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Developing a culturomic approach to build a collection of grapevine foliar microorganisms with potential applications in disease biocontrol

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ABSTRACT

Isolation and conservation of plant-associated microorganisms in pure cultures is important to be able to understand their influence on plant health. However, capturing the whole range of microbial taxa detected by culture-independent approaches remains labour-intensive and challenging since a large proportion fail to grow under laboratory conditions. Culturomic approaches that involve using a variety of culture conditions coupled with high-throughput identification methods are a recommended strategy to effectively capture plant-associated microbial diversity. A number of studies have demonstrated that using plant-based culture media rather than conventional laboratory media is an efficient strategy for increasing the cultivability of plant-associated microorganisms. This study aimed to develop a culturomic approach to build a collection of grapevine foliar microorganisms with potential applications for the biocontrol of foliar diseases. We developed and tested a custom grapevine leaf-based culture medium (GLA) to better mimic the leaf habitat and consequently try to increase the cultivability of foliar microorganisms. Of the 90 genera that were isolated, 17 were isolated exclusively on GLA including 4 bacteria, 1 yeast, and 12 filamentous fungi. Overall, using the GLA allowed for the isolation of a number of taxa that are of interest for their biocontrol potential against plant pathogens. Interestingly, it was also effective for isolating and speeding up the growth of slow-growing filamentous fungi associated with grapevine trunk diseases. Using a combination of laboratory media and the custom GLA resulted in a collection that was representative of 12.5 % of bacterial genera and 17.6 % of fungal genera detected on grapevine leaves. This collection will serve as a repository of pure cultures for conducting experiments. Candidates will be screened and tested both individually and in consortia for potential applications in microbial biocontrol against grapevine diseases, particularly *Plasmopara viticola* (grapevine downy mildew), with the ultimate goal of developing effective biocontrol products and moving towards more sustainable viticulture.

KEYWORDS: culturomics, plant-based culture media, grapevine microbiome, downy mildew, culture collection, biocontrol

INTRODUCTION

Plants are colonised by a variety of epiphytic and endophytic microorganisms. Together, a host plant and its associated microbial communities form a supra-organism called a holobiont (Müller *et al.*, 2016; Vandenkoornhuyse *et al.*, 2015). Their microbial genomes provide supplementary functions and contribute to the host's phenotype by extending the host genome (Bettenfeld *et al.*, 2022; Müller *et al.*, 2016). Grapevines are no exception. Their roots, trunks, leaves, inflorescences and fruits form distinct compartments that are colonised by distinct and diverse communities of microorganisms (Bettenfeld *et al.*, 2022). At every life stage of a grapevine, its growth, health, and fruit quality are influenced by microbe-microbe and microbe-plant interactions (Fournier *et al.*, 2022). A number of studies have used culture-independent approaches to describe the grapevine microbiome across the different plant compartments, along the plant vegetative cycle and in the surrounding soil (Jayawardena *et al.*, 2018; Knapp *et al.*, 2021; Liu & Howell, 2021; Martínez-Diz *et al.*, 2019; Palladino *et al.*, 2024; Pinto *et al.*, 2014). Others have also compared the effects of farming practices (Battie-Laclau *et al.*, 2025; Martins *et al.*, 2014; Nardi *et al.*, 2021) and plant health condition when challenged by various pathogens (Bruez *et al.*, 2020; Fournier *et al.*, 2025; Oliveira-Pinto *et al.*, 2025; Wicaksono *et al.*, 2023) on the grapevine microbiome. These culture-independent methods based on high-throughput sequencing of environmental DNA have the advantage of being rapid alongside bypassing the issues of 'uncultivability' of microorganisms within complex communities (Nichols, 2007). However, they do not provide direct access to the microorganisms themselves and cannot replace the need for microbial culture collections, which are essential for conducting experiments to unravel the mechanisms of microbe-microbe and microbe-plant interactions.

Using culture-dependent methods is necessary to procure information that can't be obtained from environmental DNA alone. Metagenomic data can be combined with metatranscriptomic, metabolomic, and metaproteomic data to gain some insights into gene expression and microbial community activity under certain environmental conditions or in response to a pathogen (Aguiar-Pulido *et al.*, 2016; Castro-Moretti *et al.*, 2020; Jin *et al.*, 2025; Kimotho & Maina, 2023). Yet, interpreting the findings of these approaches without knowledge of the underlying microbial physiology is difficult (Lewis *et al.*, 2021). Cultivation is indispensable to ascertain microbial features like optimal growth conditions, enzymatic activity, and cell functioning accurately, and the only way to understand the physiology of microorganisms that may be inactive or dormant in natural conditions (Ellis *et al.*, 2003; Youssef *et al.*, 2016). Furthermore, the isolation, purification and storage of these microorganisms in pure cultures is required for assessing and understanding their influence on crop health and productivity (Gerna *et al.*, 2022), which is a necessary first step for the development of biofertilizers, biocontrol solutions, and other biotechnological innovations in sustainable agriculture (Riva *et al.*, 2022).

Culturomics involves the use of a range of culture conditions coupled with a high-throughput method of identification to isolate and identify microorganisms in a sample (Lagier *et al.*, 2012). This approach was first developed to enable cultivation and identification of previously uncultured microorganisms in the human gut microbiome (Lagier *et al.*, 2012; Lagier *et al.*, 2015; Lagier *et al.*, 2018). It has since been used successfully for cultivating plant-associated bacterial communities (Riva *et al.*, 2022; Sarhan *et al.*, 2019). A number of studies have shown that using plant-based culture media, rather than conventional media that contain chemically synthesised or animal-based extracts, can successfully increase the cultivability of plant-associated microbiota. For instance, Youssef *et al.* (2016) demonstrated that the juices and saps of cactus and succulent plants supported the growth and recovery of rhizobacteria associated with root samples of succulent plants. Similarly, culture media made with plant powder teabags have been shown to increase the cultivability of rhizobacteria of Egyptian clover, maize and lucerne (Hegazi *et al.*, 2017; Sarhan *et al.*, 2016). Another study compared refuse of turfgrass, cactus and clover for plant-based culture medium preparation (Mourad *et al.*, 2018). Their findings demonstrated that preparing plant-based culture media using refuse from plants within the same family as the host plant was more efficient for culturing rhizobacteria. In their study on soybean, Gerna *et al.* (2022) designed a number of media containing ground germinating seeds to mimic the chemistry of the seed endosphere. Again, their fully seed-based medium allowed for isolation of a larger diversity of seed endophytic bacteria when compared with diluted and undiluted commercial selective media as well as the commercial media supplemented with germinating ground seeds.

The present study aimed to develop and use a culturomic approach to build a collection of grapevine foliar microorganisms with potential applications for the biocontrol of grapevine foliar diseases, particularly downy mildew (caused by the oomycete *Plasmopara viticola*). We developed and tested a custom grapevine leaf-based culture medium (GLA) to enhance the cultivability of microbial communities associated with grapevine leaf surface (phyllosphere) and leaf endosphere. We coupled this plant-based media with conventional diluted and undiluted laboratory media to isolate bacteria, yeast and filamentous fungi from grapevine leaves. We tested the following hypotheses: GLA allows for higher colony counts (H1) and a larger diversity of taxa (H2) compared to the conventional culture media, as it better mimics the leaf habitat compared to conventional media; GLA allows for isolation of taxa that are distinct from those isolated from conventional media, and known to be harder to isolate using conventional media (H3); using GLA in addition to conventional media increases the representativeness of the collection (H4), measured as the percentage of taxa isolated among all taxa detected using metabarcoding approaches.

MATERIALS AND METHODS

1. Grapevine leaf-based culture medium (GLA)

To increase cultivability of the grapevine foliar microbiota, we developed a custom grapevine leaf-based medium using a liquid extract of grapevine leaves. We prepared one single homogenous liquid extract for our study using leaves taken from 200 five-week-old potted vines of Cabernet-Sauvignon, grown in a greenhouse and untreated with fungicides. Between five and eight leaves were collected per plant by cutting off the petioles. The leaves were washed thoroughly with deionized water, dried using a salad spinner and finally pressed between sheets of filter paper (Cytiva Whatman™ qualitative filter paper: Grade 2 sheets, Thermo Fisher Scientific, France). The leaves were blended (Waring® laboratory blender, Merck, Germany) to create a smooth slurry with a ratio of 1 L of sterile deionised water for 500 g of leaves. The slurry was then filtered using a muslin cloth, pressing by hand to extract the liquid while eliminating as much leaf debris as possible. The liquid extract was distributed into 50 mL centrifuge tubes (Corning Inc., Falcon® Tubes, USA) and stored at –20 °C. Its chemical composition was analysed at Laboratoire AQUANAL (Pessac, France) and is available in Tables S1, S2 and S3. To make our custom grapevine leaf-based agar medium (GLA), we used 50 mL/L of the liquid extract and 20 g/L of agar (Sigma-Aldrich, France). The medium was autoclaved at 121 °C for 20 minutes.

2. Study sites

Four organic vineyard plots located in the Médoc region (Nouvelle-Aquitaine, France) were selected for this study (Table 1). Two plots were planted with the Cabernet-Sauvignon variety and two plots were planted with the Merlot variety. The plots with matching grape varieties were located in close proximity to one another and were therefore considered as a plot pair. Within each pair, the two plots had contrasting levels of downy mildew incidence and severity over several years according to the epidemiological data provided by the French Institute of Vine and Wine (IFV) (Fournier *et al.*, 2025).

3. Leaf sampling

Grapevine leaves were sampled before the first round of fungicide treatments of the growing season 2022. Both plot pairs were sampled on 21 April 2022. The date corresponded to the phenological stages between two-three unfolded leaves as well as the onset of the vine's exposure to downy mildew. In each plot, leaves were collected from two zones: the centre of the plot (CEN) and the 'untreated control' area (UC1) maintained by the IFV to monitor disease dynamics. Vines in the UC1 areas did not receive any fungicide treatments in 2021, whereas vines in the CEN areas were treated with certified organic products (Fournier *et al.*, 2025). Within each zone, we collected six leaves per vine from four adjacent vines. The leaves were pooled together to make one composite sample of approximately 24 leaves. We collected eight composite samples in total, corresponding to two zones (CEN and UC1) in each of the four plots (Table 1). All leaves were sampled using nitrile gloves and disinfected scissors. Before sampling a new plot or a new area within the plot, gloves were changed and tools were disinfected with bleach (3.6 % chlorine content) and 70 % ethanol. The leaves were placed in sterile plastic bags (NASCO™, Whirl-Pak®, USA) and kept on ice in a cooler. They were brought back to the laboratory on the same day. In the laboratory, the samples were stored at 4 °C and were processed within 24 hours to isolate their associated microbiota.

