Revisiting sour rot of grapevine through disease-associated microbiomes: a tripartite co-infection?

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ABSTRACT

Sour rot in grapevines is thought to result from berry infection by yeasts, acetic acid bacteria and vinegar flies. Our results confirm previous studies having shown that sour rot supersedes grey mould when both diseases are present in a vineyard, but also indicate that the two diseases can cohabit at an advanced stage of grape decay. To better characterize the role of each of the actors involved in sour rot expression, we conducted experiments involving the isolation of 1593 fungi and bacteria to understand the composition and dynamics of the microbiomes associated with healthy berries, diseased berries and insect vectors. As some grape clusters became symptomatic for sour rot in the absence of acetic acid bacteria, the latter do not always appear necessary for disease expression. The yeast genus *Geotrichum,* is reported here for the first time to be able to initiate sour rot in grapes, similar to other yeast genera. By allowing or denying the access of insect vectors to intact or artificially wounded grapes, we demonstrate that vinegar flies accelerate disease expression but only when berries are previously injured. Furthermore, the yeast communities identified on native and introduced vinegar flies (*Drosophila suzukii*) are very similar and yeast-dominated. This confirms the key role of both introduced and endemic vinegar flies in the transmission of sour rot fungi. However, berry skin wounds, induced by heavy rainfall leading to high water uptake and rapid berry swelling, appear to be the main factor in disease expression.

IMPORTANCE

This study sheds new light on the complex interactions between microbiomes, insect vectors and physical factors favoring the development of sour rot. While previous studies suggested that acetic acid bacteria were mandatory for sour rot expression and that the introduced *Drosophila suzukii* flies were the main insect vector, our results indicate that grape sour rot can also result solely from yeast infection and that endemic *Drosophila* flies are equally important in yeast transportation and transmission in the vineyard. The fruit flies accelerate the infection process leading to disease expression in the weeks prior to harvest, but we demonstrate here that they can do this only on the condition that the skin of grape berries shows already injuries, for example due to excessive water uptake after heavy rain. Consequently, sour rot of grapes is suggested to depend principally on climatic conditions during the maturation of grape berries.

INTRODUCTION

In grapevine (*Vitis vinifera*) production, sour rot is a disease that poses a major threat to grape quality. When more than 20 percent of grape clusters are affected by this disease, harvest becomes unsuitable for vinification as a result of the high level of volatile acetic acidity released from rotten grapes (Asplen et al. 2015). Prevalent in vineyards worldwide (Lee et al. 2011), sour rot causes substantial economic losses (Asplen et al. 2015, Ebbenga et al. 2021, Entling & Hoffmann 2020). The disease is known to arise from a co-infection of berries by yeasts and bacteria transmitted by insects such as vinegar flies. In the progression of sour rot symptoms, yeasts facilitate the conversion of sugars into alcohol, while bacteria catalyze the oxidation of alcohol into acetic acid. Most fermentative yeasts, except for *Saccharomyces cerevisiae*, require a minimum of oxygen to perform alcoholic fermentation (Visser et al. 1990), conditions fulfilled in injured berries (Hall et al. 2018). It is assumed that in the absence of drosophilids, the injured skin of berries heals rapidly, thereby limiting the access of yeasts and bacteria to the pulp and thus maintaining an anaerobic environment unsuitable for the growth of the fermentative yeasts associated with sour rot (Barata et al. 2012a).

It is widely accepted that adult flies of the genus *Drosophila* (Diptera, Drosophilidae) are the most important insect vectors of sour rot. Apart from several endemic European *Drosophila* species, notably *D. melanogaster*, the Asian *D. suzukii*, was recently introduced to Europe and North America. Both mentioned species have been shown to be involved in the development of grapevine sour rot (Hall et al. 2018, Ioriatti et al. 2018). Females of both vinegar flies typically lay their eggs in injured or overripe grape berries (Sepesy et al. 2019). The hatched larvae feed on the pulp of the berries thereby making them decay and thus unfit for consumption and winemaking (Asplen et al. 2015). It is generally assumed that native *Drosophila* species are unable to lay their eggs in intact grapes and can only do so in berries whose skins have been damaged during grape ripening by birds, insects, fungal diseases and/or heavy rainfall during grape ripening. Heavy precipitation events can cause excessive water uptake leading to rapid berry growth and skin breakdown. In contrast to native *Drosophila* species, *D. suzukii* can injure and lay its eggs in healthy grape berries with intact skins due to its serrated ovipositor (Atallah et al. 2014, Entling & Hoffmann 2020, Ioriatti et al. 2015, Kienzle et al. 2020). It is generally assumed that drosophilids transmit the microorganisms associated with sour rot from infected grapes to healthy berries during the process of egg laying (Fermaud et al. 2002). Moreover, the healing of the skin of wounded berries is delayed by the activity of hatching larvae (Barata et al. 2012a). As only *D. suzukii* is able to penetrate the skin of healthy grapes, this species is regarded as a more dangerous vector of sour rot than native vinegar flies although a recent study challenges this general assumption (Entling & Hoffmann 2020). Overall, the specific role of wounds and potential insect vectors in the development of sour rot remains unclear.

Numerous studies have extensively documented the microorganisms associated with sour rot. The yeast genera identified in sour rot symptomatic berries belong primarily to the order of Saccharomycetales. In Europe and North America, yeasts from the genera *Candida*, *Hanseniaspora*, *Pichia* (previously *Issatchenkia*), and *Saccharomyces* are predominant (Barata et al. 2008, Barata et al. 2012b, Bisiach et al. 1982, Guerzoni & Marchetti 1987, Hall et al. 2018, 2019a, Mezzasalma et al. 2017), while two additional non-yeast genera, *Magnusiomyces* (formerly *Saprochaete*) and *Colletotrichum,* are reported from China (Gao et al. 2020). In addition, the *in vitro* assay by Gao et al. (2020) suggested that fungal molds from genera *Aspergillus*, *Alternaria* and *Fusarium* could spoil grapes in China and might therefore play a role in sour rot. These same studies have also identified bacteria associated with sour rot diseased berries, predominantly acetic acid bacteria (AAB) from the genera *Acetobacter* and *Gluconobacter* in Europe and North America, as well as *Firmicutes, Cronobacter*, and *Serratia* in China. While the most abundant microorganisms in grape berries affected by sour rot are relatively well-documented (Bokulich et al. 2014, Kántor et al. 2015, Martini et al. 2009, Martins et al. 2014, Setati et al. 2012, 2015,), only one study investigated, to our knowledge, the microbial changes associated with the development

of sour rot (Hall et al. 2019a) and no study so far has analyzed the microbial community present on *Drosophila* adults that might consequently be transmitted to healthy grapes.

The authors report here on three experiments that were conducted to better understand the respective roles of the microbial communities, potential vectors and physical injuries in the development of sour rot in grapes. The first experiment aimed to differentiate between micro-organisms associated with grey mould (a disease caused by *Botrytis cinerea*) versus sour rot and, additionally, to assess whether the microbial communities associated with sour rot were similar across different regions in Switzerland. Findings are discussed and compared with those from other countries. The second experiment focused on the evolution of the microbiome on and in grape berries that were either intact or artificially wounded over the last three weeks preceding the harvest, thereby using insect-proof nets to regulate the access of potential insect vectors to the grape clusters. This bifactorial experimental design was adopted with the aim to identify the individual role of each of these two factors associated with sour rot and, also, to entangle temporal dynamics in the development of the microbiome. Finally, the third experiment analyzed and compared the fungal communities present on the surface of healthy berries, in surface-sterilized healthy berries, in sour rot affected grapes, and on the surface of *Drosophila* flies that were captured in the same vineyard. The aim of this third experiment was to determine the respective contributions of healthy berries and potential insect vectors to the mycobiome associated with sour rot.

MATERIALS AND METHODS

Experiment 1: Fungi associated with grapes symptomatic for sour rot or grey mould

An initial experiment aimed to compare the species of fungi and bacteria present in grape clusters that developed symptoms of sour rot or grey mould and to assess whether the microbial community associated with sour rot symptomatic grape clusters varied across different regions. Grape clusters were selected based on the known symptoms of each disease. In the case of grey mold, grape clusters were considered to be infected when berries were brown and covered with the greyish mycelium of *Botrytis cinerea* (Pezet et al. 2004). For the recognition of sour rot, skin discoloration, pulp discharge, a smell of vinegar and a minimum acetic acid content of 0.83 g/L in diseased berries were the required symptoms following Hall et al. (2018). To confirm that acetic acid concentration was over this threshold, symptomatic grape berries were crushed and the acetic acid content of the resulting juice was analyzed using an A25 spectrophotometric autoanalyzer (Bio System, Barcelona, Spain), together with the commercial kit "Acide Acétique" Enzytec ™ Liquid (R-Biopharm, Darmstadt, Germany).

