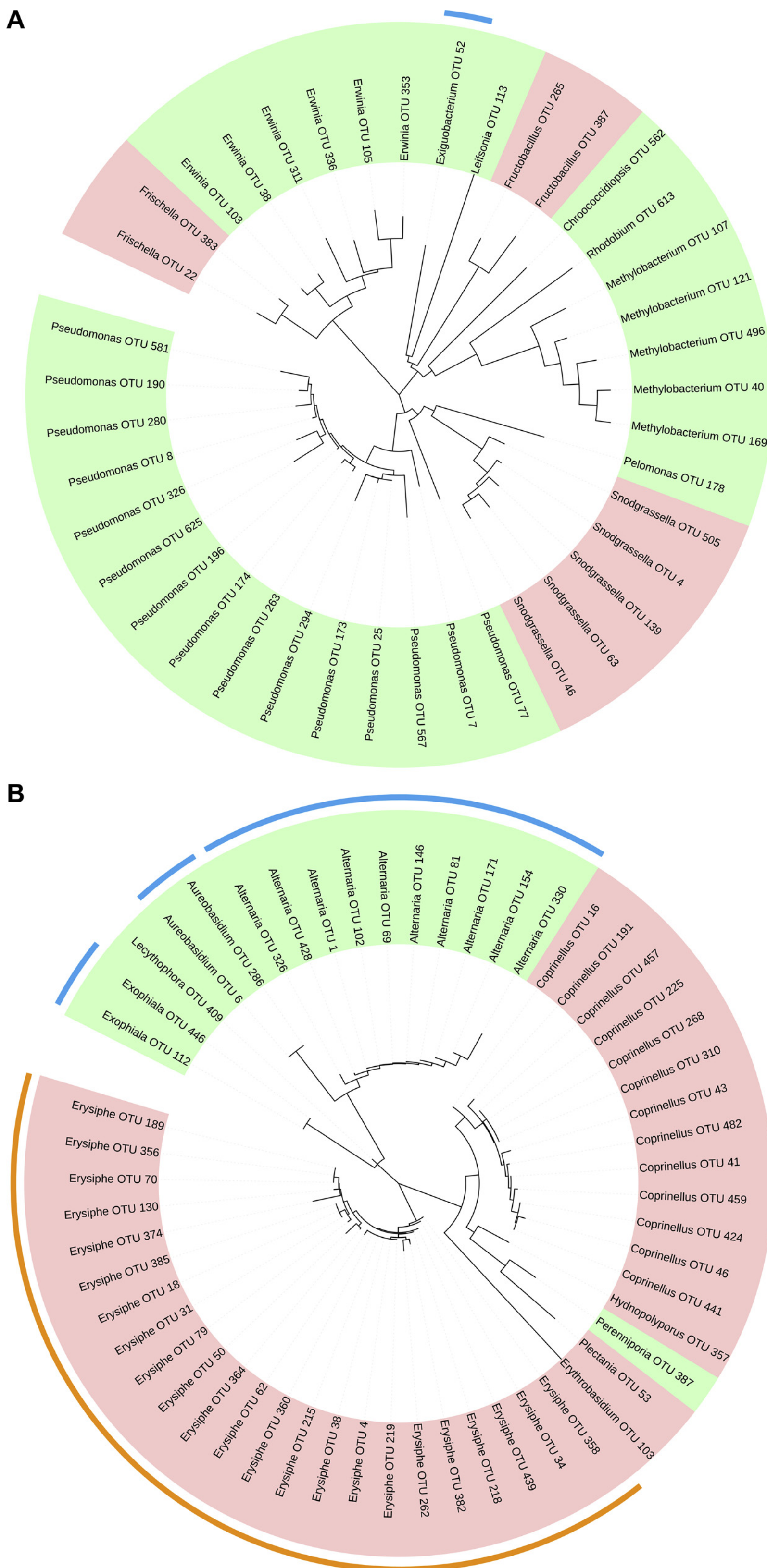

Fig. 5. Phylogenies and potential properties of genera affected by tagatose treatments. Bacterial (A) and fungal genera (B) with a significant increase (light green) or decrease (light red) in relative abundance on tagatose (TAG)-treated plants as compared with untreated plants were selected for each vineyard, according to Student's *t*-test ($P \leq 0.05$; Table S6). The neighbor-joining tree was generated based on 16S and ITS sequences using MEGA 7.0 with bootstrap and visualised using the iTOL software. Bacterial and fungal genera that comprise potential biocontrol agents [*Alternaria alternata* (Musetti et al., 2006), *Aureobasidium pullulans* (Harm et al., 2011), *Exiguobacterium acetylicum* (Lee et al., 2014) and *Exophiala jeanselmei* (Redmond et al., 2015)] and phytopathogens (*Erysiphe necator*) of grapevine are highlighted by blue and orange lines, respectively.

Cryptococcus, *Gibberella*, *Paraconiothyrium*, *Rhodosporidium* and *Sporobolomyces* were consistent among treatments and locations and they represent the conserved core microbiota of fungal leaf populations (Fig. 4B).