4. Leaf processing

The leaves within each composite sample collected from the four vineyard plots (Table 1) were placed onto sterilised filter paper and coarsely shredded into fragments of 1–1.5 cm^2 inside a laminar flow chamber using a sterilised scalpel to allow for isolation of epiphytic and endophytic microorganisms together during the subsequent washing stage. The fragments were transferred to a sterile sealable plastic bag (NASCO™, Whirl-Pak®, USA) to which sterile washing buffer (9 g/L NaCl and 100 µL/L Tween 80) was added in the ratio of 20 mL of solution per 1 g of leaf material. The bags were placed on an orbital shaker at 150 rpm for 2 hours to obtain a cell suspension of microorganisms. Under a laminar flow hood, the bags were partially unsealed near one edge to carefully decant the suspensions without any leaf fragments into sterile centrifuge tubes (Corning Inc., Falcon® Tubes, USA). These leaf washings were vortexed and plated onto solid media described below.

TABLE 1. Vineyard plots from which grapevine leaves were collected to build the microbial culture collection. Adapted from Fournier *et al.* (2025).

Plot pair	Plot name	Downy mildew incidence and severity	GPS coordinates	Geographic distance between plots	Cultivar	Management
Médoc1 (ME1)	121M	Low	45° 14' 50.7" N 0° 46' 00.4" W	1 km	Merlot	Organic
	68M	High	45° 14' 35.8" N 0° 45' 15.4" W			
Médoc2 (ME2)	33CS	Low	45° 14' 45.4" N 0° 45' 37.6" W	0.5 km	Cabernet-Sauvignon	Organic
	62CS	High	45° 14' 34.3" N 0° 45' 21.8" W			

5. Isolation of cultivable microorganisms by combining culture media

For all samples, 100 µL each of the undiluted washings as well as 1/10 diluted washings were plated in triplicate onto solid media. Three kinds of media were used to isolate bacteria: a Trypticase Soy Agar (TSA) medium (BD Difco™, Fisher Scientific, France), a Luria Bertani Broth Agar diluted 1/10 (dil. LBA) (BD Difco™, Fisher Scientific, France), and our custom Grapevine Leaf-based Agar medium (GLA). The pH was adjusted to 7 with NaOH and the media were supplemented with 150 mg/L biphenyl and 100 mg/L pimaricin, to inhibit the growth of filamentous fungi and yeast, respectively. The plates were incubated under aerobic conditions at 30 °C for two-five days without a photoperiod. To isolate yeasts and filamentous fungi, two kinds of solid media were used: a Malt Extract Agar (MEA) medium made using 15 g/L of bacteriological malt extract (Biokar Diagnostics, France) and 20 g/L of agar (Sigma-Aldrich, France) and our custom grapevine leaf-based agar medium (GLA), each of which had the pH adjusted to 7 with NaOH and was supplemented with 100 mg/L chloramphenicol to inhibit bacterial growth. The plates were incubated under aerobic conditions at 25 °C for 5–12 days with a 12-hour/day photoperiod.

For each sample, a single plate with the maximum visible morphological diversity among all the replicates was selected and we picked all or as many individual colonies as possible. These selected colonies were subcultured individually on fresh plates of their respective medium of origin by streaking (bacteria and yeast) or depositing agar plugs (filamentous fungi). Up to three successive rounds of subculturing were carried out as required to fully separate and ensure purity of all the selected Colony Forming Units (CFU) before identification.

6. Taxonomic identification of cultivable microorganisms using MALDI-TOF MS

Yeast and bacterial isolates were subcultured by streaking on fresh plates to have cells in active growth before running MALDI-TOF MS analysis. The equipment settings (microflex® LRF, Bruker, France) and direct colony spotting protocol used were as detailed by Windholtz *et al.* (2021). Taxonomic assignment to the species level was considered reliable for samples with a score of 2.00 or above. Assignment to the genus level was considered reliable for a score between 1.70 and 1.99, while isolates with a score below 1.70 were considered unidentified. When isolates could not be identified reliably, a longer extraction protocol was used as recommended by the manufacturer. A substantial proportion of isolates remained unidentified which prompted us to use Sanger sequencing for all bacteria and yeast isolates.

7. Taxonomic identification of cultivable strains using Sanger sequencing

The filamentous fungi were subcultured on their respective media of origin to recover mycelia for DNA extraction. The surface of each Petri plate was overlaid with sterilised cellophane (*feuilles couvre-confitures*, *Le Joint Français* by Hutchinson®) onto which the plugs were deposited for subculturing. The plates were incubated for 10 days at 25 °C with a 12-hour/day photoperiod. The porous filter allowed for nutrient access to the growing mycelia while providing a sturdy surface for harvesting the mycelia from the plate surface without the risk of scraping off the agar underneath (Bruez *et al.*, 2014). The harvested mycelia were stored in individual 1.2 mL microtubes with caps (Collection Microtubes, Qiagen, France) at –80 °C before lyophilisation and DNA extraction.

For both yeast and bacteria, each isolate was sub-cultured in 5 mL of the liquid medium corresponding to their media of origin in 15 mL centrifuge tubes (Corning Inc., Falcon® Tubes, USA). After five-seven days of incubation at 25 °C for yeast and 30 °C for bacteria, the tubes were centrifuged at 2,655 × g for 15 minutes at 10 °C. The pellets were transferred with approximately 1 mL of the supernatant to 1.2 mL microtubes (Collection Microtubes, Qiagen, France). The tubes were then centrifuged at 1,454 × g for 15 minutes and the supernatant discarded before storage at –80 °C for subsequent lyophilisation and DNA extraction.

Two 3.0 mm glass beads (VWR® International, France) were added to each the lyophilised sample for grinding with a TissueLyser II (Qiagen, Hilden, Germany) for two-three cycles at 30 Hz for 30 s before genomic DNA extraction using the protocol detailed by Bruez *et al.* (2020). Extracted genomic DNA was dosed using a DeNovix DS-11 series spectrophotometer (DeNovix Inc., USA) and diluted for PCR amplification, which was performed using the 8F (5′-AGA GTT TGA TCC TGG CTC AG-3′) forward primer (Schütte *et al.*, 2008) and 1115R (5′-AGG GTT GCG CTC GTT G-3′) reverse primer (Reysenbach & Pace, 1995) for bacteria and using the ITS1F (5′-CTT GGT CAT TTA GAG GAA GTA A-3′) forward primer (Gardes & Bruns, 1993) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) reverse primer (White, 1990) for fungi. The reaction mixes for amplification contained 10 µL of 2× Invitrogen™ Platinum™ II Hot Start PCR Master Mix (Thermo Fisher Scientific, France), 0.5 µL of each 10 µM primer, 1 µL of 1–100 ng genomic DNA and 13 µL of PCR grade water for a total volume of 25 µL. The cycle for bacteria was 94 °C for 2 minutes, 25 cycles of 94 °C for 30 s, 55 °C for 30 s, 68 °C for 30 s followed by 68 °C for 10 minutes and finally, holding at 15 °C. The cycle for fungi was 94 °C for 2 minutes, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 68 °C for 30 s followed by 68 °C for 10 minutes and finally, holding at 15 °C. Samples were sent to Eurofins Genomics EU GmbH (Cologne, Germany) for Sanger sequencing.

The 16S and ITS sequences obtained from Sanger sequencing were trimmed at the ends to remove the first and last 20 to 40 bases that were not well resolved. Using the chromatograms for each sequence, errors resulting from issues such as dye blobs were corrected manually on BioEdit (Hall, 1999) before taxonomic assignments using QIIME 2 2023.5, with the VSEARCH algorithm (Rognes *et al.*, 2016) and

the q2-feature-classifier (Bokulich *et al.*, 2018) plugin against the UNITE v9.0 (Abarenkov *et al.*, 2020) and SILVA 138 (Robeson II *et al.*, 2021) databases for fungi and bacteria, respectively. To potentially improve assignment at the species level, the sequences were also BLASTn searched against the NCBI RefSeq release 221 databases for 16S rRNA and ITS sequences of type strains using Geneious Prime 2024.0.2 (https://www.geneious.com). In case of mismatches between QIIME 2 and Geneious Prime results, the latter were chosen as the final assignment because of the database being more recently updated at the time of analyses. With both methods, fungal isolates were assigned at the species level only if the BLAST hit with the highest max score and the lowest *e*-value (usually 0.0 but no lower than 1e-13) showed a percentage identity above 97 % (Lücking *et al.*, 2020). Similar criteria were used for the bacteria except the threshold for percentage identity was over 99 % for species level assignment (Madden, 2013). To dereplicate and assess the number of unique sequences we obtained in the total collection, we used the *–derep_fullength* function with the *–sizeout* parameter within VSEARCH (Rognes *et al.*, 2016).

8. Final collection taxonomy and review of literature

The Sanger sequencing was the only method used for identification and taxonomic assignments of filamentous fungi. To finalize taxonomic assignments of bacteria and yeast isolates, we compared the results obtained by initial MALDI-TOF MS analyses followed by Sanger sequencing. A small proportion of bacterial isolates were assigned to specific groups (*Bacillus cereus* group and *Pseudomonas fluorescens* group) since they matched with one or more species within the same group that are difficult to distinguish at the species level with both MALDI-TOF MS and 16S rRNA sequencing. For bacteria and yeast isolates that could successfully be sequenced, we retained the final Sanger taxonomic assignment and the MALDI-TOF MS assignment was kept for isolates where sequencing failed or the obtained results were of insufficient quality.

For the finalised collection of microorganisms, we looked at published literature for information on their role in plant health. To select the taxa of interest for biocontrol, we looked at culture-dependent studies that tested biocontrol potential of isolates or specific strains through confrontation assays *in vitro* or *in planta*, across various plant and pathogen species. We also looked at culture-independent studies to select taxa that were suspected to have potential antagonistic activity toward a pathogen from network analyses or enriched in healthier conditions such as asymptomatic plants or associated with suppressive soils.