Sour rot symptomatic grapes were sampled from two vineyard areas in Switzerland, namely Nyon (Vaud) and Landquart (Grisons). In early October 2019, 18 sour rot symptomatic grape clusters were sampled from seven cultivars: Pinot noir (3 from Landquart, 2 from Nyon), Diolinoir (2 from Landquart), Pinot blanc (2 from Landquart, 2 from Nyon), Pinot gris (1 from Landquart), Sauvignon blanc (2 from Landquart), Chardonnay (2 from Nyon), and Chasselas (3 from Nyon). In addition, three clusters of Chasselas infected with grey mould in Nyon were sampled to compare them with sour rot symptomatic grape clusters from the same vineyard plot. Grape clusters were harvested using pruning shears and immediately placed in individual sterile sealed plastic bags. They were then left at room temperature in the plastic bags for two weeks before being crushed. Subsequently, 1 ml of the material was spread onto Petri dishes (9 cm Ø) containing potato dextrose agar (PDA). Petri dishes were then kept at room temperature for three weeks. We examined dishes daily over four weeks and as soon as fungi or bacteria became visible, they were isolated onto new PDA Petri dishes (6 cm Ø) to obtain them in pure culture.

Experiment 2: Evolution of the microbial community on grapes in respect of wounds and vectors

To assess the impact of fungi, bacteria, insect vectors and wounds on the evolution of the microbial communities of berries and the expression of sour rot, an exclusion experiment was conducted in 2019. The evolution of the microbial community was monitored over the course of

three weeks on healthy and artificially wounded grape clusters in the presence or absence (protected by an insect-proof nets) of *Drosophila* flies. In a Chasselas vineyard of Agroscope at Nyon, 36 grape clusters were selected for the experiment. In early September, 18 grape clusters were protected from insects with iron cages covered with insect-proof nets, while the remaining 18 grape clusters were left unprotected. In each of these two groups, ten berries on half (9) of the grape clusters were artificially wounded using a scalpel sterilized with 70% ethanol, The experiment consisted thus of four different treatments repeated on nine independent grape clusters: protected and unwounded, protected and wounded, unprotected and unwounded as well as unprotected and wounded clusters. Grape clusters were sampled at a weekly interval for three weeks. The first sampling was carried out one week after the set-up of the experiment and consisted in pruning three clusters per modality which were then placed individually in sterile, airtight plastic bags. Back in the laboratory, two artificially wounded berries and two unwounded berries adjacent to the wounded berries were collected from each of the six wounded grape clusters. Similarly, two berries were randomly picked from each of the six unwounded grape clusters. This sampling procedure was repeated in week two and three following the wounding of the berries. To obtain the microbial community on collected berries, each sampled berry was rolled in a Petri dish filled with PDA (9 cm Ø). As in the survey described above, plates were controlled daily for the development of fungi and bacteria. For four weeks, emerging fungi and bacteria were transferred to new PDA Petri dishes (6 cm Ø) as soon as they became visible to obtain them in pure culture. To capture *Drosophila* flies, insect traps were positioned around the experimental Chasselas vineyard of Agroscope. These homemade traps were made of a plexiglass cylinders (20 cm in height and 10 cm Ø) in which a vinegar fly attractant was placed (Gasser lure®, RIGA AG - Paul Gasser, Ellikon an der Thur, Switzerland). The top of the cylinder was sealed with an insect-proof net, and small insects could enter through 10 small holes of circa 3 mm diameter drilled around the cylinder approximately 3 cm from the top. Traps were sampled once a week and dead drosophilids were identified under a stereo microscope. Specimens of the introduced *D. suzukii* could easily be distinguished from native *Drosophila* species by the black spot on the wings of males and the characteristic strongly serrated ovipositor of females.

Experiment 3: Comparison of the fungal communities associated with sour rot on healthy and symptomatic berries as well as drosophilids

To determine if the fungi present on the surface of healthy berries or on the bodies of *Drosophila* species may act as potential reservoirs for the mycobiome associated with sour rot symptomatic berries, a vineyard of the Gamay cultivar affected by this disease was surveyed in Begnins near Nyon (Switzerland) in 2020. A total of 18 grape clusters (eight symptomatic and ten asymptomatic) were collected in airtight sterile plastic bags between September 14th and September 23rd, 2020. To isolate only the microorganism community present inside of the berries, the eight symptomatic grape clusters were rinsed in tap water for one hour immediately upon arrival in the laboratory. Following this step, eight sour rot affected berries per grape cluster were sampled under a laminar flow, with seven small pieces of each berry placed equidistantly on a PDA Petri dish (9 cm Ø). From the ten healthy grape clusters eight berries were randomly collected per cluster. Half of these berries were rinsed under tap water for half an hour, then their surface was sterilized in 70% ethanol for 1 minute. Once their surface was dry, the berries were cut into small pieces with a sterile scalpel under a laminar flow. Seven samples of each of these berries (skin and pulp) were placed on PDA Petri dish (9 cm Ø). The other half of the berries were rolled individually, without surface sterilization, within the same type of Petri dishes. As before, and for each treatment (sour rot symptomatic berries, asymptomatic surface-sterilized berries, and asymptomatic non-surface-sterilized berries), fungi were isolated in pure culture. To capture *Drosophila* adults alive, three homemade insect traps were placed in the Gamay vineyard at Begnins, with a second cylinder added inside. The bottom of this second cylinder was removed, and fitted into the cylinder that contained the fly attractant with an insect net separating the two cylinders to prevent specimens from drowning in the attractant. The traps were checked daily from September 8th to 14th 2020.

After identification of the fruit flies, five individuals were placed equidistantly on a PDA Petri dish (9 cm Ø), with *D. suzukii* and endemic species placed in separate dishes (57 individuals for D. suzukii and 52 for endemic drosophilid spp.). The vinegar flies were pressed into the culture medium to immobilize them. As in the previous experiment, the Petri dishes were surveyed daily for four weeks to isolate the emerging fungi and bacteria in pure culture as soon as they were visible.

Molecular characterization and identification of the fungal and bacterial strains

As soon as the fungi isolated in pure culture were sufficiently developed (0.5 cm^2), direct amplification of the ribosomal nuclear DNA internal transcribed spacers 1 and 2 plus the 5.8S (ITS) was performed according to Hofstetter et al. (2012). For the bacteria isolated in pure culture, we amplified the ribosomal nuclear small subunit (16S) using two different primer pairs to amplify this locus in order to maximize the number of successful PCRs: 8F/1492R designed by Galkiewicz & Kellogg (2008) and 341f/785r designed by Thijs *et al.* (2017). Direct PCR was performed using a sterile pipettor tip (10 µl) to aseptically transfer a tiny amount of mycelium in a PCR tube and to squash it manually with the tip in the PCR mix (25 µl mix, reagents, and conditions of the Taq PCR core kit (QIAGEN, Inc., Valencia, California, USA). When direct amplification of fungal ITS or bacterial 16S did not work, we used ITS1 instead of ITS1F for fungal amplifications or performed genomic DNA extractions of both organism types following Hofstetter et al. (2012) from samples collected from pure cultures (60-70 mg) and placed in an Eppendorf tube containing 500 µl of CTAB buffer (1x) before amplification. The strategy adopted for the amplification was to first use the fungal primer pair ITS1F-ITS4 on all isolates and then adopt ITS1-ITS4. We used then the bacterial primers for those isolates where the fungal primers did not work. PCR products were sequenced in both directions by ©Eurofins Genomics (Ebersberg, Germany) or by Fasteris Life Science Genesupport (Geneva, Switzerland) using the same primer pair as for amplification.

The obtained sequences were assembled using the Sequencher v. 4.9 software (Gene Codes Corp., USA). Once assembled, they were verified by eye and sequences were then imported in MacClade v. 4 (Maddison & Maddison 2005) and for each locus (ITS and 16S) aligned manually. Since the primers used to amplify ITS are designed at the end of the small nucleic ribosomal subunit (SSU) and at the beginning of the large ribosomal subunit (LSU) for ITS1(or ITS1F) and ITS4 respectively, sequence alignment allowed ITS delimitation. The sequences of each locus were then subjected to a similarity search in Sequencer with an assembly parameter of 100% to determine the number of genotypes present in our samplings. Two different nucleotide similarity searches (https://blast.ncbi.nlm.nih.gov/) were performed for each genotype in the GenBank (National Center of Biotechnology Information). The first sequence similarity search used the "blastn" options (Megablast) and excluded "uncultured/environmental" sequences. The second was conducted using the option “Sequences from type material”. The name associated with the most similar sequence(s) ("BLAST top score(s)" expressed as %) in GenBank was adopted to name our isolates, favoring, when possible, the names associated with sequences from type material, this when BLAST top score of type sequence(s) exhibited a similarity percentage equal to the Megablast top score sequence(s). The similarity threshold values of BLAST top score(s) adopted to name sequences at systematic ranks followed Hofstetter et al. (2019) but using higher sequence similarity thresholds for species ranks. Species name associated to the BLAST top score(s) of our ITS or 16S sequences was only adopted when similarity with a GenBank sequence was 100%. When the isolated fungal species belonged to Ascomycota species complexes in which several cryptic species share identical ITS sequences, we retained only the genus rank. The suffixes cf. (likely that species) and aff. (close but might not be that species) were used for 99.5-99.9% and for 99.0-99.49% sequence similarity intervals respectively. Below 99% of sequence similarity, the isolates were assigned to genus, family or higher ranks. When assigning family and order ranks to our taxa, the classification presented in GenBank was followed. For species names we followed GenBank, but we checked for the species current name in Mycobank.