The relative abundance of the dominant genera *Erysiphe* decreased by TAG treatments in vineyard 2 and it was low in UNT and TAG-treated plants in vineyard 1 (Fig. 4B), in agreement with the reduction of powdery mildew severity in vineyard 2 and the absence of visible symptoms in vineyard 1. Moreover, the pairwise comparison between TAG-treated and UNT leaves within each vineyard demonstrated also

the decrease in relative abundance of the rare genera (relative abundance ≤ 2 %) *Coprinellus* and *Plectania* in vineyard 1, *Erythrobasidium* and *Hydnopolyporus* in vineyard 2 (Table S6). Moreover, the increase in relative abundance of two (*Lecythophora* and *Perenniporia*) and three (*Alternaria*, *Aureobasidium* and *Exophiala*) genera was found on TAG-treated leaves of vineyard 1 and vineyard 2, respectively. In particular, TAG increased the relative abundance of three genera that comprised potential biocontrol species against grapevine pathogens in at least one vineyard, such as *Alternaria*, *Aureobasidium* and *Exophiala* (Fig. 5B and Table S6).

4. Discussion

Dynamics of the phyllosphere microbiota and the ratio between pathogenic and beneficial microorganisms could influence grapevine health and production (Bokulich et al., 2016; Perazzoli et al., 2014; Pinto et al., 2015, 2014; Zarraonaindia et al., 2015). In particular, proportions of beneficial taxa can be affected by plant treatments, environmental conditions and anthropogenic factors (Fort et al., 2016; Perazzoli et al., 2014; Pinto et al., 2014; Vitulo et al., 2019). The plant microbiota engineering has been proposed as possible approach to optimize microbial consortia that harbour beneficial microbes, but this potential biocontrol strategy has not been applied yet (Syed Ab Rahman et al., 2018; Toju et al., 2018). In particular, the possibility of stimulating phyllosphere indigenous biocontrol agents and plant defences by leaf treatments with a protein-derived product raised the question whether beneficial plant microbiota can be artificially shaped by the application of selected nutritional substrates (Cappelletti et al., 2016). TAG is a rare sugar, which is metabolised only by certain microbial taxa and has an inhibitory activity against some important crop pathogens (Ohara et al., 2019). TAG was recognised as GRAS with no negative effects on human health (Levin, 2002; Vastenavond et al., 2011), suggesting a low risk in field application as a fungicide in agriculture. In addition, this rare sugar showed prebiotic properties on the human gut microbiota (Oh, 2007; Vastenavond et al., 2011) letting us hypothesise that it might positively affect the grapevine phyllosphere microbiota. TAG influenced the growth of culturable plant-associated bacteria and fungi *in vitro* and affected the diversity and taxonomic structure of leaf bacterial and fungal populations under field conditions. In particular, the contrasting TAG effects on culturable bacteria and fungi between the *in vitro* and field condition assay indicated a strong effect in the bacterial stimulation and fungal inhibition during the short incubation *in vitro* and suggested possible selection of plant associated microorganisms to TAG treatments under field conditions. Specifically, we showed here that the relative abundance of a fungal genus that comprises grapevine pathogens (*Erysiphe* spp.) was decreased by TAG treatments, in agreement with the reduction of powdery mildew symptoms on grapevine leaves under field conditions. Conversely, the relative abundance of bacterial and fungal genera comprising potential biocontrol agents against grapevine pathogens was increased by TAG under field conditions, such as *Alternaria* spp. (Musetti et al., 2006), *Aureobasidium* spp. (Harm et al., 2011), *Exiguobacterium* spp. (Lee et al., 2014) and *Exophiala* spp. (Redmond et al., 2015). Therefore, TAG treatments could have a role in the reduction of two grapevine diseases (*i.e.* downy mildew and powdery mildew) and in the consequent increase in relative abundance of potential beneficial microorganisms. However, three replicates for each condition have been analysed in this study and the promising preliminary results obtained need further validation. In particular, our amplicon sequencing approach allowed a taxonomic identification at the level of genera and, in some cases, the same genus comprises both pathogenic and beneficial microorganisms of grapevine, as in the case of *Pseudomonas*. The potential properties of TAG-affected genera were assigned in relation to the possible grapevine interaction and some taxa can also include potential pathogens of other plant species, as for example *Alternaria*, *Erwinia* and *Exophiala*. Thus, potential properties of plant-associated microorganisms cannot be easily assigned with a taxonomic identification at the level of genus and only putative effects can be hypothesized. Further molecular analyses are therefore required to identify TAG-stimulated microorganisms at deep taxonomic resolution on a large study with several grapevine cultivars and locations, in order to asses TAG effects under different environmental conditions.