9. Phylogenetic trees for cultivable microorganisms

To build maximum likelihood (ML) trees of the unique Sanger sequences for the bacteria on one hand, and for the filamentous fungi and yeast on the other hand, we aligned the sequences using MAFFT version 7 (Katoh & Standley, 2013) using default (Auto) parameters of the online server (https://mafft.cbrc.jp/alignment/server/) and then built trees using the IQ-TREE software (Nguyen *et al.*, 2015) using the model and parameters described by Zhou *et al.* (2022). The trees were annotated with the total counts of identical sequences in the collection and the culture media on which the corresponding isolates were obtained by uploading to iTOL (https://itol.embl.de/).

10. Culture-independent approach to assess collection representativeness

To assess the representativeness of the culture collection, we analysed leaf bacterial and fungal communities using metabarcoding approaches, in order to uncover the whole leaf microbiota and compare it with the cultivable fraction. Leaf samples used for metabarcoding were collected from the same sites and the same areas within the sites, on the same date as the leaves used to build the culture collection. Detailed protocols are described in the study by Fournier *et al.* (2025). We sequenced a total of 64 samples, representing 4 phyllosphere samples and 4 leaf endosphere samples for each of the two zones (CEN and UC1) in each of the four plots (Table 1). Sequencing results are described in Fournier *et al.* (2025).

11. Statistical analyses

We performed all statistical analyses for this study using R v4.4.2 (R Core Team, 2024) and generated figures using *ggplot2* v3.5.1 (Wickham, 2016), *ggpubr* v0.6.0 (Kassambara, 2016), *ggtext* v0.1.2 (Wilke & Wiernik, 2020), *cowplot* v1.1.3 (Wilke, 2015), *patchwork* v1.3.0 (Sandberg *et al.*, 2023), and *microViz* v0.12.0 (Barnett *et al.*, 2021).

To compare the colony counts obtained across the different culture media and test hypothesis H1, we used a negative binomial generalised linear mixed-effects model (GLMM) with the CFU/mL as the dependent variable and the culture medium as a fixed factor. We considered eight independent sampling locations in the study, each corresponding to one of the two zones within each of the four plots. This variable was included as a random effect to account for the non-independence of plates inoculated with washing solutions from the same location. The models were built using the *glmer.nb* function from the *lme4* v1.1-36 package (Bates *et al.*, 2003) and evaluated using the *performance* v0.13.0 (Lüdecke *et al.*, 2021) and *car* v3.1.3 packages (Fox *et al.*, 2001). Subsequent pairwise comparisons for the three bacterial culture media were performed using the *emmeans* v1.10.7 package (Lenth, 2017).

To compare the taxonomic diversity obtained across the different culture media and test hypothesis H2, we calculated the Shannon index using the *vegan* v2.6.10 package (Oksanen *et al.*, 2001). We used Linear Models (LM) with culture medium and the sampling location as fixed factors. Subsequent pairwise comparisons were performed using the *emmeans* v1.10.7 package.

To compare the community composition obtained across the different culture media and test hypothesis H3, we performed permutational analysis of variance (PERMANOVAs) with the compositional dissimilarity as the dependant variable and the culture medium as a fixed factor using the *adonis2* function from *vegan* v2.6.10 package. Dissimilarity between communities was calculated using the Bray–Curtis index. Permutations were constrained within each sampling location using the *strata* option to account for the non-independence of communities cultivated from the same leaf washing solution. Following this, we generated a permutation matrix based on the sampling location stratification and ran pairwise comparisons with the precomputed permutations to compare the different culture media. Differences in community composition across media were represented on heatmaps using *phyloseq* v1.50.0 (McMurdie & Holmes, 2013) and Principal Coordinates Analysis (PCoA) using the *vegan* v2.6.10 package.

Finally, to assess the representativeness of the collection and test hypothesis H4, we analysed the overlap between the sequencing data obtained from Sanger sequencing of the collection isolates and the metabarcoding analyses of samples taken simultaneously at the same sampling locations (Fournier *et al.*, 2025). We retained only the Amplicon Sequence Variants (ASVs) from the metabarcoding dataset and isolates from the culturomics dataset that could be taxonomically assigned to the level under consideration for the comparison (either genus or species). Analyses were conducted separately for bacteria and fungi. Within each kingdom, results were analysed independently at both the genus and species levels. Microbial taxa were classified into three categories: those identified exclusively by metabarcoding, those identified exclusively by culturomics, and those detected by both methods. To visualize the results, we used taxonomic trees coloured according to category. These were generated using the *heat_tree* function from the *metacoder* v0.3.8 package (Foster, 2016).

RESULTS

1. Description of the collection

We isolated a total of 1,090 microorganisms across the 8 sampling locations and the 4 culture media, including 543 bacteria, 243 yeasts and yeast-like fungi, and 304 filamentous fungi. The taxa of interest for their biocontrol potential *in vitro* and *in planta* from culture-dependent studies are listed in Table S4 for bacteria, Table S5 for yeasts/yeast-like fungi, and Table S6 for filamentous fungi. Apart from these taxa of interest, we also found a mix of generally ubiquitous fungi and bacteria in vineyards, saprotrophs, Plant Growth Promoting Rhizobacteria (PGPR), pathogens, and opportunistic pathogens. A complete table of isolates with their taxonomic assignments, associated metadata and information on their role in plant health from published literature is available on Recherche Data Gouv (https://doi.org/10.57745/PJD7Z3).

2. Comparison of Sanger sequencing and MALDI-TOF MS for taxonomic identification

Sanger sequencing allowed us to identify 84 % of the isolates at least to the family level. The combination of Sanger sequencing with MALDI-TOF MS for bacteria and yeasts slightly increased this rate, with 90 % of all isolates identified (Figure 1). A total of 90 genera, including 43 bacterial genera and 47 fungal genera, were identified with both methods across all samples and 4 culture media. MALDI-TOF MS allowed for a slight increase in the number of identified genera, with two genera being identified exclusively using this method (the bacterial genus *Exiguobacterium* and the fungal genus *Metschnikowia*), suggesting some complementarity between both methods. All analyses of the diversity or representativeness of the collection were henceforth based on the combined results of MALDI-TOF MS and Sanger sequencing.

The overall efficiency and accuracy of MALDI-TOF MS was much lower than those of Sanger sequencing (Figure 1). We suspected that the culture medium chosen to grow the isolates for identification by direct colony spotting could affect the spectrum generated during the MALDI-TOF MS analyses. We investigated this by subcloning a small subset of bacteria and yeast isolated originally on GLA and repeating the protocol. We were able to successfully identify three bacterial taxa and two yeast taxa with a change in medium, but a large majority of the tested isolates remained unidentified (Tables S7 and S8). We also saw a marginal improvement in taxonomic identification to the species level for bacteria when subcloning on LBA and TSA (Figure S1). Sanger sequencing allowed us to identify 73 % of bacterial isolates to at least the genus level, compared to only 22 % for MALDI-TOF MS. In the case of yeasts and yeast-like strains, Sanger sequencing allowed us to identify 86 % of strains to the species level, compared to only 16 % for MALDI-TOF MS. Interestingly, MALDI-TOF MS had a much lower performance for microorganisms isolated on GLA than on conventional media. Only 38 % of bacterial isolates obtained on GLA were identified reliably by MALDI-TOF MS, compared to 51 % and 69 % for bacteria isolated on dil. LBA and TSA, respectively. Similarly, only 8 % of yeast isolates obtained on GLA were identified reliably by MALDI-TOF MS, compared to 49 % for yeasts isolated on MEA.

3. Comparison of colony counts between GLA and conventional culture media

We observed that the colonies obtained on the GLA were smaller and translucent or lighter in colour compared to the conventional and diluted media. Interestingly, when subcloning bacterial colonies originally grown on GLA on richer media like TSA and Yeast extract Peptone Glucose agar (YPG), we observed that some did not grow well on the richer media even with a prolonged incubation period (Table S9).

In contrast to our hypothesis (H1), GLA did not allow for isolation of higher colony counts compared to conventional culture media. The number of bacterial colonies on the GLA medium was significantly lower than on the diluted LBA

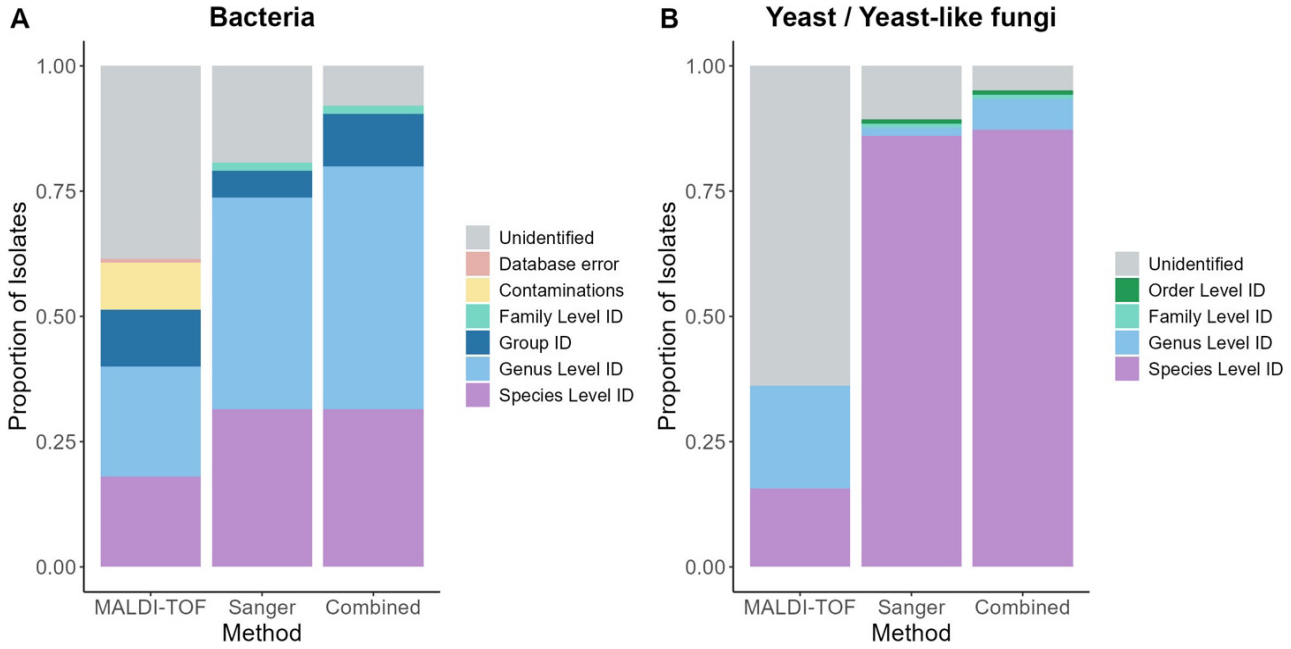


FIGURE 1. Proportion of isolates identified using MALDI-TOF MS, Sanger sequencing, and both methods combined for (A) bacteria and (B) yeast/yeast-like fungi. Taxa assigned to order, family, genus, or species level. A small proportion of bacterial isolates that were difficult to distinguish at the species level assigned to specific groups (*Bacillus cereus* group and *Pseudomonas fluorescens* group) represented as "Group ID". "Database error" indicated that the database matched the isolate with an unreliable low score to a non-plant associated microorganism within the MALDI-TOF database. "Contaminations" in bacterial MALDI-TOF MS analyses were a consequence of cross-contaminations from runny colonies on the Petri plates.

medium (negative binomial model, $p = 0.008$, Tables S10 and S11) and not significantly different from the number of colonies obtained on the TSA medium ($p = 0.5$, Tables S10 and S11) (Figure 2A). For filamentous fungi and yeast, the number of colonies obtained on GLA was not significantly different from the number obtained on MEA ($p = 0.1$, Table S10) (Figure 2B).