Statistical analyses

Exploratory analyses of the data were carried out using R (R Core team 2024) and the metacoder package (Foster et al. 2017). The used datasets consisted of the mycobiomes isolated from the surfaces of healthy berries, from berries with sour rot symptoms, and from the surface of *Drosophila* flies We compared the abundance of ITS genotypes of the isolated fungi, identified according to the results of the GenBank similarity searches, between these three datasets. All analyses and graphs produced with R as well as the original data are available online (https://github.com/agroscope-ch/sour-rot), in particular the script used to reproduce the graphs.

RESULTS

Experiment 1: Fungi associated with grapes symptomatic for sour rot and grey mould

Having sampled grape clusters symptomatic for sour rot (i.e., acetic acid concentration in berries ≥ 0.83 g/L) in two regions (e.g., Vaud and Grisons) and for grey mould in one region of Switzerland (e.g., Vaud), a total of 78 fungi and bacteria were isolated in pure culture. The barcode sequences obtained from these isolates (ITS for fungi and the 16S for bacteria) corresponded to 35 different genotypes, where 11 could be assigned to the species rank and 25 to the genus rank based on GenBank BLAST top score results (Table S1, https://github.com/agroscope-ch/sour-rot/ in the "Data" folder; click on "view raw" to download the file). Out of these 35 genotypes, 31 were fungi that were assigned to 12 different genera. The four remaining were bacteria that were assigned to two distinct genera. Considering the region and disease symptoms, 12 out of these genotypes were obtained from sour rot symptomatic grapes in the Grisons, 16 from sour rot symptomatic berries in Vaud and 12 from grey mould symptomatic berries in Vaud (Table S1).

As the ITS and 16S sequences did not provide sufficient resolution to identify most of the fungal strains and half of the bacterial strains at the species level (Table S1), we inferred the composition of the microorganism communities based on the genus rank (Figure 1).

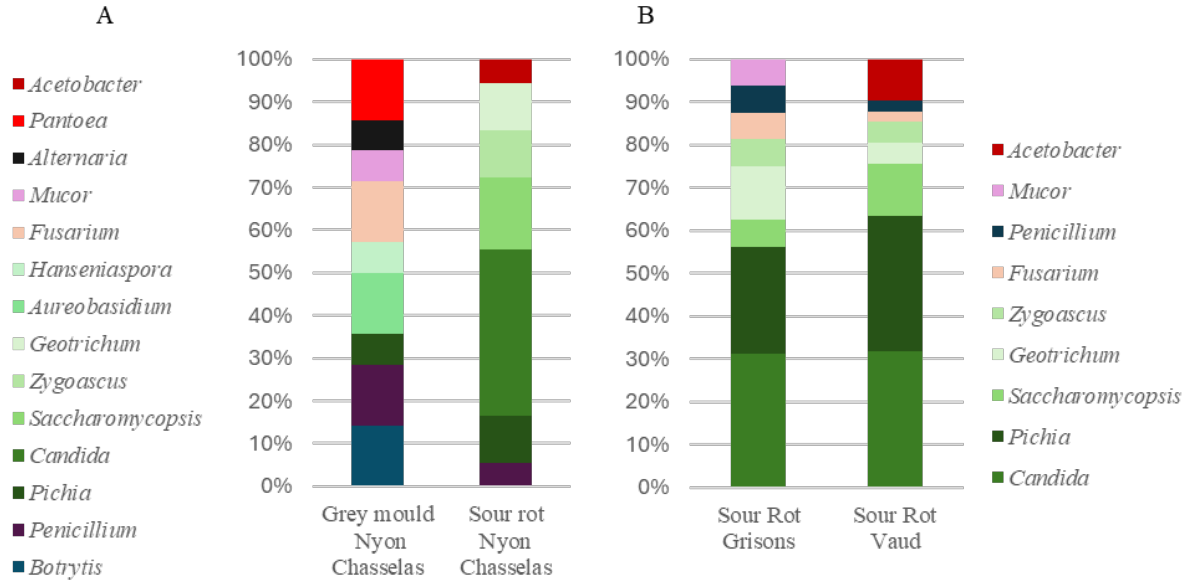

Figure 1. Relative abundances (expressed in %) of fungal and bacterial genera associated with grape clusters. A. Associated with the cultivar Chasselas with net-protected clusters symptomatic for grey mould and unprotected clusters symptomatic for sour rot in Nyon; B. Associated with several cultivars symptomatic for sour rot in the two regions (Vaud and Grisons). Generic names followed the name(s) associated to BLAST top score(s) sequence(s) in GenBank (see Table S1).

By comparing the microbial communities associated with sour rot and grey mould at the genus rank in a single Chasselas plot in Nyon, significant differences were observed (Table S1; Figure 1A). Only two out of the 14 genera isolated were present in both sour rot and grey mould symptomatic berries, namely the fungal genus *Penicillium* (relative abundance of 5.6% for sour rot

and of 14.3% for grey mould) and *Pichia* (relative abundance of 11.1% and 7.1%). The genera *Candida*, *Geotrichum*, *Saccharomycopsis* and *Zygoascus* (all Saccharomycetales according to GenBank) were exclusively isolated from sour rot symptomatic grape clusters with relative abundances of 38.9%, 11.1%, 16.7% and 11.1%, respectively. Together, these four genera , accounted for 88.8% of the microbial community of sour rot symptomatic clusters. In contrast, in the microbial community associated with grey mould, Saccharomycetales accounted only for 14.2% and was represented by the genera *Hanseniaspora* (7.1%), absent in sour rot symptomatic clusters, and *Pichia* (7.1%). The fungal genera *Alternaria* (with a relative abundance of 7.1%), *Aureobasidium* (14.3%), *Botrytis* (14.3%), *Mucor* (7.1%) and *Fusarium* (7.1%) were exclusively found in grey mould symptomatic grape clusters. Of the two bacterial genera identified, the genus *Acetobacter* (with a relative abundance of 5.6%) was isolated only from grapes symptomatic for sour rot, whereas *Pantoea* (7.1%) was exclusively isolated from grey mould symptomatic berries.

When comparing the microbial communities associated with sour rot symptomatic berries between the two cantons, Grisons and Vaud, the seven fungal genera *Candida*, *Fusarium*, *Geotrichum*, *Penicillium, Pichia*, *Saccharomycopsis* and *Zygoascus* were present in both regions (Figure 1B). The Saccharomycetales yeasts *Candida*, *Geotrichum*, *Pichia*, *Saccharomycopsis* and *Zygoascus* accounted for 81.3% of the microbial community associated with sour rot symptomatic berries in the Grisons and for 85.4% in Vaud (Table S1). The remaining fungal genera, namely *Penicillium*, *Fusarium* and *Mucor*, represented each less than 6.3% in both regions, whereas *Mucor* was only present in the Grisons. Overall, the microbial communities isolated from both regions were highly similar at the genus rank. However, looking at the level of species and genotypes, the two regions appeared less similar (Table S1). In fact, the two regions shared only four out of the 31 fungal genotypes, namely *Candida californica*, *Fusarium* sp. 4, *Penicillium crustosum* and *Zygoascus meyerae*. Species of *Pichia* differed between regions, with *P. kluyveri* and *P. membranifaciens* (with two isolates cf. *membranifaciens*) only isolated in the Grisons, and *P.* aff. *manshurica*, *P.* aff. *kluyveri*, *P.* cf. *kudriavzevii* and *P.* sp. 1 only isolated in Vaud. Two different species of *Geotrichum* were isolated in each region, while different genotypes of *Saccharomycopsis crataegensis* and of *Mucor* cf. *circinelloides* were present in each of the two regions. Looking at the bacteria, only the genus *Acetobacter* was isolated in Vaud with a relative abundance of 9.8%. while no bacteria were isolated in the Grisons (Figure 1B).