TAG effects on the balance between potential pathogenic and beneficial microorganisms observed on grapevine leaves was in agreement with previous studies on human-associated microorganisms. For example, TAG increased and reduced the abundance of beneficial (*Enterococcus* spp. and *Lactobacillus* spp.) and pathogenic bacteria (coliform bacteria) in the human gut microbiota, respectively (Vastenavond et al., 2011). TAG was not metabolised by some human pathogens, such as *Bacillus* spp., *Escherichia* spp., *Listeria* spp., *Salmonella* spp., *Staphylococcus* spp. and *Streptococcus* spp. (Bautista et al., 2000; Hasibul et al., 2018; Lobete et al., 2017; Sawada et al., 2015). Although limited information is available on the TAG catabolic pathways, it can be used as carbohydrate source by only certain microbial taxa (Hayer et al., 2013; Komoń-Zelazowska et al., 2007; Van der Heiden et al., 2013). To be more precise, TAG can be either transported into the cell by phosphotransferase uptake systems or used as an intermediate in the lactose, galactose and galactitol catabolism by some bacterial species (Van der Heiden et al., 2013). For example, the *Lactobacillus* spp. metabolism includes the TAG-6-phosphate pathway (Martinussen et al., 2013; Wu and Shah, 2017) and the incubation with TAG caused a complex transcriptional reprogramming of the carbohydrate metabolism in *L. rhamnosus* with activation of the phosphotransferase system (Koh et al., 2013). Moreover, *Erwinia persicinus* (Hao et al., 1990) and *Exiguobacterium aurantiacum* (Raichand et al., 2012) were able to produce acid from the TAG fermentation and a TAG epimerase implicated in the TAG metabolism was cloned in *Pseudomonas cichorii* (Ishida et al., 1997), in line with the TAG-dependent stimulation of the phyllosphere *Erwinia*, *Exiguobacterium* and *Pseudomonas* genera in our samples. It was also reported that TAG supported the growth of *Trichoderma aggressivum* and *T. pleuroticola* (Komoń-Zelazowska et al., 2007), but not that of *Aspergillus niger* (Hayer et al., 2013), indicating that TAG prebiotic properties are possibly related to its nutritional and anti-nutritional effects on specific microbial taxa. Therefore, TAG could have a double effect on plant health: it could act as an anti-nutritional molecule on some phytopathogens (direct effect) and as a nutritional factor on some indigenous biocontrol microorganisms (indirect effect). TAG could increase the relative abundance of some natural beneficial microorganisms and they could partially contribute to disease control by competing for space, antagonism or plant resistance activation, as shown for leaf microorganisms stimulated by a protein-derived product (Cappelletti et al., 2016). However, further functional studies are required to better characterize nutritional and anti-nutritional properties of TAG on culturable plant-associated pathogenic and beneficial microorganisms *in vitro* and on grapevine leaves. Moreover, additional investigations are required to understand potential TAG impacts on insects and soil-borne microorganisms. Although TAG residues were detectable on grapevine leaves at the collection date, further studies are required to characterise the TAG persistence on grapevine leaves, its rain fastness and possible routes of degradation by leaf-associated microorganisms.

TAG-dependent changes on leaf microbial populations different in the two vineyards tested, indicating that TAG effects were influenced by the indigenous communities originally residing on grapevine leaves, consistently with previous findings with a protein-derived product (Cappelletti et al., 2016). Plants of the ‘Schiava gentile’ cultivar located in vineyard 2 on a low hill with north-west exposition showed high relative abundance of the bacterial families *Bifidobacteriaceae*, *Leuconostocaceae*, *Neisseriaceae* and *Orbaceae* and the fungal families *Dothioraceae*, *Erysiphaceae* and *Mytilinidiaceae*. Conversely, the ‘Pinot gris’ plants in vineyard 1 in the valley floor with a different training system from vineyard 1 hosted high relative abundance of *Micrococcaceae*,

Enterobacteriaceae, *Pseudomonadaceae* and *Xanthomonadaceae* bacteria and *Amphisphaeriaceae*, *Agaricaceae*, *Botryobasidiaceae*, *Cystofilobasidiaceae*, *Hydnodontaceae*, *Sarcosomataceae* and *Xylariaceae* fungi. At the genus level, UNT plants of vineyard 2 showed greater relative abundance of three dominant bacterial (*Gilliamella*, *Frischella* and *Snodgrassella*) and one dominant fungal (*Erysiphe*) genera, while vineyard 1 showed greater relative abundance of *Pseudomonas*, *Pantoea* and *Stenotrophomonas*. In agreement with previous findings (Fort et al., 2016; Mezzasalma et al., 2018; Perazzolli et al., 2014; Pinto et al., 2014; Varanda et al., 2016), the structure of the phyllosphere microbiota differed according to the grapevine location. Although the two vineyards were at less than 5 km from each other, differences in the host genotype, microclimatic conditions, agricultural practices and soil properties could exert a selective pressure and consequent adaptation of the phyllosphere microbiota of grapevine (Fort et al., 2016; Mezzasalma et al., 2018; Perazzolli et al., 2014; Pinto et al., 2014; Vitulo et al., 2019; Zarraonaindia et al., 2015). However, some dominant bacterial (*Acinetobacter*, *Enterobacter*, *Lactococcus*, *Orbus* and *Variovorax*) and fungal genera (*Chalastospora*, *Cryptococcus*, *Gibberella*, *Paraconiothyrium*, *Rhodosporidium* and *Sporobolomyces*) were not affected by the grapevine location and they represent a conserved core microbiota of the analysed vineyards.