4. Comparison of taxonomic diversity of cultivable communities isolated on GLA and conventional culture media

In contrast to our hypothesis (H2), GLA did not allow for isolation of more diverse cultivable communities, compared to conventional culture media. For bacteria, the highest taxonomic diversity per sample was obtained on the dil. LBA medium. Taxonomic diversity obtained on the GLA medium was significantly lower than on the dil. LBA medium (linear model, $R^2 = 0.780$, $p = 0.028$, Tables S12 and S13), but not significantly different from taxonomic diversity obtained on the TSA medium ($R^2 = 0.780$, $p = 0.76$, Tables S12 and S13) (Figure 3A). For filamentous fungi and yeast, taxonomic diversity on GLA was not significantly different from diversity obtained on MEA ($R^2 = 0.401$, $p = 0.39$, Table S12) (Figure 3B).

5. Comparison of microbial genera isolated on GLA and conventional culture media

In accordance with our hypothesis (H3), GLA allowed for isolation of taxa that had not been isolated from conventional media. Of the 90 genera identified, 17 were isolated exclusively on GLA, representing 19 % of the genera in the collection. Of the 43 genera of bacteria, 4 were obtained uniquely on GLA, 7 uniquely on TSA, and 12 uniquely on dil. LBA. Overall, 9 genera were seen across all three media (Figure 4A). Of the 47 identified genera of fungi, 12 genera of filamentous fungi and one yeast genus *(Udeniomyces)* were obtained exclusively from GLA. Exclusive to MEA were 5 yeast genera and 7 genera of filamentous fungi (Figure 4B), while 11 genera of filamentous fungi and 11 genera of yeasts/yeast-like fungi were isolated on both culture media.

6. Comparison of unique sequences recovered from GLA and conventional culture media

Among the 543 bacterial isolates, 437 were identified to at least the family level using Sanger sequencing. Dereplication of these 437 Sanger sequences yielded 213 unique sequences. Among these, 175 sequences came from isolates obtained on only one of the three media used. The largest number of unique sequences was obtained on GLA (67 unique sequences) followed by 62 on dil. LBA and finally 46 on TSA. Looking at the different classes of bacteria by medium, we obtained Actinobacteria and Gammaproteobacteria on all three media, Alphaproteobacteria on GLA and dil. LBA only, Bacilli only on TSA and dil. LBA and finally, Bacteroidia exclusively on dil. LBA (Figure 5).

Among the 243 yeast or yeast-like isolates, 217 could be identified to at least the order level using Sanger sequencing.

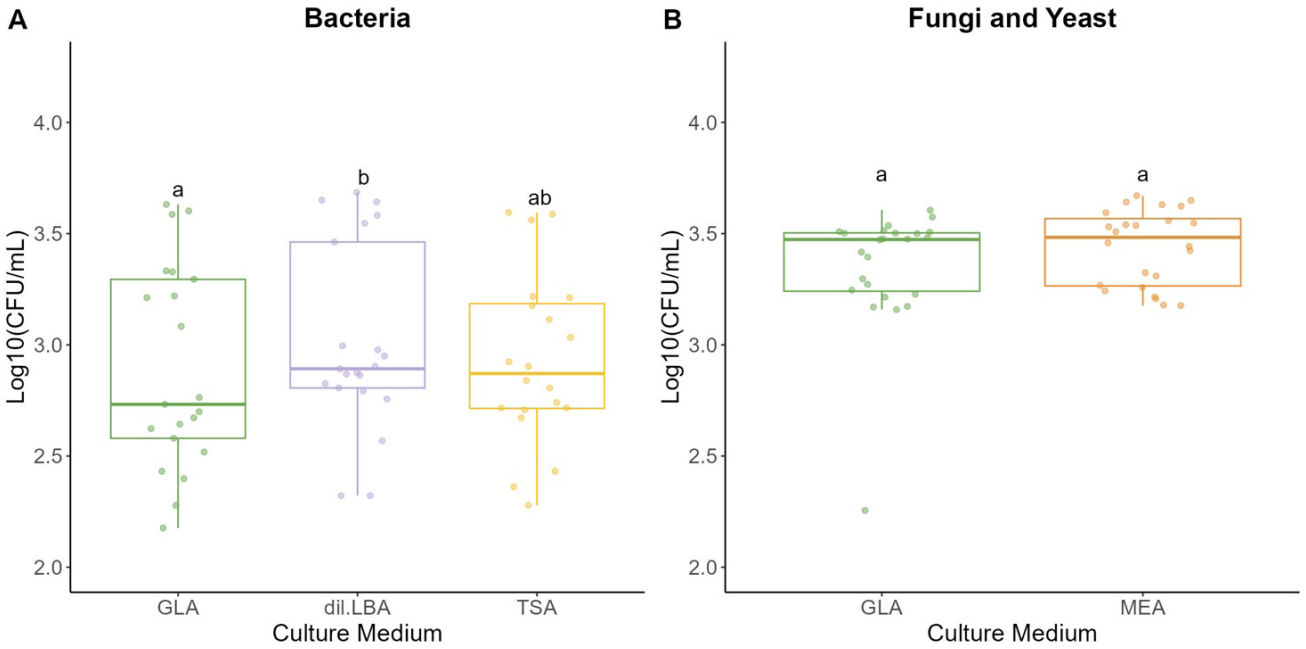


FIGURE 2. Comparison of the number of colonies (CFU/mL) obtained across the culture media. (A) Log CFU/mL of bacteria across the three culture media used – grapevine leaf-based culture medium (GLA), LBA diluted 1/10 and TSA. (B) Log CFU/mL of filamentous fungi.

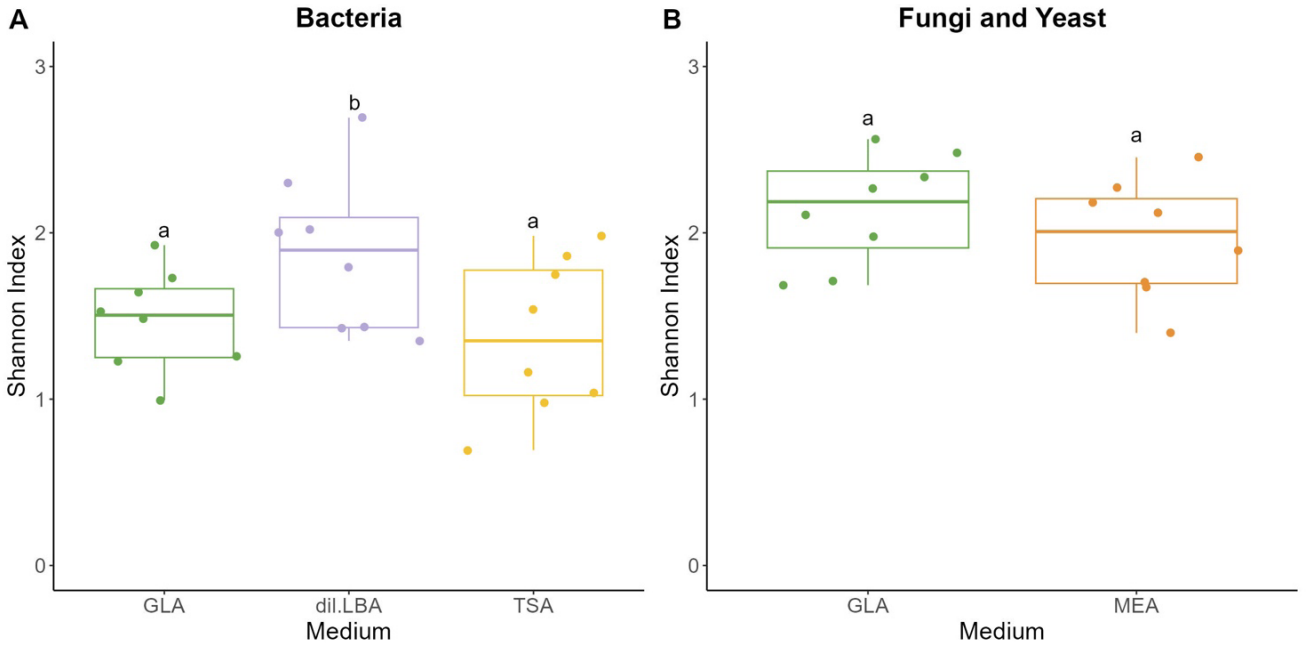


FIGURE 3. Comparison of alpha diversity of cultivable communities across the culture media. Alpha-diversity was measured for each sample using the Shannon index. (A) Bacterial community diversity across three culture media – grapevine leaf-based culture medium (GLA), LBA diluted 1/10 and TSA. (B) Fungal community diversity across two culture media – GLA and MEA. Results of pairwise statistical comparisons between culture media are shown with letters.