Experiment 2: Evolution of the microbiome on grapes in respect of wounds and vectors

The aim of this experiment was to follow the evolution of fungal and bacterial communities preceding the expression of sour rot and to determine the contribution of wounds and insect vectors on disease expression. Berries were sampled from grape clusters treated under four different modalities: undamaged berries protected from insect vectors by a net, protected by a net and artificially wounded, unprotected and undamaged, and unprotected and artificially wounded. Sampling was conducted one, two, and three weeks after the wounding of grape berries. Trap captures confirmed that potential insect vectors such as *Drosophila* spp. and *D. suzukii* were present over the experimental period.

A total of 495 microorganisms (fungi and bacteria) were obtained in pure culture after sampling these four modalities (Table S2, https://github.com/agroscope-ch/sour-rot/ in the "Data" folder; click on "view raw" to download the file). In total, we obtained 469 fungal ITS sequences representative of all the cultured morphotypes, except one (anamorphic mycelium named fungal sp.), whereas a total of 25 sequences for the 16S locus were obtained for bacterial strains. Overall, 90 different sequence genotypes were obtained, 81 ITS for fungi and nine 16S for bacteria. BLAST top score(s) results (Table S2) assigned the ITS genotypes to 34 fungal genera and the 16S genotypes to three bacterial genera. Out of the 81 ITS fungal genotypes, only 28 allowed an identification at the species rank, while merely two out of the nine bacterial 16S genotypes could be assigned at species rank.

As sour rot has been shown to result from a tripartite co-infection involving fungi, bacteria and insect vectors, we looked at the evolution of the microbial community over time at

genus/species rank (Table S2) considering only the wounded and unprotected berries as these are susceptible to express sour rot symptoms (Figure 2). Among these grape clusters, only the three clusters sampled after three weeks were symptomstic of sour rot (acetic acid content = 1.4, 2.2 and 4.6 g/L). Detection of sour rot associated fungi started after three weeks when the AAB reported to be associated with sour rot were still absent. Representatives of four yeast or yeast-like genera were isolated, namely one *Zygoascus* (*Z. meyerae*; Table S2) accounting for 15% of the microbial community, one *Saccharomycopsis* (a cf. sp. likely to be *S. vini*) with a relative abundance of 20% and two unidentified species of *Geotrichum* (sp. 1 and 2) representing 35% of the microbial community.

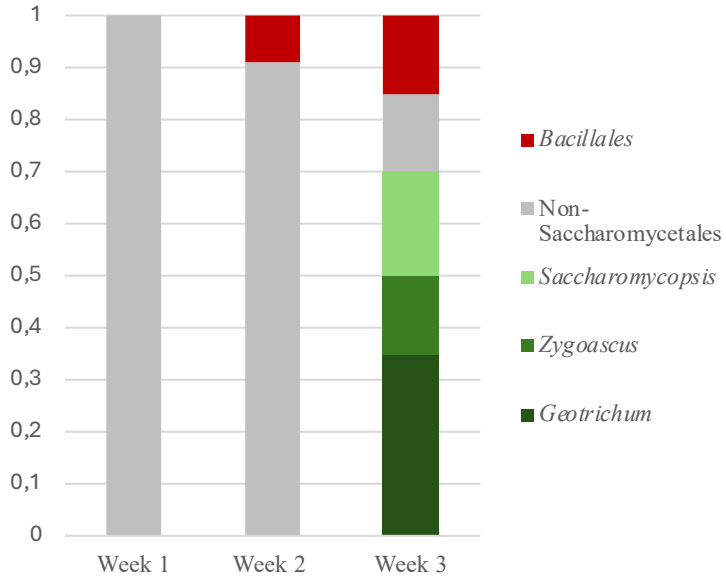

Figure 2. Evolution of the relative abundance (expressed in %) of sour rot associated microorganisms in wounded and unprotected grape berries of Chasselas over a three week period after their wounding. Sour rot involved yeasts (or yeast-like fungi) are indicated, while all fungi that are not responsible for sour rot expression are grouped as "Non-Saccharomycetales" (see Table S2 for the species/genera names assigned to our sequences based on BLAST results).

To determine whether sour rot affected berries transmit disease-causing microorganisms to adjacent unwounded berries in the presence of insect vectors such as drosophilids, we compared the identified microbial communities associated with wounded berries affected by sour rot to the microbial community on unwounded berries adjacent to symptomatic berries as well as with healthy berries sampled from unwounded grape clusters three weeks after the starting of the experimentation in unprotected grape clusters (Figure 3). The microbial community structure of symptomatic berries differed strongly from that of unwounded berries. This was especially evident for yeasts and yeast-like fungi, which represented 70.0% of the microbial community in sour rot affected berries, but only 14.3% and 16.7% for those associated with adjacent and healthy berries, respectively. In addition, two other orders of fungi were present in the three different set-ups, namely Hypocreales (symptomatic: 10.0 %, adjacent: 38.1% and healthy: 25.0%) and Agaricales (5.0%, 9.5% and 25.0% respectively). Unwounded berries, adjacent to wounded berries or taken from healthy clusters, shared the fungal order Eurotiales (19.5% and 8.3% respectively), an order absent in symptomatic grapes. The fungal orders Polyporales (9.5%), Glomerellales (4.8%), and Mucorales (4.8%) were present only on berries adjacent to injured berries and Pleosporales was only found on berries from healthy clusters (25.0%). Considering the bacterial community, the order of Bacillales was only present in symptomatic berries with an abundance of 15.0%. Overall, the microbial communities isolated from unwouded healthy berries were more similar to each other than to the community isolated from symptomatic berries because of the dominance of yeasts (Saccharomycetales) and the presence of bacteria (Bacillales), already present the second week, in sour rot symptomatic berries.

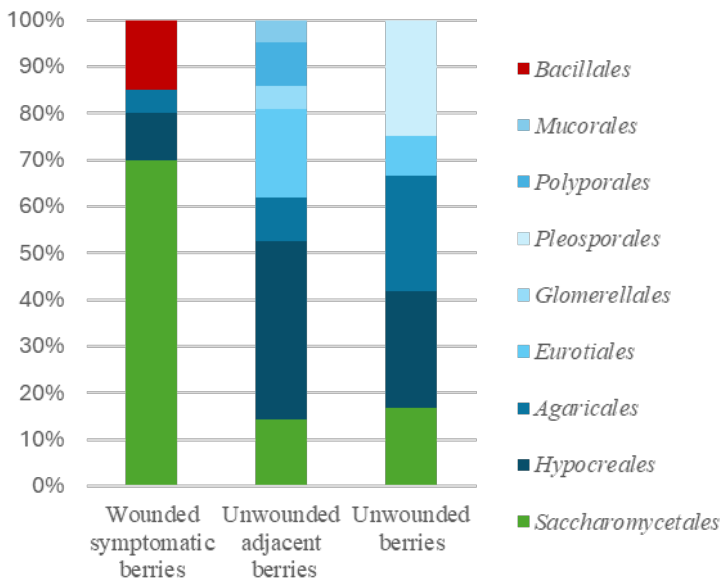

Figure 3. Comparison of the relative abundance of microorganisms isolated from unprotected grape clusters of Chasselas three weeks after the start of the experiment (see Table S2 for the classification assigned to our sequences).

We also tested for the impact of insect vectors on the development of sour rot in the absence of wounds, comparing the evolution of the microbial communities on unwounded healthy berries protected or not by a net over the three weeks (Figure 4). Although insect vectors such as *Drosophila* spp. could access grape clusters when unprotected, none of the unwounded berries developed symptoms of sour rot over the three weeks of exposure. One week after the installation of the protective nets, the microbial community was still the same as for unprotected berries and dominated by Hypocreales (Figure 4). In addition to Hypocreales, the two fungal orders Xylariales and Eurotiales (both orders at 7.2% relative abundance) were also present on the surface of protected and unwounded berries, whereas the bacteria belonging to Xanthomonadales (3.4%) were only present on the surface of unprotected berries. After two weeks, Hypocreales on unprotected berries strongly decreased and the order were largely replaced by fungi belonging to Agaricales (37.1%), Microascales (20.0%), Eurotiales (8.6%) and Glomerellales (5.7%), while the abundance of the bacteria (Xanthomonadales) remained constant (2.9%). On the surface of net-protected berries, however, Hypocreales remained dominant (66.7%) but were partly replaced by Eurotiales (20.8%) and Agaricales (12.5%), while the order of Xylariales disappeared completely. After three weeks, species of Pleosporales and Saccharomycetales appeared, particularly on the surface of unprotected berries (25.0% and 16.7%, respectively), while the Xylariales (23.8%) reappeared and the bacterial order Enterobacterales (4.8%) was identified for the first time on net protected berries. Although the microbial communities changed over time, they were relatively similar between the two set-ups after three weeks (Figure 4). They both included the five fungal orders Agaricales, Eurotiales, Hypocreales, Pleosporales and Saccharomycetales, with the additional fungal Xylariales and bacterial Enterobacterales in the net-protected modality.