Although the structure of the phyllosphere microbiota differed according to the plant genotype, climatic conditions and anthropogenic factors, common features with microorganisms residing on grapevine leaves can be recognised (Fort et al., 2016; Perazzolli et al., 2014; Pinto et al., 2014; Zarraonaindia et al., 2015). For example, leaf communities of this study were dominated by Ascomycota, Basidiomycota and Proteobacteria, in agreement with previous findings (Cappelletti et al., 2016; Fort et al., 2016; Perazzolli et al., 2014; Pinto et al., 2014; Zarraonaindia et al., 2015). The relative abundance of Proteobacteria commonly increased during the growing season (Singh et al., 2018), supporting the high proportions of these bacteria at the collection date of this study. The large proportion of Gammaproteobacteria was mainly due to the high relative abundance of *Enterobacteriaceae*, *Moraxellaceae*, *Orbaceae*, *Pseudomonadaceae* and *Xanthomonadaceae*. In particular, *Frischella*, *Gilliamella* and *Orbus* (Orbaceae), *Enterobacter* and *Pantoea* (*Enterobacteriaceae*), *Pseudomonas* (*Pseudomonadaceae*), *Stenotrophomonas* (*Xanthomonadaceae*), *Acinetobacter* (*Moraxellaceae*) were found on grapevine leaves. Moreover, *Neisseriaceae* (*e.g.Snodgrassella*), belonging to the Betaproteobacteria, were also highly represented. *Acinetobacter*, *Enterobacter, Leuconostocaceae*, *Pantoea, Pseudomonas* and *Stenotrophomonas* have been commonly found on grapevine leaves (Cappelletti et al., 2016; Perazzolli et al., 2014; Pinto et al., 2014). On the other hand, *Frischella*, *Gilliamella*, *Orbus* and *Snodgrassella* were usually hosted by honey bee, bumble bee and fruit fly (Kwong and Moran, 2013; Stathopoulou et al., 2019; Yong et al., 2019). However, a genus phylogenetically related to *Snodgrassella* was isolated from poplar bark (Li et al., 2017) and plant-mediated transmission of *Snodgrassella* was found in wild bees (McFrederick et al., 2017), suggesting possible microbial exchanges among insects and plants. Possible insect-derived taxa could be only temporary present and they are probably not persistent colonisers of grapevine leaves. In consequence to the presence of possible insect-derived microorganisms, the relative abundance of some typical grapevine-associated taxa was lower than expected, as for example that of *Acetobacter*, *Comamonadaceae*, *Curtobacterium*, *Serratia* and *Sphingomonas* (Cappelletti et al., 2016; Perazzolli et al., 2014; Pinto et al., 2014). Possible microaerophilic bacteria belonging to *Bifidobacteriaceae* and *Leuconostocaceae* were found on grapevine leaves and the relative abundance *Fructobacillus* was affected by TAG. Interestingly, OTUs belonging to the *Lactobacillaceae* family (*e.g.Lactobacillus*) were previously found on grapevine leaves (Singh et al., 2018), suggesting that microaerophilic bacteria can be hosted by the leaf environment. In agreement with previous studies, fungal communities were dominated by Dothideomycetes, Microbotryomycetes and Tremellomycetes classes (Fort et al., 2016); *Erysiphaceae* (Cappelletti et al., 2016), *Pleosporaceae* and Tremellales (Perazzolli et al., 2014) family, as well as by *Alternaria*, *Areobasidium*, *Erisiphe*, *Cryptococcus* genus (Fort et al., 2016; Pinto et al., 2014) (Cappelletti et al., 2016; Castañeda et al., 2018).

The role and ecology of the phyllosphere microbiota is only partially understood (Bulgarelli et al., 2013; Lindow and Brandl, 2003), but many practical applications on plant health and grape quality are affected by the equilibrium between pathogenic and beneficial microorganisms (Bokulich et al., 2016; Perazzolli et al., 2014; Pinto et al., 2015, 2014; Zarraonaindia et al., 2015). Beneficial communities of the gut microbiota display a barrier effect against human pathogens (Hooper, 2009). Likewise, a protective effect was assumed for the phyllosphere microbiota (Vorholt, 2012) and beneficial communities of the plant microbiota were described as plant probiotic agents (Berlec, 2012). TAG has prebiotic benefits for the human gut microbiota (Vastenavond et al., 2011) and, in our study, changed the structure of phyllosphere communities of grapevine. Thus, TAG could have plant prebiotic properties and it may represent a novel strategy for sustainable plant protection that will possibly gain deeper investigations in the future for the control of grapevine pathogens. This work represents onestep forward on research studies focusing on the engineering of indigenous phyllosphere populations by the application of selected nutritional factors to control phytopathogens and to increase the relative abundance of plant beneficial microorganisms. In particular, TAG seems able to shift the relative abundance of phyllosphere pathogenic and beneficial microorganisms, possibly thanks to its possible nutritional/anti-nutritional properties for specific microbial taxa. Further functional analyses of TAG-dependent stimulatory and inhibitory effects are required on selected phytopathogenic and biocontrol microorganisms, in order to better understand genetic determinants and metabolic pathways affected by this rare sugar.

Author contributions

AN and OG carried out the experiments under filed conditions and collected the samples, AN and GP characterised culturable microorganisms. MP, LA and GP analysed the amplicon sequencing data. MP, GP, LA and AN contributed to data interpretation and manuscript writing. IP and MP conceived the study, designed the experiment and coordinated all research activities. All the authors revised and approved the final manuscript.

Funding

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 773431 (project RELACS).

Declaration of Competing Interest

All authors declare no conflict of interest.