Dereplication of these 217 Sanger sequences yielded 45 unique sequences. For filamentous fungi, we obtained 64 unique Sanger sequences, out of the 255 identified sequences obtained from the 304 isolates. Overall, 44 unique sequences were obtained exclusively from MEA and 38 were from GLA. Only 27 were sequences from isolates obtained on both media (Figure 6). Of the 109 unique sequences, 61 were ascomycetes and 48 were basidiomycetes. The majority of unique ascomycete sequences corresponded to filamentous fungi, with only 5 sequences being from yeasts or yeast-like fungi. Among these 5 unique sequences, 1 sequence assigned to the yeast-like fungus *Aureobasidium pullulans* represented 84 isolates in the collection (Figure 6). In contrast, the majority of unique basidiomycete sequences corresponded to yeasts, with only 8 sequences being from filamentous fungi (Figure 6).

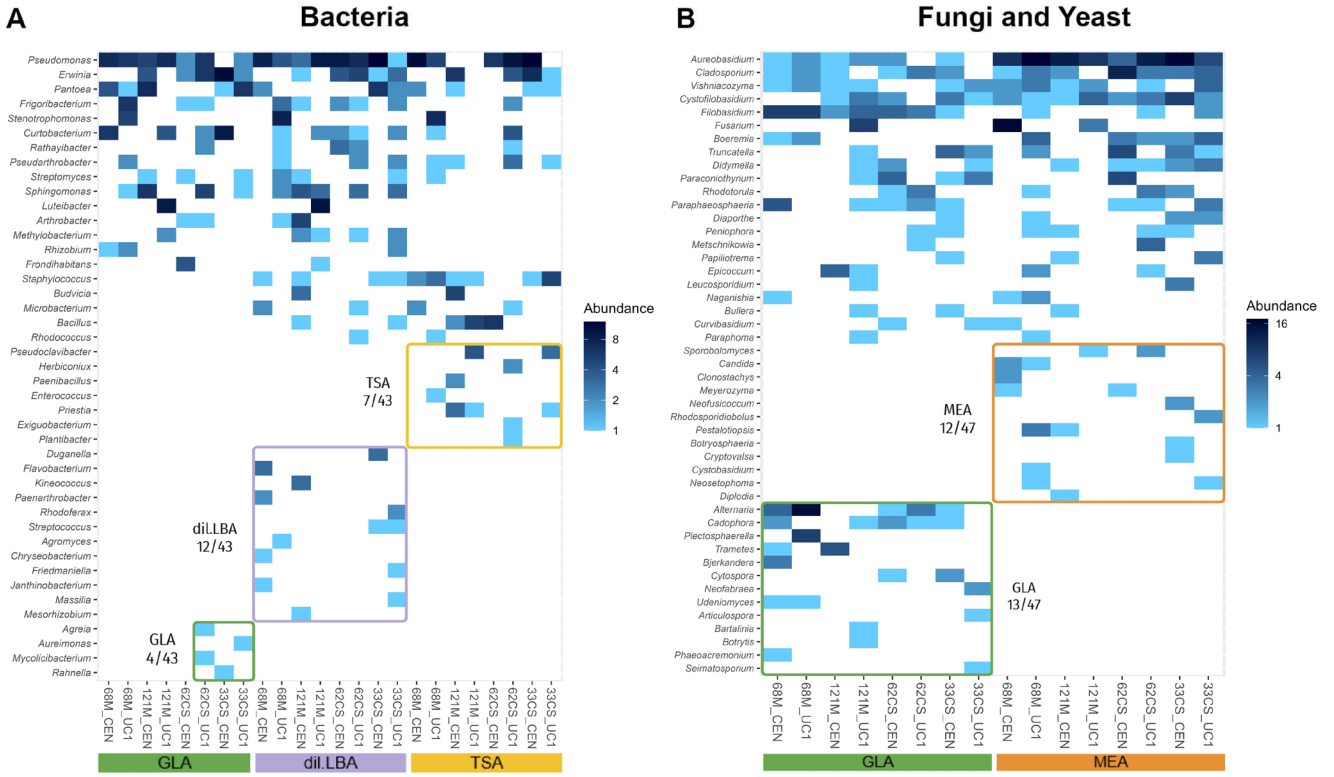


FIGURE 4. Heatmaps showing the microbial genera isolated from each of the eight sampling locations grouped by culture medium. (A) Bacterial genera (*n* = 43) identified on three culture media (grapevine leaf-based culture medium (GLA), dil. LBA, and TSA). (B) Filamentous fungi and yeast genera (*n* = 47) identified on two culture media (GLA and MEA). The abundance represents the number of isolates per genus.

7. Comparison of composition of cultivable communities isolated on GLA and conventional culture media

In line with previous results (Figure 4B) and in accordance with our hypothesis (H3), GLA allowed us to isolate fungal communities that were distinct from those isolated on the conventional MEA medium. The principal coordinates analysis (PCoA) revealed marked differences in the composition of cultivable fungal communities between GLA and MEA (Figure 7B), which were significant according to the PERMANOVA (Table S15, R^2 = 0.23448, p = 0.005). In contrast, GLA did not allow us to recover distinct communities in the case of bacteria. The PCoA (Figure 7A) revealed substantial overlap between bacterial communities isolated on GLA and dil. LBA, while TSA appeared to have isolated distinct communities in approximately half of the samples. The PERMANOVA confirmed that there were no significant differences in community composition between GLA and dil. LBA (Table S16, R^2 = 0.06828; p = 0.1), while there were significant differences between community composition between TSA and GLA (Table S17, R^2 = 0.13877, p = 0.01) as well as TSA and dil. LBA (Table S18, R^2 = 0.12042, p = 0.01).

8. Representativeness of the collection

In line with hypothesis (H4), GLA increased the representativeness of the collection, measured as the percentage of taxa isolated among all taxa detected using metabarcoding approaches. For the bacteria at the genus level, we found a 12.5 % overlap between the metabarcoding and culturomic datasets, which decreased to 12 % when excluding the GLA (Figures S2B and S3B). At the species level, we found a 6.6 % overlap, which decreased to 5.9 % when excluding GLA (Figures S4B and S5B). For the fungi at the genus level, we found a 17.6 % overlap between the metabarcoding and culturomic datasets (Figure 8B), which decreased to 12.6 % (Figure 9B) when excluding the GLA. At the species level, we found 14.6 % overlap, which decreased to 9.9 % when excluding GLA (Figures S6B and S7B).

DISCUSSION

The objective of the present study was to develop a culturomic approach to build a collection of grapevine foliar microorganisms with potential applications for the biocontrol of foliar diseases. We developed and tested a custom grapevine leaf-based culture medium (GLA) to better mimic the leaf habitat and consequently try to increase the cultivability of foliar microorganisms. The key result of our study was that GLA allowed us to increase the representativeness of the collection measured as a percentage of overlap between the culturomic and metabarcoding analyses carried out on leaf samples taken simultaneously from the same plots. The increase was particularly pronounced for fungi, with genus-level overlap rising from 12.6 % when using conventional medium alone to 17.6 % when combining conventional media with GLA. Overall, our study yielded six novel findings, which are discussed in detail in the following sections.

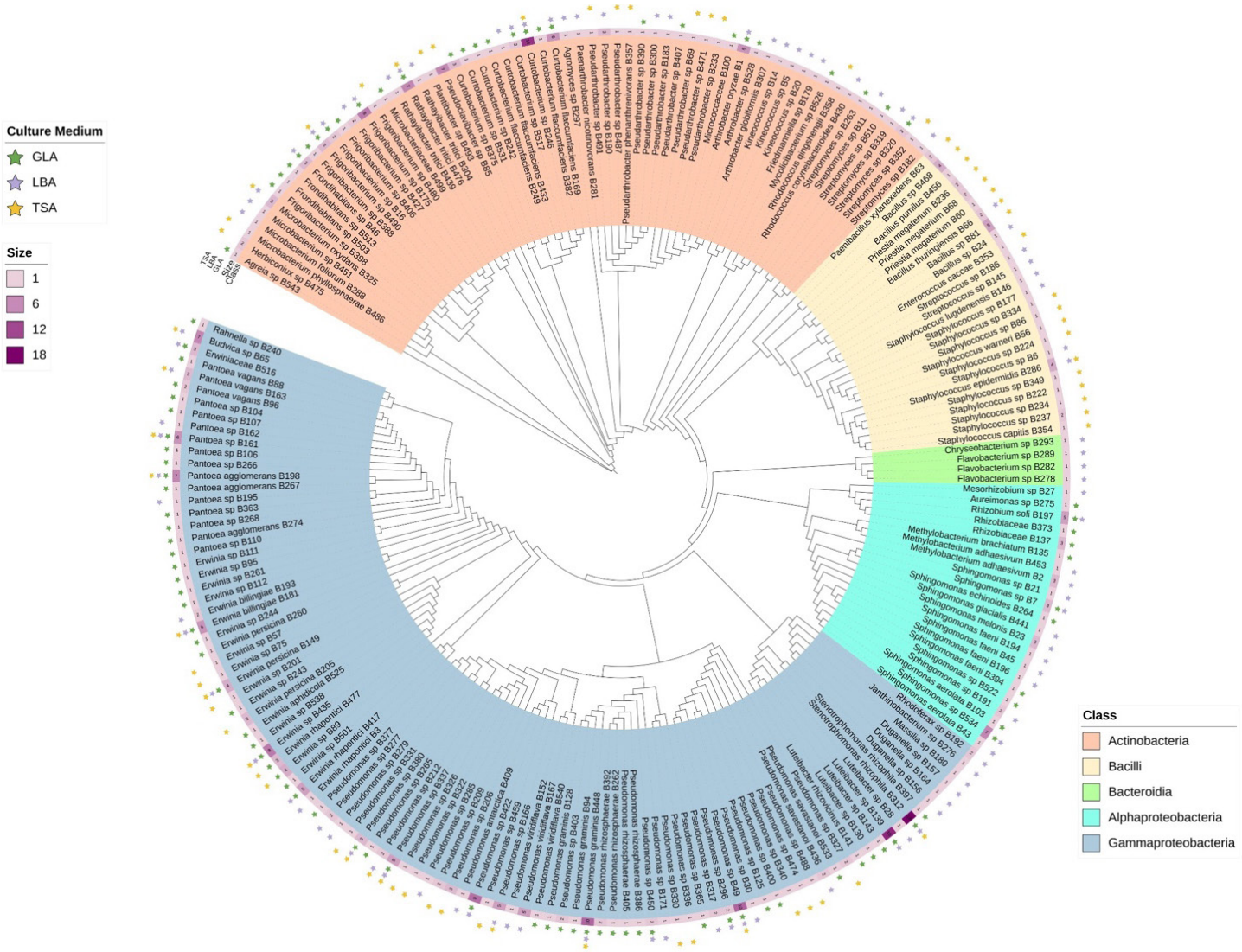


FIGURE 5. Phylogenetic tree of unique sequences of bacteria (*n* = 213) coloured by taxonomic class. The heatmap shows the number of isolates per unique sequence (Size). The coloured stars indicate the culture media on which the isolates were obtained (grapevine leaf-based culture medium (GLA), dil. LBA, or TSA).