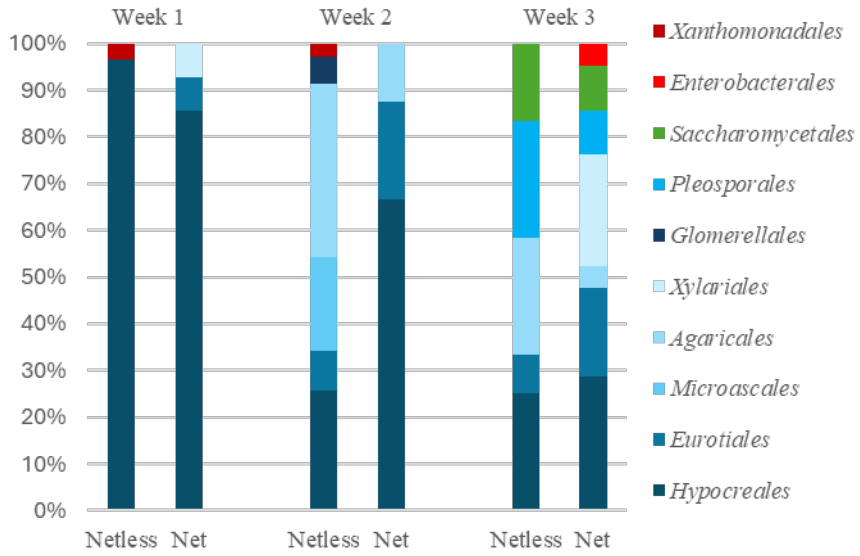

Figure 4. Three-week evolution of the microbial community inferred from the relative abundance (expressed in %) of fungal and bacterial orders present on the surface of unwounded berries from grape clusters of cv. Chasselas protected or not by a net in the vineyard plot of Nyon (see Table S2 for the classification of isolates at order rank).

Interested in the effect of wounding on the evolution of the microbial community, we compared the evolution of the microbial communities associated with wounded and healthy berries, all sampled from unprotected grape clusters over the three weeks before harvest (Figure 5). Already one week after wounding, the microbial communities on wounded and healthy berries differed considerably. While the microbial community on the surface of healthy berries was entirely composed of Hypocreales (96.6 %) and Xanthomonadales (3.4%) , the microbial community on the wounded berries was more diverse. Agaricales accounted for 48.0% of the community, followed by Hypocreales (28.0%), Microascales (20.0%) and Eurotiales (4.0%). After two weeks, the microbial community on the surface of intact berries closely resembled that of wounded berries after one week, with the same four orders comprising more than 90% of the microbiome. However, on wounded berries the fungal order Pleosporales appeared with an incidence of 26.7%, while the Microascales disappeared. By week three, the community on the surface of unwounded berries closely resembled that of wounded berries after two weeks, being composed of the four fungal orders Hypocreales (25.0%), Eurotiales (8.3%), Agaricales (25.0%) and Pleosporales (25.0%) and yeasts (order Saccharomycetales, 16.7%). The yeasts largely dominated the microbial community associated with wounded berries after three weeks (70.0%) and was accompanied by the two fungal orders Hypocreales and Agaricales. Yet, only the bacterial order Bacillales was isolated from wounded berries in the third week with a relative abundance of 15.0%. No acidic acid bacteria (AAB) were isolated even though three grape clusters became sour rot symptomatic according to their acetic acid content (≥ 0.83 g/L).

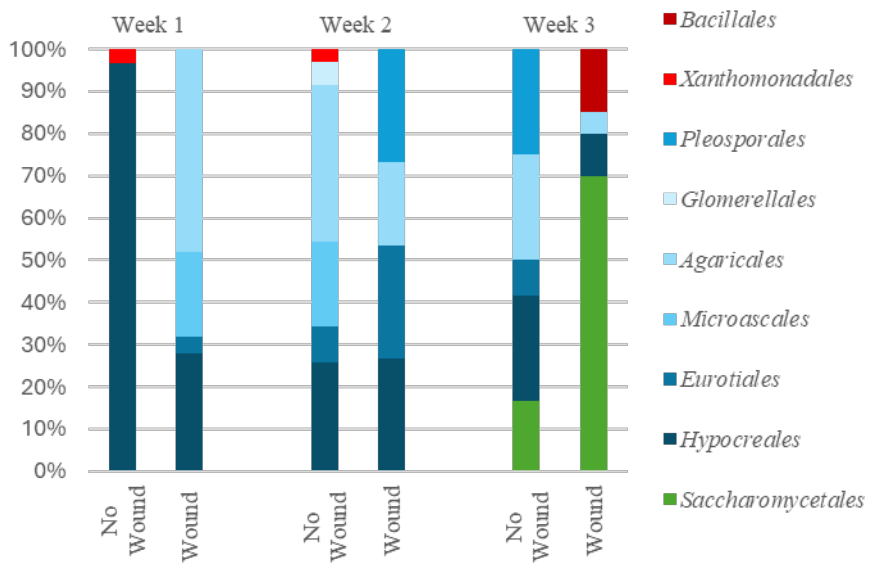

Figure 5. Three-week evolution of the microbial community inferred from the relative abundance of fungi and bacteria present in berries of Chasselas sampled from unprotected grape clusters of artificially wounded or intact berries in the vineyard plot of Nyon (see Table S2 for the classification of the isolates).

We also tested for the impact of insect vectors on wounded berries. For this, we compared the evolution of the microbiomes associated with wounded berries protected or not by a net over the three weeks (Table S2, colums A-F). Over the first two weeks, the evolution of the microbial communities on wounded berries in the presence or absence of potential insect vectors was similar in respect of the fungal orders present, although there existed some difference in their relative abundances (Figure 6A; Table S2). Hypocreales (genera *Fusarium* and *Sarocladium*) dominated both modalities after one week, followed by Agaricales (*Coprinopsis*). Together, these three genera accounted for 77.0% of the microbial community isolated from net-protected wounded berries, and for 83.0% of that from unprotected wounded berries. After the second week, the two communities shared four more fungal orders (Eurotiales, Xylariales, Glomerellales and Sordariales) as well as the bacterial order Bacillales. Together all seven orders represented 85.2% and 91.0% of the microbial communities isolated from wounded berries protected with a net or unprotected, respectively. However, at the genus rank, the microbiomes of the two modalities shared only the five fungal genera *Aspergillus* (Eurotiales), *Arthrinium* (Xylariales) *Sarocladium* (Hypocreales) *Coprinopsis* (Agaricales) and *Gibellulopsis* (Glomerellales) as well as the bacterial genus *Bacillus*. These six genera accounted for 73.1% of the microbiome on net-protected berries and for 54.6% on unprotected berries. Three weeks after the wounding of berries, the communities of the two modalities were not only more diverse but clearly different among the two set-ups sharing only three out of the 26 isolated genera. Pleosporales was represented by six genera (*Alternaria*, *Preussia*, *Pithomyces*, *Epicoccum*, *Pseudopithomyces* and *Torula*) and totalled 37.7% of the microbiome on net-protected berries, while this order was completely missing on unprotected berries. With a relative abundance of 41.5%, the yeasts of order Saccharomycetales, represented by the genera *Geotrichum*, *Saccharomycopsis* and *Zygoascus,* were the most abundant group in the unprotected modality, while in the net-protected berries these same three genera were absent but replaced by the yeast genus *Hanseniaspora*. In the third week, Bacillales (genus *Bacillus*) was only present in wounded and unprotected berries, with a slightly lower relative abundance than after two weeks.