Acknowledgments

We thank Dr. Massimo Pindo and the technical staff of the Sequencing Platform at Fondazione Edmund Mach for sequencing and Dr. Mario Malacarne of the Chemistry Unit at Fondazione Edmund Mach for tagatose quantification.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2019.126387.

References

Anderson, M.J., Willis, T.J., 2003. Canonical analysis of principal coordinates: a useful method of constrained ordination for ecology. Ecology 84, 511–525.

Armijo, G., Schlechter, R., Agurto, M., Muñoz, D., Nuñez, C., Arce-Johnson, P., 2016. Grapevine pathogenic microorganisms: understanding infection strategies and host response scenarios. Front. Plant Sci. 7, 382.

Baker, G.C., Smith, J.J., Cowan, D.A., 2003. Review and re-analysis of domain-specific 16S primers. J. Microbiol. Methods 55, 541–555.

Bautista, D.A., Pegg, R.B., Shand, P.J., 2000. Effect of L-glucose and D-tagatose on bacterial growth in media and a cooked cured ham product. J. Food Prot. 63, 71–77.

Bengtsson-Palme, J., Ryberg, M., Hartmann, M., Branco, S., Wang, Z., Godhe, A., De Wit, P., Sanchez-Garcia, M., Ebersberger, I., de Sousa, F., Amend, A.S., Jumpponen, A., Unterseher, M., Kristiansson, E., Abarenkov, K., Bertrand, Y.J.K., Sanli, K., Eriksson, K.M., Vik, U., Veldre, V., Nilsson, R.H., 2013. Improved software detection and extraction of ITS1 and ITS2 from ribosomal ITS sequences of fungi and other eukaryotes for analysis of environmental sequencing data. Methods Ecol. Evol. 4, 914–919.

Berlec, A., 2012. Novel techniques and findings in the study of plant microbiota: search for plant probiotics. Plant Sci. 193, 96–102.

Bokulich, N.A., Collins, T.S., Masarweh, C., Allen, G., Heymann, H., Ebeler, S.E., Mills, D.A., 2016. Associations among wine grape microbiome, metabolome, and fermentation behavior suggest microbial contribution to regional wine characteristics. mBio 7, e00631–16.

Bray, J.R., Curtis, J.T., 1957. An ordination of the upland forest communities of Southern Wisconsin. Ecol. Monogr. 27, 325–349.

Bulgarelli, D., Schlaeppi, K., Spaepen, S., van Themaat, E.V.L., Schulze-Lefert, P., 2013. Structure and functions of the bacterial microbiota of plants. Annu. Rev. Plant Biol. 64, 807–838.

Buonassisi, D., Colombo, M., Migliaro, D., Dolzani, C., Peressotti, E., Mizzotti, C., Velasco, R., Masiero, S., Perazzolli, M., Vezzulli, S., 2017. Breeding for grapevine downy mildew resistance: a review of “omics” approaches. Euphytica 213, 103.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Tumbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335–336.

Cappelletti, M., Perazzolli, M., Antonielli, L., Nesler, A., Torboli, E., Bianchedi, P.L., Pindo, M., Puopolo, G., Pertot, I., 2016. Leaf treatments with a protein-based resistance inducer partially modify phyllosphere microbial communities of grapevine. Front. Plant Sci. 7, 1053.

Castañeda, L.E., Miura, T., Sánchez, R., Barbosa, O., 2018. Effects of agricultural management on phyllosphere fungal diversity in vineyards and the association with adjacent native forests. PeerJ 6, e5715.

Cataldi, T.R.I., Campa, C., De Benedetto, G.E., 2000. Carbohydrate Analysis by High-Performance Anion-Exchange Chromatography With Pulsed Amperometric Detection: The Potential is Still Growing 368. pp. 739–758.

Edgar, R.C., 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat. Methods 10, 996–998.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27, 2194–2200.

EPPO, 2001. European and Mediterranean Plant Protection Organization. Guidelines for the efficacy evaluation of fungicides: *Plasmopara viticola*. EPPO Bull. 31, 313–317.

EPPO, 2002. European and Mediterranean Plant Protection Organization. Guidelines for the biological evaluation of fungicides: *Uncinula necator*. EPPO Bull. 32, 315–318.

Fort, T., Robin, C., Capdevielle, X., Delière, L., Vacher, C., 2016. Foliar fungal communities strongly differ between habitat patches in a landscape mosaic. PeerJ 4, e2656.

Gadoury, D.M., Cadle-Davidson, L., Wilcox, W.F., Dry, I.B., Seem, R.C., Milgroom, M.G., 2012. Grapevine powdery mildew (*Erysiphe necator*): a fascinating system for the study of the biology, ecology and epidemiology of an obligate biotroph. Mol. Plant Pathol. 13, 1–16.

Gaspar, J.M., Thomas, W.K., 2015. FlowClus: efficiently filtering and denoising pyrosequenced amplicons. BMC Bioinformatics 16, 105.

Gessler, C., Pertot, I., Perazzolli, M., 2011. *Plasmopara viticola*: a review of knowledge on downy mildew of grapevine and effective disease management. Phytopathol. Mediterr. 50, 3–44.

Granström, T.B., Takata, G., Tokuda, M., Izumori, K., 2004. Izumoring: a novel and complete strategy for bioproduction of rare sugars. J. Biosci. Bioeng. 97, 89–94.