1. GLA is nutritionally poorer but the number of colonies obtained on GLA was comparable to the conventional undiluted media

Among the tested media, GLA was the poorest nutritionally. There were clear morphological differences between isolates of the same taxa grown on GLA and conventional media, which is consistent with observations in other studies that used plant-based media (Mourad *et al.*, 2018; Sarhan *et al.*, 2016; Youssef *et al.*, 2016). Despite its lower nutritional content, the GLA was comparable in the number of colonies counted to both the undiluted media we used in this study (the TSA for bacteria and MEA for the fungi and yeasts).

We observed weak growth for some isolates originally obtained on GLA when subcloning on richer media like TSA and YPG. Losses at the subcloning stage are not uncommon since some specific microorganisms may require specific nutrients or conditions to be cultured which can be lost with changes in media and some may not be adapted to growing on media containing animal-based or synthetic chemical nutrient sources (Connon & Giovannoni, 2002; Sarhan *et al.*, 2019). This is what drove the choice of continuing to subculture the isolates on their respective media of origin during the purification step to try and minimize potential losses before being able to identify the isolates by either MALDI-TOF MS or Sanger sequencing.

2. Using MALDI-TOF MS for a culturomic approach is constrained by the limited scope of the available reference database, making Sanger sequencing essential

MALDI-TOF MS has been demonstrated to be a useful tool for microbial identification and has applications as a diagnostic tool in the medical field (Singhal *et al.*, 2015). It is well-suited to applications in environmental microbiology but environmental microbes are poorly represented in the associated databases (Lamontagne *et al.*, 2021). The Bruker MALDI-TOF MS database that we used contained mainly medically relevant microorganisms rather than

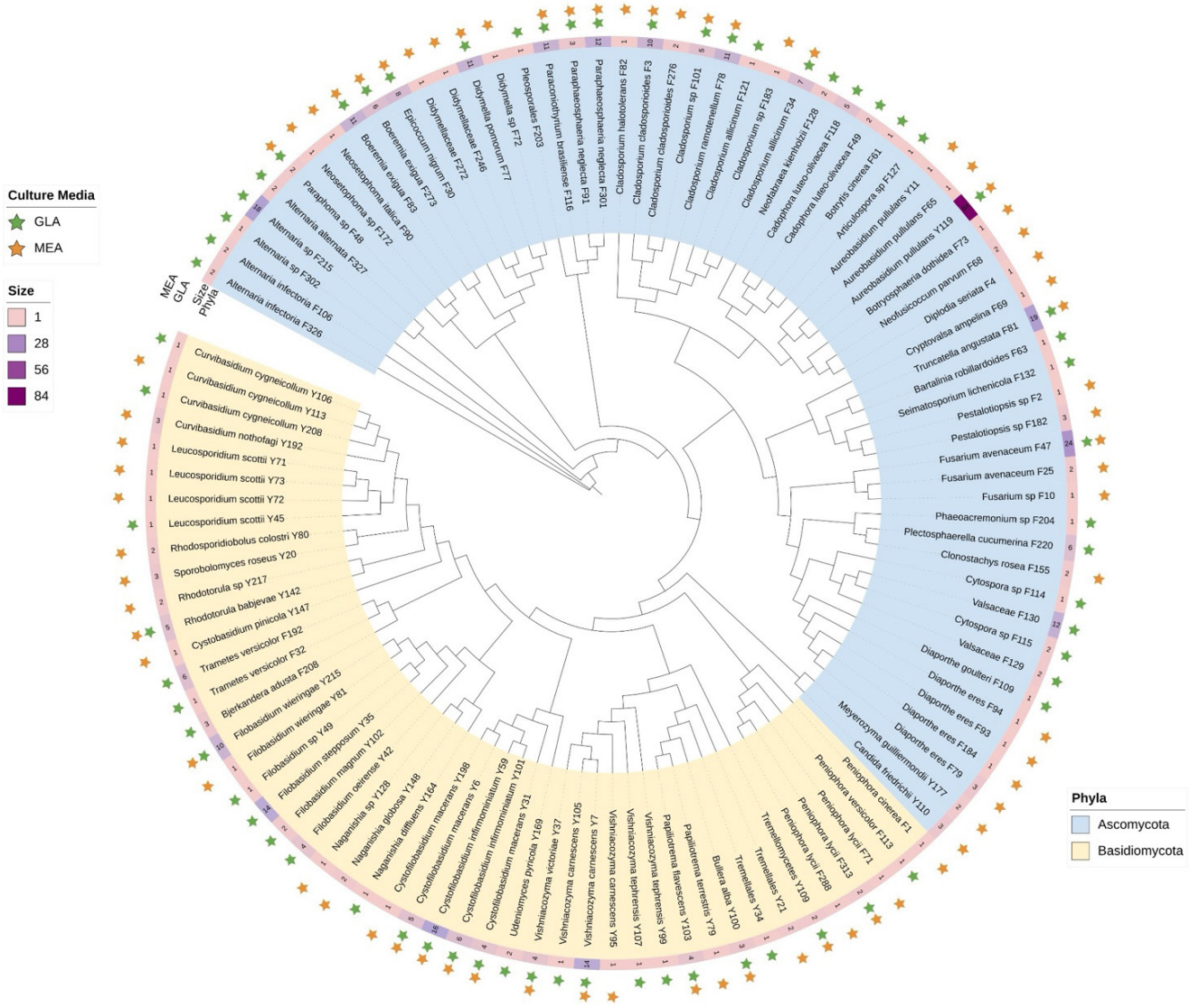


FIGURE 6. Phylogenetic tree of unique sequences of filamentous fungi (n = 64) and yeasts (n = 45) in the culture collection coloured by taxonomic phylum. The heatmap shows the number of isolates per unique sequence (Size). The coloured stars indicate the culture media on which the isolates were obtained (grapevine leaf-based culture medium (GLA) or MEA).

environmental ones. As many authors have reported, laboratory-specific databases are highly effective in identifying colonies at the genus or species level in the context of food and environmental microbiota (Lamontagne *et al.*, 2021; Windholtz *et al.*, 2021). MALDI-TOF MS allowed us to identify only two genera that we didn't manage to with Sanger sequencing. The first of these two genera was the *Exiguobacterium* genus which are classified among plant growth promoting rhizobacteria (PGPR) (Chauhan *et al.*, 2015). The second was *Metschnikowia* which is an interesting genus in both viticulture and oenology since it contains some pulcherrimin-producing subspecies that are known for potential biocontrol effects post-harvest (Lombardo *et al.*, 2023) and others known for their impact on the organoleptic qualities of wine in spontaneous fermentations or fermentations including the use of non-*Saccharomyces* yeasts (Vicente *et al.*, 2023). Notably, due to the large number of ambiguous nucleotide sites randomly distributed across the ITS1 barcode in pulcherrimin-producing *Metschnikowia* yeasts, using Sanger sequencing of the ITS region isn't recommended for this group (Sipiczki *et al.*, 2024) which could explain why we were able to identify them only using MALDI-TOF MS in the present study.

However, for our collection, Sanger sequencing proved indispensable to identify the large proportion of isolates that remained unidentified with MALDI-TOF MS. Sanger sequencing has the advantage of not needing to reculture fresh isolates and is reliable irrespective of culture medium used for microbial isolate growth. MALDI-TOF MS has the advantage of being faster and cost effective at the expense of being limited to the scope of the reference database available, and requiring fresh culturing of isolates before analyses. It performed poorly for strains isolated from GLA in comparison to strains isolated on conventional media.