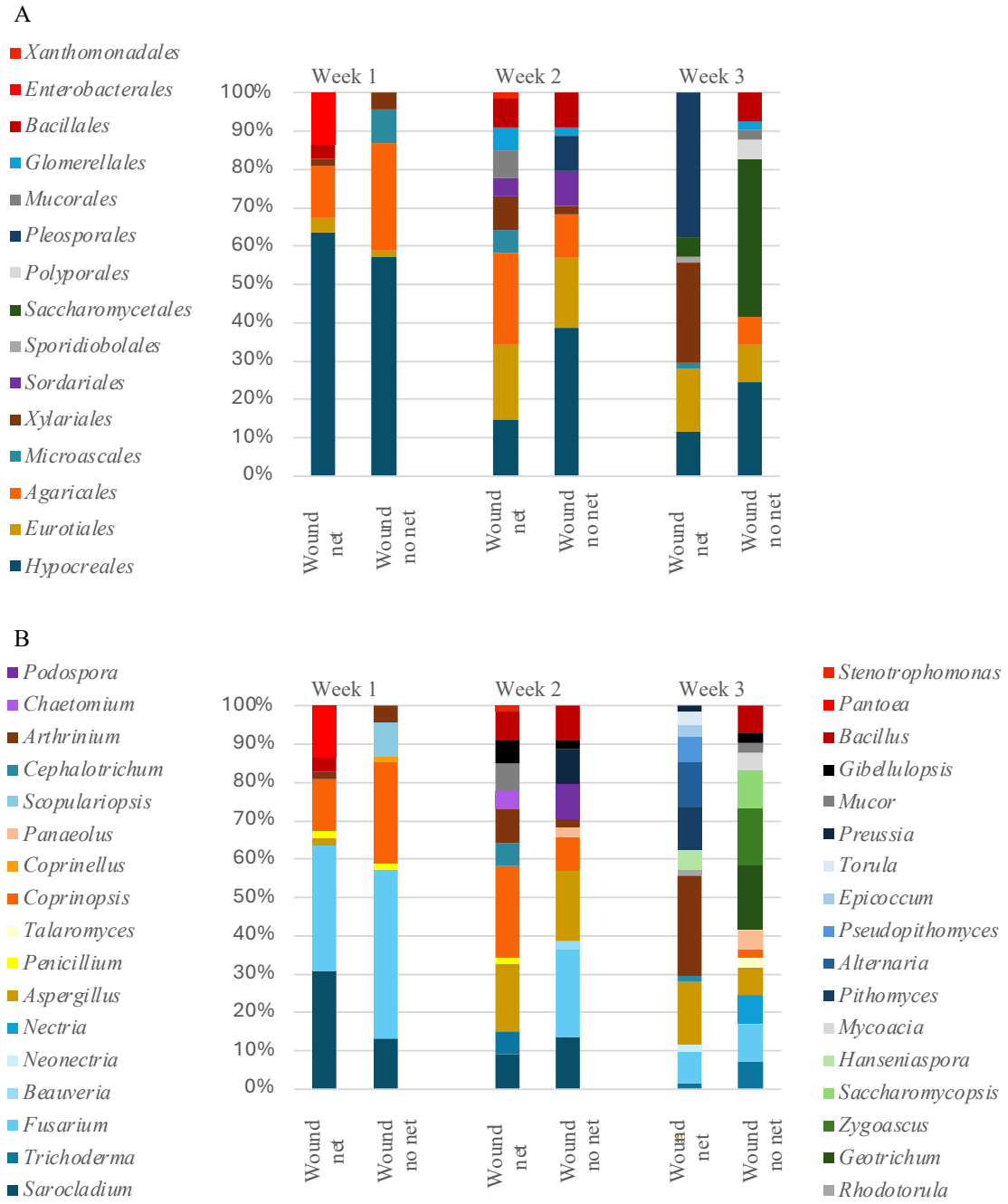

Figure 6. Evolution over time of the microbial community inferred from the relative abundance of fungal and bacterial orders (A) and genera (B) in berries of Chasselas grapes sampled from artificially wounded grape clusters protected or not by a net in a vineyard plot at Nyon (see Table S2 for the classification of the isolates).

Experiment 3: Comparison of the fungal communities associated with sour rot symptomatic berries, with healthy berries (surface-sterilized or not), and on the surface of Drosophila flies.

A sour rot infected vineyard of the cultivar Gamay in Begnins was used to compare the fungal community found on and in healthy grapes, in sour rot diseased grapes and on the surface of potential insect vectors. Fungi were isolated from the surface of healthy berries, from the surface of surface sterilized healthy berries, from sour rot affected berries and from the surface of captured drosophilids in the experimental vineyard. A total of 1020 fungal isolates were obtained in pure culture, 29 from surface-sterilized healthy berries, 265 from non-surface-sterilized healthy berries, 507 from berries affected by sour rot and 219 from the surface of *Drosophila* spp. (Table S3, https://github.com/agroscope-ch/sour-rot/ in the "Data" folder; click on "view raw" to download the file). Among these isolates, ITS sequences were obtained from 999 isolates. The 100%

similarity assembly performed on the 999 obtained ITS sequences showed that our isolates comprised 136 different ITS genotypes. Further BLAST analyses of these genotypes in GenBank enabled the identification of 40 genotypes at the species rank, 92 at the genus rank, one at the family rank, two at the order rank and one at the class rank. Yet, BLAST analyses gave results that did not allow us to assign two ITS genotypes to an order. The top score sequence for two genotypes were only assigned to a fungal class in GenBank (Table S3; Gen 58:is the parasitic basidiomycete *Curvibasidium pallidicorallinum* to Urediniomycetes, and Gen 109: an ascomycete as Sordariomycetes sp.). Yet, ITS sequences could not be obtained for one fungal morphotype (21 isolates) and it was consequently designated as "fungal sp.".

The structure of the fungal communities isolated from pulp and skin of healthy surface-sterilized berries, from the surface of healthy non-surface-sterilized berries, from sour rot symptmatic berries and from the surface of *Drosophila* spp. was first examined at the order rank (Figure 7). Healthy surface-sterilized berries harbored very few fungi, having less than 13.4% of the fungal community in common compared with the communities isolated from the surface of healthy non-surface-sterilized berries or the *Drosophila*, and even less than 0.6% with the community isolated from sour rot-affected berries (Table S3). While the fungi isolated from the skin and pulp of healthy surface-sterilized berries was dominated by Xylariales and Dothideales with relative abundances of 41.4% and 27.6%, respectively, the community associated with the surface of unsterilized healthy berries was dominated by Pleosporales (33.1%) and Saccharomycetales (36.5%). With 81.5% and 77.1%, the order Saccharomycetales represented the vast majority of fungi associated with sour rot affected berries and those present on the surface of *Drosophila* spp., respectively. Notably, yeasts (Saccharomycetales), typically reported to be associated with sour rot, were completely absent from surface-sterilized berries.

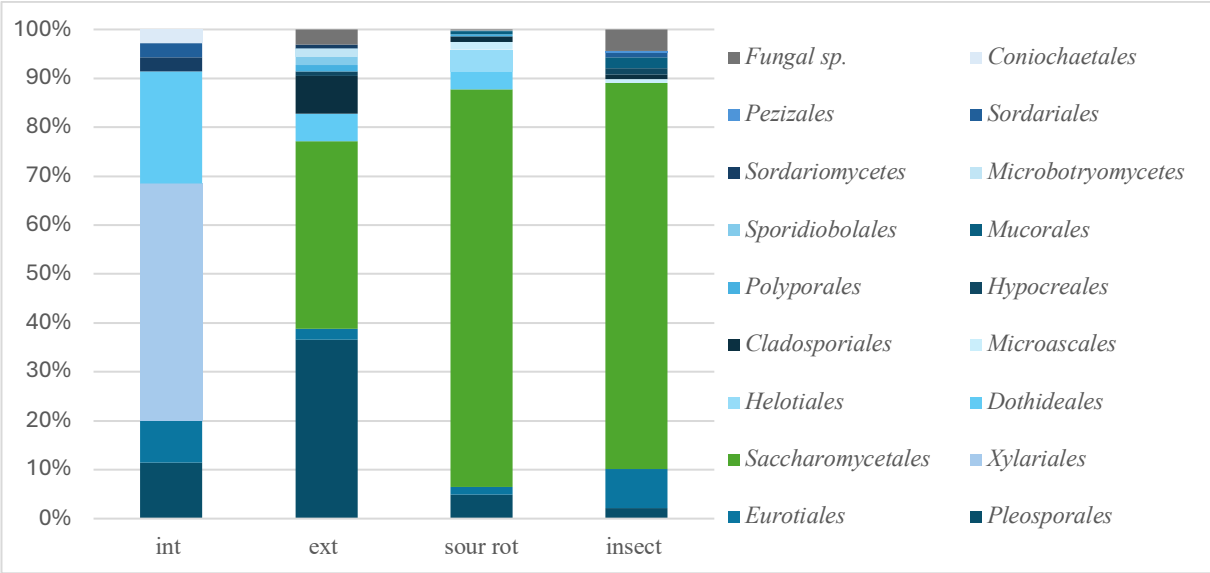

Figure 7. Relative abundance of fungal orders, where this classification rank was indicated in GenBank, in healthy berries post-surface sterilization (internal), on the surface of healthy non-sterilized berries (external), in berries affected by sour rot (sour rot) of grapes cv. Gamay, and on the surface of *Drosophila* spp. (insects) captured in a commercial vineyard in Begnins.