Hao, M.V., Brenner, D.J., Steigerwalt, A.G., Kosako, Y., Komagata, K., 1990. *Erwinia persicinus*, a new species isolated from plants. Int. J. Syst. Evol. Microbiol. 40, 379–383.

Harm, A., Kassemeyer, H.-H., Seibicke, T., Regner, F., 2011. Evaluation of chemical and natural resistance inducers against downy mildew (*Plasmopara viticola*) in grapevine. Am. J. Enol. Vitic. 62, 184–192.

Hartmann, M., Howes, C.G., Abarenkov, K., Mohn, W.W., Nilsson, R.H., 2010. V-Xtractor: an open-source, high-throughput software tool to identify and extract hypervariable regions of small subunit (16 S/18 S) ribosomal RNA gene sequences. J. Microbiol. Methods 83, 250–253.

Hasibul, K., Nakayama-Imaohji, H., Hashimoto, M., Yamasaki, H., Ogawa, T., Waki, J., Tada, A., Yoneda, S., Tokuda, M., Miyake, M., Kuwahara, T., 2018. D-Tagatose Inhibits the Growth and Biofilm Formation of Streptococcus mutans 17. pp. 843–851.

Hayer, K., Stratford, M., Archer, D.B., 2013. Structural features of sugars that trigger or support conidial germination in the filamentous fungus *Apergillus niger*. Appl. Environ. Microbiol. 79, 6924–6931.

Hooper, L.V., 2009. Do symbiotic bacteria subvert host immunity? Nat. Rev. Micro. 7, 367–374.

Ishida, Y., Kamiya, T., Itoh, H., Kimura, Y., Izumori, K., 1997. Cloning and characterization of the D-tagatose 3-epimerase gene from *Pseudomonas cichorii* ST-24. J. Ferment. Bioeng. 83, 529–534.

Izumori, K., 2006. Izumoring: a strategy for bioproduction of all hexoses. J. Biotechnol. 124, 717–722.

Kim, Y.-H., Nagata, R., Ohkubo, A., Ohtani, N., Kushibiki, S., Ichijo, T., Sato, S., 2018. Changes in ruminal and reticular pH and bacterial communities in Holstein cattle fed a high-grain diet. BMC Vet. Res. 14, 310.

Koh, J.H., Choi, S.H., Park, S.W., Choi, N.-J., Kim, Y., Kim, S.H., 2013. Synbiotic impact of tagatose on viability of *Lactobacillus rhamnosus* strain GG mediated by the phosphotransferase system (PTS). Food Microbiol. 36, 7–13.

Kõljalg, U., Larsson, K.-H., Abarenkov, K., Nilsson, R.H., Alexander, I.J., Eberhardt, U., Erland, S., Høiland, K., Kjøller, R., Larsson, E., Pennanen, T., Sen, R., Taylor, A.F.S., Tedersoo, L., Vrålstad, T., 2005. UNITE: a database providing web-based methods for the molecular identification of ectomycorrhizal fungi. New Phytol. 166, 1063–1068.

Kõljalg, U., Nilsson, R.H., Abarenkov, K., Tedersoo, L., Taylor, A.F.S., Bahram, M., Bates, S.T., Bruns, T.D., Bengtsson-Palme, J., Callaghan, T.M., Douglas, B., Drenkhan, T., Eberhardt, U., Duenas, M., Grebenc, T., Griffith, G.W., Hartmann, M., Kirk, P.M., Kohout, P., Larsson, E., Lindahl, B.D., Luecking, R., Martin, M.P., Matheny, P.B., Nguyen, N.H., Niskanen, T., Oja, J., Peay, K.G., Peintner, U., Peterson, M., Poldmaa, K., Saag, L., Saar, I., Schuessler, A., Scott, J.A., Senes, C., Smith, M.E., Suija, A., Taylor, D.L., Telleria, M.T., Weiss, M., Larsson, K.H., 2013. Towards a unified paradigm for sequence-based identification of fungi. Mol. Ecol. 22, 5271–5277.

Komoń-Zelazowska, M., Bissett, J., Zafari, D., Hatvani, L., Manczinger, L., Woo, S., Lorito, M., Kredics, L., Kubicek, C.P., Druzhinina, I.S., 2007. Genetically closely related but phenotypically divergent *Trichoderma* species cause green mold disease in oyster mushroom farms worldwide. Appl. Environ. Microbiol. 73, 7415–7426.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.

Kwong, W.K., Moran, N.A., 2013. Cultivation and characterization of the gut symbionts of honey bees and bumble bees: description of *Snodgrassella alvi* gen. nov., sp. nov., a member of the family *Neisseriaceae* of the Betaproteobacteria, and *Gilliamella apicola* gen. nov., sp. nov., a member of *Orbaceae* fam. nov., Orbales ord. nov., a sister taxon to the order ‘Enterobacteriales’ of the Gammaproteobacteria. Int. J. Syst. Bacteriol. 63, 2008–2018.

Lee, S.H., Kim, I.S., Kim, Y.C., 2014. Identification and characterization of novel biocontrol bacterial strains. Res. Plant Dis. 20, 182–188.

Legendre, P., Legendre, L., 1998. Numerical ecology. In: Legendre, P., Legendre, L. (Eds.), Developments in Environmental Modelling. Elsevier Science B.V, Amsterdam, pp. 852.