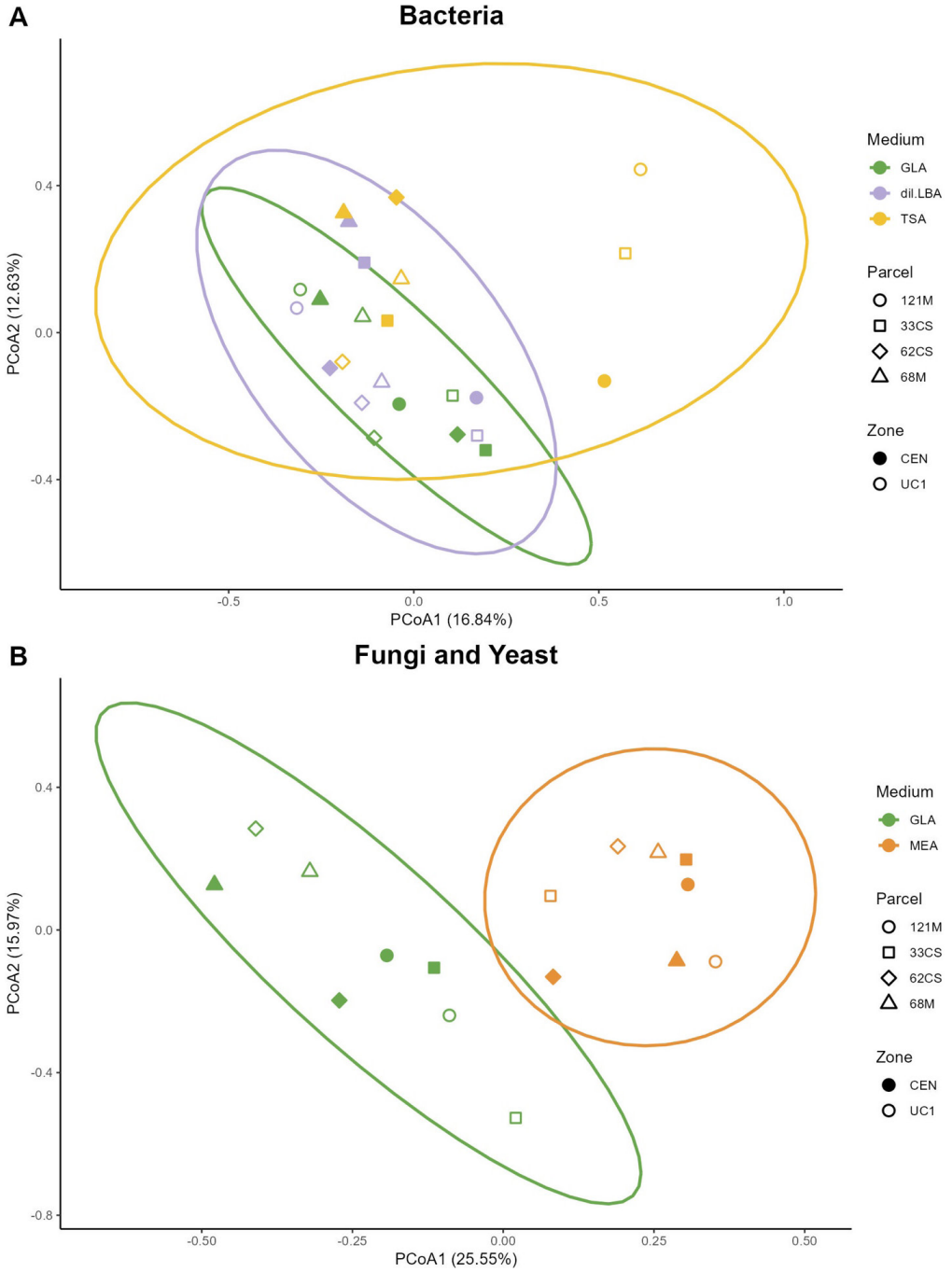


FIGURE 7. Principal coordinates analysis (PCoA) showing variation in the composition of cultivable communities across culture media used for (A) bacteria (grapevine leaf-based culture medium (GLA), dil. LBA, and TSA) and (B) filamentous fungi and yeasts (GLA and MEA).

Changing from GLA to a conventional medium like TSA and LBA for bacteria did allow for improved identification of a few taxa. This could indicate that using a culture medium that is nutritionally similar to the medium used for entering the reference spectrum into the MALDI-TOF MS database is probably advisable for better identification of bacteria. Nevertheless, the large proportion of bacteria and yeast isolates that remained unidentified in our study indicate that the MALDI-TOF MS database being insufficient is the main cause for our observations. Consequently, for future research, if the use of high-throughput techniques like MALDI-TOF MS is planned, it would be advisable to first expand the existing MALDI-TOF databases with environmental microbes. We would also recommend subcloning isolates onto similar media as the ones used for the reference spectra in the databases for better identification.

3. GLA allowed for isolation of a few bacterial genera known for potential biocontrol effects

Four bacterial genera, *Agreia*, *Rahnella*, *Mycolicibacterium*, and *Aureimonas*, were isolated exclusively on the GLA medium. Three of them, the *Rahnella, Mycolicibacterium*, and *Aureimonas* genera, include species known for potential

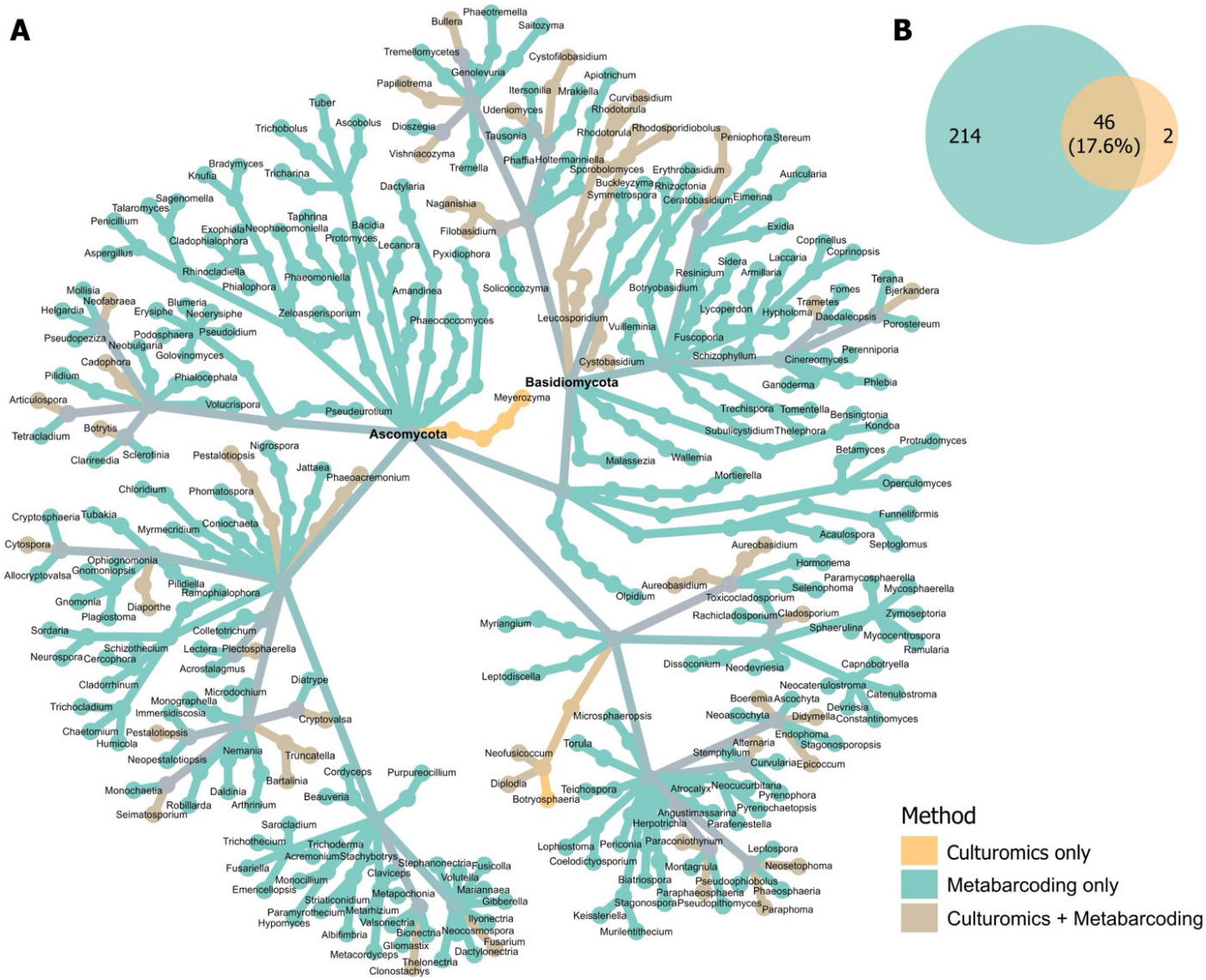


FIGURE 8. (A) Taxonomic tree showing overlap between the metabarcoding and culturomics datasets for fungal genera obtained on both grapevine leaf-based culture medium (GLA), and MEA. (B) Venn diagrams show the number of taxa obtained by each method as well as the percentage of overlap between both methods.

biocontrol effects. Within the *Rahnella* genus, we were able to isolate and identify the species *Rahnella aquatilis*. This species has been demonstrated to show antagonistic activity against grapevine crown gall both *in vitro* and *in planta* (Habbadi *et al.*, 2017) and it is known to grow readily on laboratory media (Brady & Denman, 2024). Certain *Aureimonas* strains have shown potential for suppression of ash dieback (Burghard *et al.*, 2023). Some species within the genus can require selective media and long incubation periods for isolation from environmental samples but most can grow well on laboratory media once isolated (Li *et al.*, 2017; Tuo & Yan, 2019). The *Mycolicibacterium* genus is known to contain both fast and slow growing species (Kim *et al.*, 2013) with some strains showing potential both alone and in consortia for biocontrol of *Pythium aphanidermatum* root rot diseases in lettuce (Stouvenakers *et al.*, 2023). *Agreia* species, while not known for biocontrol, are known to grow well at neutral pH on laboratory media containing peptone, yeast extract, and glucose (Evtushenko, 2015). Despite all of the GLA exclusive taxa being capable of growth on standard laboratory media, it is notable that all except the *Aureimonas* are among the rarer taxa associated with grapevine leaves (confirmed by mining the leaf microbiome dataset published by Barroso-Bergadà *et al.* (2023)), suggesting that the limited nutrient content of the GLA allowed for them not to be outcompeted by more abundant and fast growing bacteria.

4. GLA increased the cultivability of filamentous fungi, particularly those associated with grapevine trunk diseases (GTDs)

Of the 12 genera of filamentous fungi that were obtained exclusively from GLA, we found a number of fungi associated with trunk diseases as well as a few pathogens. The genera of fungi associated with trunk diseases were *Neofabraea, Phaeoacremonium*, and *Seimatosporium.* Growth rates for *Neofabraea* and *Seimatosporium* can vary by species and