When comparing the three remaining modalities among each other at the genus/genotype level (Figure 8; Table S3, columns B-D), our data indicate that some of the genera found in sour rot symptomatic berries were not shared between the surface of healthy berries and fruit flies. For instance, the genera *Metschnikowia* and *Aureobasidium* were not detected on *Drosophila* spp., while present on the surface of healthy berries and in sour rot affected berries. On the opposite, the genera *Mucor* and *Candida* were absent from the surface of healthy berries but present on flies and in diseased grapes. Looking at sour rot associated fungi, the two most abundant genera were the yeast genera *Hanseniaspora* and *Pichia*. Together, these yeasts accounted for more than 85% of the

Saccharomycetales isolated in this experiment. Fungi from the genus *Hanseniaspora* were isolated from all three modalities, but for example the genotypes *H. uvarum* gen. 8, *H. cf. uvarum* gen 1, gen 4 and gen 6 were more abundant in sour rot affected berries than on healthy non-surface-sterilized berries or drosophilids (see Table S3 and Figure 8). Some *Hanseniaspora* genotypes were exclusively present in sour rot symptomatic berries or only in two out of the three modalities (e.g., *H*. aff. *uvarum* gen 10, *H*. cf. *uvarum* gen 12 and 3). The genus *Pichia* consisted out of 37 ITS genotypes and BLAST top scores identified three different species, namely *P. kluyveri* and close allies [cf.], *P. terricola* and close allies [cf.] as well as *P. sporocuriosa*. In addition, two potentially other species (i.e., *P. kudriavzevii* [cf. or aff.], *P.* cf. *fermentans*) were identified along with 12 other unidentified *Pichia* ITS genotypes. From the 37 ITS genotypes obtained for the genus *Pichia*, 18 were only isolated from sour rot affected berries. *Pichia terricola*, the most abundant genotype in sour rot affected berries, was also isolated from the surface of *Drosophila* spp.. Four other *Pichia* genotypes were shared among the three modalities, although three of them were more abundant in sour rot diseased grapes than on healthy berries or on flies (e.g., *P.* cf. *terricola* gen 1, *P.* cf. *kluyveri* gen 2, and *P. kluyveri*). Among the five ITS genotypes of *P. kudriazewii* and allies, only one was shared among the three modalities (i.e. *P.* cf. gen. 1), but with a lower abundance in sour rot affected berries than on healthy non surface-sterilized berries or on *Drosophila* spp.. The remaining four genotypes were isolated exclusively from the surface of healthy berries (cf. gen. 3), from the surface of *Drosophila* spp. (cf. gen. 2 and 4) or from sour rot affected berries (aff.). In addition, we isolated seven other genera of Saccharomycetales, namely *Candida*, *Geotrichum*, *Metschnikowia*, *Saccharomycopsis*, *Starmerella*, *Zygoascu*s as well as an unidentified genus in Dipodascaceae. Isolates of *Candida californica* were present in relatively high abundance in sour rot affected berries but rare on the surface of *Drosophila* spp. and absent on the surface of healthy berries. Representatives of *Geotrichum* were mostly associated with sour rot diseased berries but they were also isolated from the flies and from the surface of healthy berries. Representatives of *Metschnikowia*, completely absent from the surface of *Drosophila* spp., were more abundant on the surface of healthy berries than in sour rot affected berries with half of the genotypes shared between the two modalities. Members of the genus *Saccharomycopsis* were more abundant on the surface of drosophilids than in the other two modalities. *Zygoascus* and Dipodascaceae were each represented by a single isolate present in sour rot affected berries and on the surface of healthy berries respectively. Four isolates of *Starmerella* were identified on drosophilids and one in sour rot affected berries.

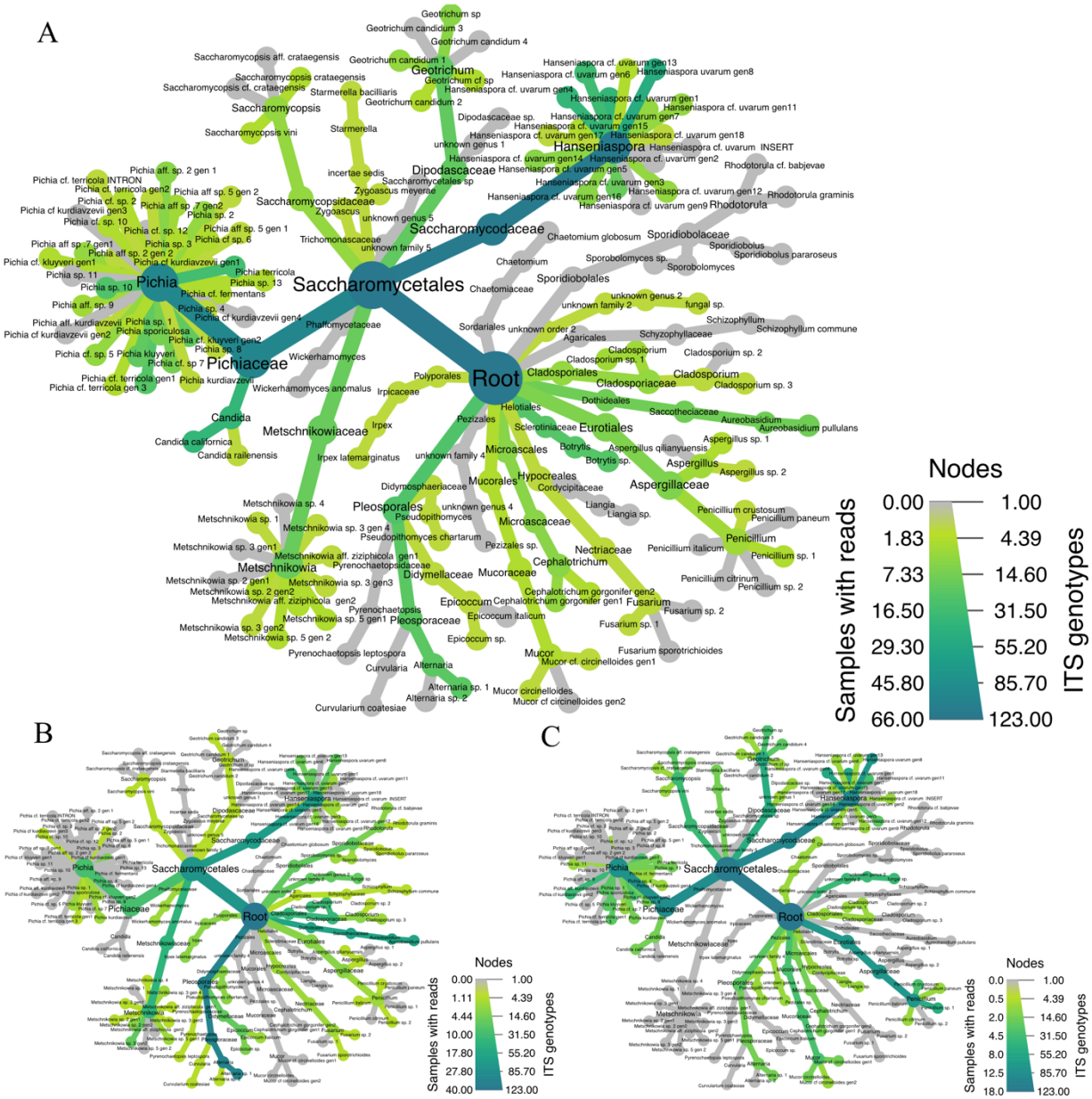

Figure 8. Abundance (in counts) of the fungal ITS genotypes in the three modalities (A. sour rot symptomatic berries, B. surface of healthy berries, C. surface of *Drosophila* spp.). The width of the edges denotes the number of counts in the whole experiment, while the color indicates the number of counts in the considered modality. The color scale is different for each group. The counts are not normalized, i.e. not proportional.

Two additional species commonly considered as pathogens were also isolated (Table S3, Figure 8). One of these was a *Botrytis* species, which was exclusively present in berries affected by sour rot. Yet, our ITS sequences could not be assigned at the species rank, as four *Botrytis* species have identical ITS sequences in GenBank, namely *B. cinerea*, *B. eucalypti*, *B. fabae* and *B. pelargonii*. Also, an unidentified *Alternaria* species (sp. 1) was particularly abundant on the surface of healthy berries, with an ITS sequence 100% identical to type sequences deposited in GenBank for three different species (i.e., *A. alstromeriae*, *A. cerealis* and *A. aborescens*) and to several sequences deposited under the name *A. alternata*. Yet for the latter species, no type sequence is flagged in GenBank.