Letunic, I., Bork, P., 2011. Interactive tree of Life v2: online annotation and display of phylogenetic trees made easy. Nucleic Acids Res. 39, W475–W478.

Levin, G.V., 2002. Tagatose, the new GRAS sweetener and health product. J. Med. Food 5, 23–36.

Li, Y., Xue, H., Sang, S.-q., Lin, C.-l., Wang, X.-z., 2017. Phylogenetic analysis of family *Neisseriaceae* based on genome sequences and description of *Populibacter corticis* gen. nov., sp. nov., a member of the family *Neisseriaceae*, isolated from symptomatic bark of *Populus* × *euramericana* canker. PLoS One 12, e0174506.

Li, Z., Gao, Y., Nakanishi, H., Gao, X., Cai, L., 2013. Biosynthesis of rare hexoses using microorganisms and related enzymes. Beilstein J. Org. Chem. 9, 2434–2445.

Lindow, S.E., Brandl, M.T., 2003. Microbiology of the phyllosphere. Appl. Environ. Microbiol. 69, 1875–1883.

Lobete, M.M., Noriega, E., Batalha, M.A., de Beurme, S., Van de Voorde, I., Van Impe, J.F., 2017. Effect of tagatose on growth dynamics of *Salmonella* typhimurium and *Listeria monocytogenes* in media with different levels of structural complexity and in UHT skimmed milk. Food Control 73, 31–42.

Martins, G., Lauga, B., Miot-Sertier, C., Mercier, A., Lonvaud, A., Soulas, M.-L., Soulas, G., Masneuf-Pomarede, I., 2013. Characterization of epiphytic bacterial communities from grapes, leaves, bark and soil of grapevine plants grown, and their relations. PLoS One 8, e73013.

Martinussen, J., Solem, C., Holm, A.K., Jensen, P.R., 2013. Engineering strategies aimed at control of acidification rate of lactic acid bacteria. Curr. Opin. Biotechnol. 24, 124–129.

McFrederick, Q.S., Thomas, J.M., Neff, J.L., Vuong, H.Q., Russell, K.A., Hale, A.R., Mueller, U.G., 2017. Flowers and wild megachilid bees share microbes. Microb. Ecol. 73, 188–200.

McMurdie, P.J., Holmes, S., 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8, e61217.

Mezzasalma, V., Sandionigi, A., Guzzetti, L., Galimberti, A., Grando, M.S., Tardaguila, J., Labra, M., 2018. Geographical and Cultivar Features Differentiate Grape Microbiota in Northern Italy and Spain Vineyards 9. pp. 946.

Müller, T., Ruppel, S., 2014. Progress in cultivation-independent phyllosphere microbiology. FEMS Microbiol. Ecol. 87, 2–17.

Musetti, R., Vecchione, A., Stringher, L., Borselli, S., Zulini, L., Marzani, C., D'Ambrosio, M., di Toppi, L.S., Pertot, I., 2006. Inhibition of sporulation and ultrastructural alterations of grapevine downy mildew by the endophytic fungus *Alternaria alternata*. Phytopathology 96, 689–698.

Oh, D.-K., 2007. Tagatose: properties, applications, and biotechnological processes. Appl. Microbiol. Biotechnol. 76, 1.

Ohara, T., Ishida, Y., Kudou, R., Kakibuchi, K., Akimitsu, K. Plant disease control agent comprising d-tagatose as active ingredient, and plant disease control method 2010. vol. Patent No.: US9125409B2.

Ondov, B.D., Bergman, N.H., Phillippy, A.M., 2011. Interactive metagenomic visualization in a web browser. BMC Bioinformatics 12, 385.

Ottesen, A.R., Gorham, S., Pettengill, J.B., Rideout, S., Evans, P., Brown, E., 2015. The

impact of systemic and copper pesticide applications on the phyllosphere microflora of tomatoes. J. Sci. Food Agric. 95, 1116–1125.

Perazzolli, M., Antonielli, L., Storari, M., Puopolo, G., Pancher, M., Giovannini, O., Pindo, M., Pertot, I., 2014. Resilience of the natural phyllosphere microbiota of the grapevine to chemical and biological pesticides. Appl. Environ. Microbiol. 80, 3585–3596.

Perazzolli, M., Moretto, M., Fontana, P., Ferrarini, A., Velasco, R., Moser, C., Delledonne, M., Pertot, I., 2012. Downy Mildew Resistance Induced by Trichoderma harzianum T39 in Susceptible Grapevines Partially Mimics Transcriptional Changes of Resistant Genotypes 13. pp. 660.

Pinto, C., Pinho, D., Cardoso, R., Custódio, V., Fernandes, J., Sousa, S., Pinheiro, M., Egas, C., Gomes, A.C., 2015. Wine fermentation microbiome: a landscape from different Portuguese wine appellations. Front. Microbiol. 6, 905.

Pinto, C., Pinho, D., Sousa, S., Pinheiro, M., Egas, C., Gomes, A.C., 2014. Unravelling the diversity of grapevine microbiome. PLoS One 9, e85622.

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 Res. 41, D590–D596.