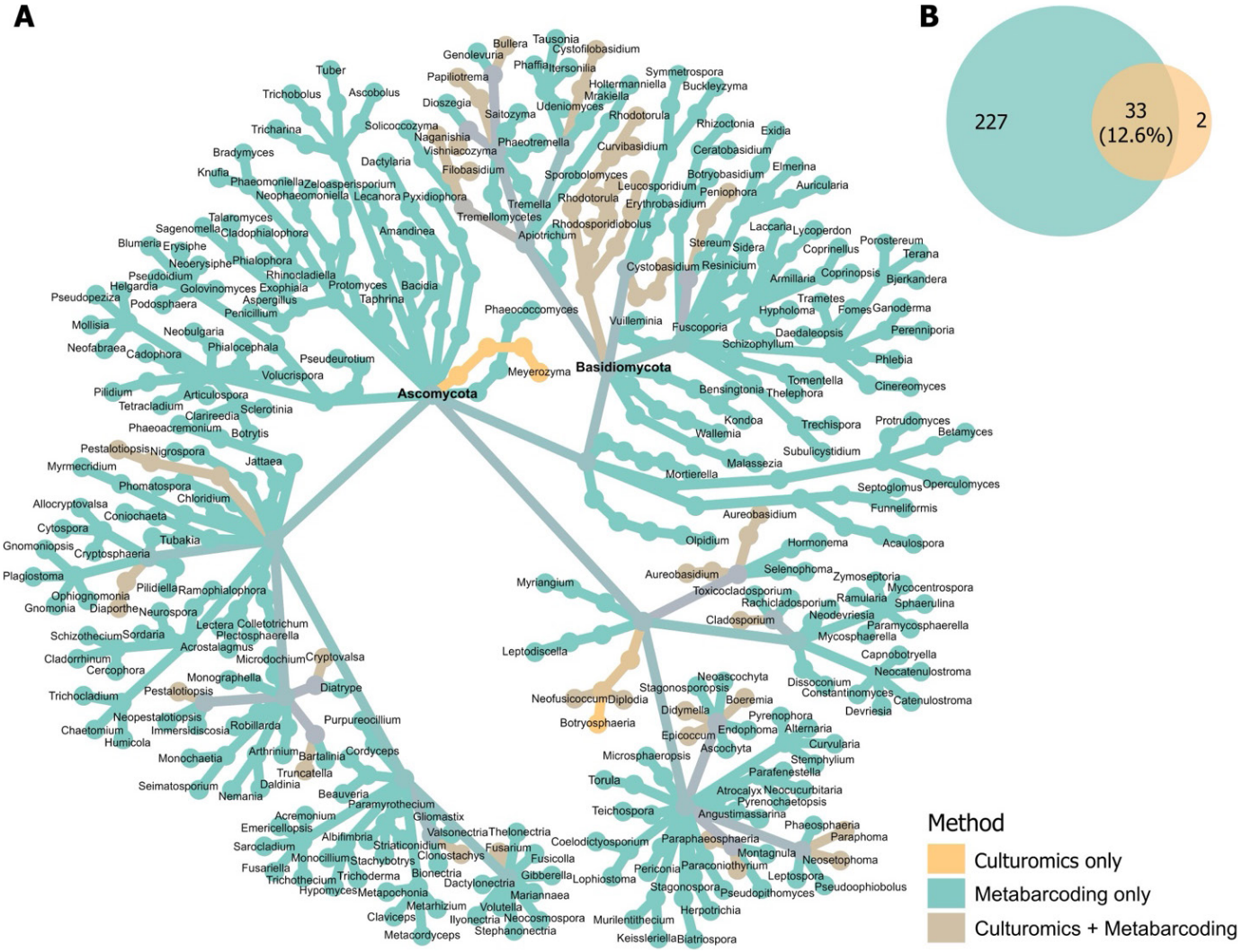


FIGURE 9. (A) Taxonomic tree showing overlap between the metabarcoding and culturomics datasets for fungal genera obtained on MEA only. (B) Venn diagrams show the number of taxa obtained by each method as well as the percentage of overlap between both methods.

they generally tend to be moderate growers on laboratory media (Kanetis *et al.*, 2022; Testempasis *et al.*, 2024; Vukotić *et al.*, 2022). On the other hand, *Phaeoacremonium*, which we managed to isolate over a 5-day incubation period on GLA, are known to be slow growing requiring up to 20 days on enriched media leading it to be frequently outcompeted by other microorganisms (Aigoun-Mouhous *et al.*, 2021; Aroca & Raposo, 2007). Other moderate/slow growers we obtained included *Articulospora* (Seena *et al.*, 2018) and *Bartalinia robillardoides* (Nguyen *et al.*, 2019). Among the fungal pathogens were fast growers on laboratory media like *Botrytis cinerea* (Nicot *et al.*, 1996) and slower growers like wood pathogen *Cadophora luteo-olivacea* which can require 8–15 days of incubation (Aigoun-Mouhous *et al.*, 2021) and canker causing *Cytospora* whose growth rate can vary by species but tends to be moderate (He *et al.*, 2024).

In addition to the pathogens and GTD-associated fungi, we found a few fungal taxa of interest for biocontrol effects exclusively on the GLA. One notable species was the basidiomycete *Trametes versicolor,* which is known for its potential effects against *Fusarium langsethiae* in cereals (Parroni *et al.*, 2019). The *Alternaria, Bjerkandera*, and *Plectosphaerella* genera were also obtained only on GLA. They are known for various effects, being opportunistic pathogens or disease associated in some plant systems, while being known for potential biocontrol effects against pathogens in others (Table 4). For instance we isolated *Alternaria alternata* which can cause leaf spot in soybean (Li *et al.*, 2023), but is also known for biocontrol of *P. viticola* (causing downy mildew) (Musetti *et al.*, 2006). Similarly, some strains of *Bjerkandera adusta* are known for biocontrol of *Fusarium graminearum* in wheat (Li *et al.*, 2022) while others are associated with apple tree dieback (Jacobs *et al.*, 2024). The species *Plectosphaerella cucumerina* is associated with root and collar rot in horticultural crops (Carlucci *et al.*, 2012) but has shown promising results against potato cyst nematodes (Jacobs, 2000) and as a bioherbicide (Bailey *et al.*, 2017). Comparing their abundance in grapevine leaves and growth rates, the *Alternaria* genus is abundant on grapevine leaves (Barroso-Bergadà *et al.*, 2023; Bettenfeld *et al.*, 2022) and the growth rate on laboratory media varies by species.

Similarly *Trametes* species are known to grow on various laboratory media without specific growth factor requirements (Veena & Pandey, 2012), suggesting that these two may have been isolated by chance on GLA exclusively. On the other hand, *Bjerkandera* species are known to be slow growing on MEA requiring a long incubation period for isolation from environmental samples (Oses *et al.*, 2008), while *Plectosphaerella cucumerina* generally shows moderate growth on laboratory media (Carlucci *et al.*, 2012) and both are among the rarer genera on grapevine leaves (Barroso-Bergadà *et al.*, 2023). As such, it is very likely that we would not have been able to isolate these taxa without the use of the GLA.

5. GLA did not enhance the cultivability of yeasts but enabled the isolation of several biocontrol candidates, similarly to conventional media

The GLA did not allow us to increase the cultivability of yeasts. Only one basidiomycete yeast species, *Udeniomyces pyricola,* was isolated exclusively on GLA. *U. pyricola* is not among the abundant taxa on grapevine leaves (Barroso-Bergadà *et al.*, 2023). It shows moderate growth on laboratory media and requires milder temperature conditions (17–24 °C) (Takashima & Nakase, 2011) and is known for biocontrol of *Erysiphe necator* (powdery mildew) under controlled conditions (Pauvert *et al.*, 2020). Isolating basidiomycete yeasts was of particular interest for our objective of developing biocontrol against grapevine foliar diseases. These yeasts are known for their biocontrol potential, especially for post-harvest diseases (Freimoser *et al.*, 2019; Liu *et al.*, 2013; Ruiz-Moyano *et al.*, 2020). Moreover, comparing pairs of plots with contrasted levels of incidence and severity of downy mildew symptoms, Fournier *et al.* (2025) found that basidiomycete yeast-like fungi were significantly more abundant in plots with lower disease incidence and severity. We successfully obtained a number of these biocontrol candidates from the *Cystofilobasidium*, *Curvibasidium*, *Leucosporidium*, *Filobasidium*, *Naganishia*, *Papiliotrema*, *Rhodotorula*, and *Vishniacozyma* genera (Table S5) on both MEA and GLA.

6. GLA increased the representativeness of collection, particularly for fungi

The percentage of bacteria that are known to be cultivable from the phyllosphere can tend to be similar to other environments, in the range of 0.1 to 5 % (Ritz, 2007). The range for fungi varies more substantially by plant host, environment and by method used; however, they tend to be easier to cultivate than bacteria on selective media (Ritz, 2007). We measured the representativeness of the collection as a percentage of taxa isolated using our culturomic approach among all taxa detected using metabarcoding approaches. We demonstrated that using conventional media as well as our own custom plant-extract based medium helped us successfully cultivate 12.5 % of bacteria genera and 17.6 % of fungal genera. With the exclusion of the GLA from the culturomics dataset, we observed a 5 % drop in representativeness of filamentous fungi and yeasts at the genus level. In comparison, the drop we observed with the bacteria was only 0.5 % at the genus level, confirming that the use of GLA was more efficient for fungi. It should be noted that we excluded the unidentified isolates from the culturomics dataset as well as the unassigned ASVs from the metabarcoding dataset. The overlap between the culturomic and metabarcoding datasets could therefore change in the future with new identifications.

CONCLUSION

The objective of this study was to develop a culturomic approach to build a collection of grapevine foliar microorganisms with potential applications in disease biocontrol. We developed a custom grapevine leaf-extract-based culture medium (GLA) to test alongside diluted and undiluted commercial culture media. Our results indicated that using a plant-extract-based culture medium increases the cultivability of foliar micro-organisms. The GLA allowed us to isolate several microbial taxa that are of interest for their biocontrol potential. Moreover, the GLA was surprisingly effective for isolating and speeding up the growth of filamentous fungi, particularly those known to be associated with grapevine trunk diseases. We therefore recommend using the GLA or developing similar custom plant-based media for the targeted isolation of rare and slow-growing taxa from different grapevine organs, particularly wood. Using other modified protocols to make the leaf extract could also be tested. For instance, filter-sterilising the grapevine leaf extract instead of autoclaving it would likely be better to preserve the leaf metabolites in the final culture medium. Overall, despite the combination of nutritionally rich, diluted, and custom plant-based media, only a limited fraction of the existing microbial diversity on grapevine leaves could be cultivated. Multiplying the nutrient, pH and incubation conditions, through classical isolation methods, would likely be prohibitively time-consuming and labour-intensive. A more efficient strategy to improve representativeness of the microbial collection would be to combine custom plant-based media with high-throughput isolation methods, like dilution to extinction with 96-well plates. Based on our results, we recommend including MALDI-TOF MS for isolate identification in a culturomic approach only if reference databases provide sufficient coverage of environmental microorganisms. Expanding these databases is critical to fully exploit the potential of this high-throughput identification approach for environmental microbiology. The microbial collection built in this study will serve as a repository of pure cultures for conducting biocontrol experiments. Isolates will be screened and tested both individually and in consortia for their activity against grapevine diseases, with a particular focus on *P. viticola* (grapevine downy mildew). Synthetic microbial Community (SynCom) approaches will be used to elucidate the interactions between foliar micro-organisms. This knowledge will inform the development of microorganism-based biocontrol products and contribute to the move towards more sustainable viticulture.

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Availability of data

Sequences were deposited in the National Center for Biotechnology Information (NCBI) under the accession numbers PQ348104-PQ348540 for bacteria and PQ350423-PQ350897 for fungi and yeasts. Codes and metadata for statistical analyses, as well as the complete table of isolates with their taxonomic assignments and information on their role in plant health from published literature, are available on Recherche Data Gouv: DOI https://doi.org/10.57745/PJD7Z3.

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