Raichand, R., Pareek, S., Singh, N.K., Mayilraj, S., 2012. *Exiguobacterium aquaticum* sp. nov., a member of the genus *Exiguobacterium*. Int. J. Syst. Evol. Microbiol. 62, 2150–2155.

Rastogi, G., Coaker, G.L., Leveau, J.H.J., 2013. New insights into the structure and function of phyllosphere microbiota through high-throughput molecular approaches. FEMS Microbiol. Lett. 348, 1–10.

Redmond, J.C., Marois, J.J., MacDonald, J.D., 2015. Biological control of *Botrytis cinerea* on roses with epiphytic microorganisms. Plant Dis. 71, 799–802.

Sawada, D., Ogawa, T., Miyake, M., Hasui, Y., Yamaguchi, F., Izumori, K., Tokuda, M., 2015. Potent inhibitory effects of D-tagatose on the acid production and water-insoluble glucan synthesis of *Streptococcus mutans* GS5 in the presence of sucrose. Acta Med. Okayama 69, 105–111.

Schmieder, R., Edwards, R., 2011. Quality control and preprocessing of metagenomic datasets. Bioinformatics 27, 863–864.

Simpson, E.H., 1949. Measurement of diversity. Nature 163, 688.

Singh, P., Santoni, S., This, P., Péros, J.-P., 2018. Genotype-environment interaction shapes the microbial assemblage in grapevine's phyllosphere and carposphere: an NGS approach. Microorganisms 6, 96.

Stathopoulou, P., Asimakis, E.D., Khan, M., Caceres, C., Bourtzis, K., Tsiamis, G., 2019. Irradiation effect on the structure of bacterial communities associated with the oriental fruit fly, *Bactrocera dorsalis*. Entomol. Exp. Appl. 167, 209–219.

Syed Ab Rahman, S.F., Singh, E., Pieterse, C.M.J., Schenk, P.M., 2018. Emerging microbial biocontrol strategies for plant pathogens. Plant Sci. 267, 102–111.

Sylla, J., Alsanius, B., Krüger, E., Reineke, A., Bischoff-Schaefer, M., Wohanka, W., 2013a. Introduction of *Aureobasidium pullulans* to the phyllosphere of organically grown strawberries with focus on its establishment and interactions with the resident microbiome. Agronomy 3, 704–731.

Sylla, J., Alsanius, B.W., Kruger, E., Reineke, A., Strohmeier, S., Wohanka, W., 2013b. Leaf microbiota of strawberries as affected by biological control agents. Phytopathology 103, 1001–1011.

Toju, H., Peay, K.G., Yamamichi, M., Narisawa, K., Hiruma, K., Naito, K., Fukuda, S., Ushio, M., Nakaoka, S., Onoda, Y., Yoshida, K., Schlaeppi, K., Bai, Y., Sugiura, R., Ichihashi, Y., Minamisawa, K., Kiers, E.T., 2018. Core microbiomes for sustainable agroecosystems. Nat. Plants 4, 247–257.

Vacher, C., Hampe, A., Porté, A.J., Sauer, U., Compant, S., Morris, C.E., 2016. The phyllosphere: microbial jungle at the plant–climate interface. Annu. Rev. Ecol. Evol. Syst. 47, 1–24.

Van der Heiden, E., Delmarcelle, M., Lebrun, S., Freichels, R., Brans, A., Vastenavond, C.M., Galleni, M., Joris, B., 2013. A pathway closely related to the d-tagatose pathway of gram-negative enterobacteria identified in the gram-positive bacterium *Bacillus licheniformis*. Appl. Environ. Microbiol. 79, 3511–3515.

Varanda, C.M.R., Oliveira, M., Materatski, P., Landum, M., Clara, M.I.E., Félix, Md.R., 2016. Fungal endophytic communities associated to the phyllosphere of grapevine cultivars under different types of management. Fungal Biol. 120, 1525–1536.

Vastenavond, C., Bertelsen, H., Hansen, S., Laursen, R., Saunders, J., Eriknauer, K., 2011. Tagatose (D-tagatose)Alternative Sweeteners, fourth edition. CRC Press, pp. 197–222.

Vitulo, N., Lemos, W.J.F., Calgaro, M., Confalone, M., Felis, G.E., Zapparoli, G., Nardi, T., 2019. Bark and Grape Microbiome of Vitis vinifera: Influence of Geographic Patterns and Agronomic Management on Bacterial Diversity 9. pp. 3203.

Vorholt, J.A., 2012. Microbial life in the phyllosphere. Nat. Rev. Microbiol. 10, 828–840.

Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R., 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. 73, 5261–5267.

White, T., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M., Gelfand, D., Sninsky, J., TJ, W. (Eds.), PCR Protocols: A guide to Methods and Applications. Academic Press, Inc., New York, NY, pp. 315–322.

Wu, Q., Shah, N.P., 2017. The potential of species-specific tagatose-6-phosphate (T6P) pathway in *Lactobacillus casei* group for galactose reduction in fermented dairy foods. Food Microbiol. 62, 178–187.

Yong, H.-S., Song, S.-L., Chua, K.-O., Lim, P.-E., Eamsobhana, P., 2019. Microbiota and potential opportunistic pathogens associated with male and female fruit flies of Malaysian *Bactrocera carambolae* (Insecta: Tephritidae). Meta Gene 19, 185–192.

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, e02527–14.