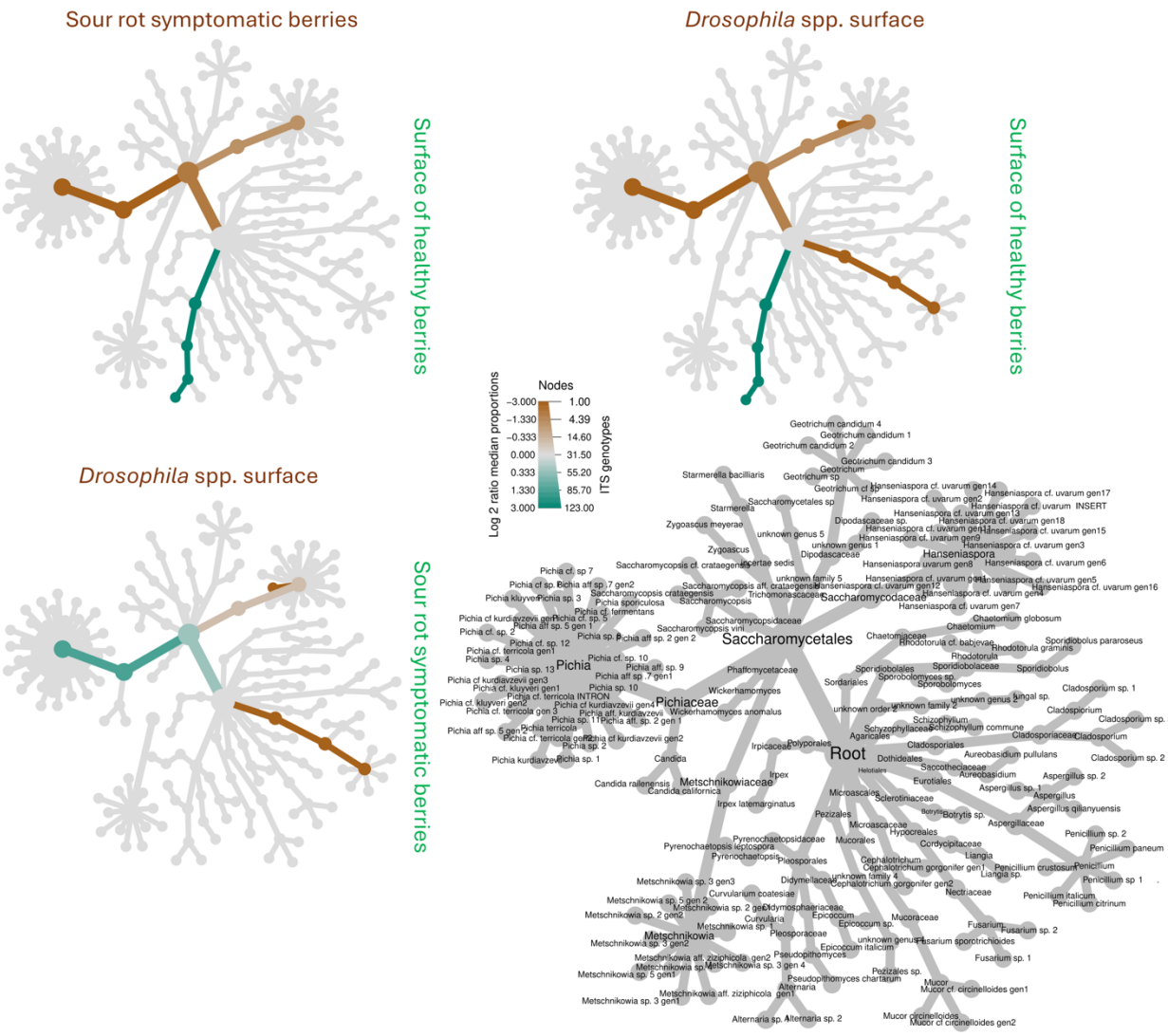

Figure 9. Pairwise differences of log (in base 2) median relative abundance of ITS genotypes in the three groups (healthy berries, sour rot symptomatic berries and *Drosophilia*). For each edge (i.e. taxon), the median of the proportion of the corresponding taxon in the modality considered is computed. Then, the log (in base 2) of the ratio of the two medians (or equivalently, of the differences of the logs) is represented on the color scale. Note that, by convention, if any of the two medians is 0, then 0 is displayed. See this hyperlink for implementation details and further clarifications. A taxon colored in brown is more abundant in the modality in the column while a taxon colored in green is more abundant in the modality of the row (see the color of the labels of rows and columns).

Pairwise comparisons of the relative abundance of the fungal ITS genotypes between the three mycobiomes also indicated that genus *Alternaria* was more abundant on the surface of healthy berries than in the two other modalities (Figure 9). The genera *Penicillium* and *Hanseniaspora* were inferred to be essentially associated with the surface of insects, while *Pichia* was found to be more abundant in sour rot symptomatic berries than on insects' surface. Overall, these three pairwise comparisons indicate that the mycobiome of sour rot symptomatic berries is somehow closer to the one isolated from the surface of the insects than to the one isolated on healthy berries.

Finally, we sought to determine whether the introduced *D. suzukii* carried the same or different sour rot associated fungi as endemic *Drosophila* species. Our data show that the surfaces of *D. suzukii* and endemic fruit flies (Table S3; Figure 10) carry a similar load of yeasts, 78.6% versus 78.9% of isolates, respectively. On the surface of all *Drosophila* species the genus *Hanseniaspora* was the most abundant with 42.1% for *D. suzukii* and 31.7% for endemic species, followed by the genus *Pichia* with 19.8% and 31.8% for the introduced and native species,

respectively. These two genera accounted for over 60% of the yeasts present on the surface of introduced and endemic *Drosophila* species. The other Saccharomycetales genera present on the surface of *D. suzukii* were *Geotrichum* (10.3%) and *Saccharomycopsis* (4.8%), two genera also present on the surface of endemic species (3.9% and 11.5%), while the genus *Starmerella* (1.6%) was only present on the surface of *D. suzukii*, and the genera *Candida* and the representative of *Dipodascaceae* were only isolated from endemic *Drosophila* species.

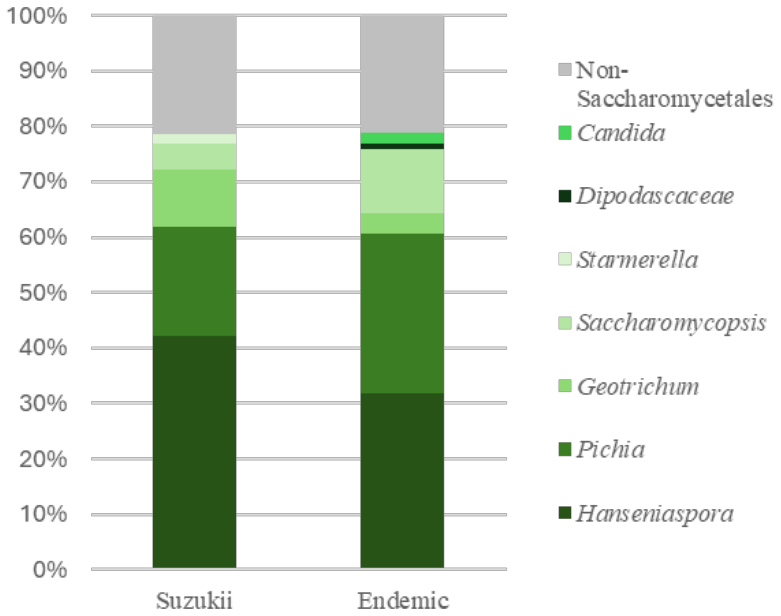

Figure 10. Comparison of Saccharomycetales genera present on the surface of *D. suzukii* and on the surface of endemic *Drosophila* species. Fungal genera not associated with sour rout were coded as non-Saccharomycetales.

DISCUSSION

Sequence-based identification of the isolated microorganisms

In this study, we assigned names to the isolated microorganisms based on the results of sequence similarity searches (BLAST top score sequences) in GenBank for ITS, the fungal barcode locus (Schoch et al. 2012) and for the 16S, the bacterial barcode locus (Lebonah et al. 2014). By using a molecular based identification of these organisms and adopting a 100% sequence similarity threshold, we were able to identify less than one third of the fungi and half of the bacteria at the species rank (Tables S1-S3). The 16S locus has been shown to offer resolution at genus rank for bacteria but in most cases not below (Bartoš et al. 2024). The ITS locus is known to fail in the recognition and delimitation of cryptic species in many Ascomycota genera. As examples (see Tables S1-S3), identical ITS sequences have been deposited in GenBank under different species names and this locus offers very low identification power for the discrimination of cryptic species in genera such as *Alternaria* (Dettman & Eggertson 2022), *Botrytis* (Plesken et al. 2021), *Cladosporium* (Schubert et al. 2007), or *Fusarium* (Crous et al. 2021). To unambiguously identify these cryptic species, a multi-locus phylogenetic analysis using a representative sampling of species in each genus will be necessary. The poor resolution offered by ITS for the discrimination between cryptic mold species also applies to yeasts, the order Saccharomycetales representing more than 95% of the fungi isolated in this study.

About ten years ago, Saccharomycetales consisted of 70 genera (Kurtzman & Robnett 2013) versus circa 100 genera today according to the GenBank taxonomy (https://www.ncbi.nlm.nih.gov/Taxonomy). However, the sequences deposited in GenBank for the most frequently isolated genera in the present study (i.e., *Hanseniaspora*, *Metschnikowia*, and *Pichia*), have often been deposited with the suffixes cf., aff. or sp. and we therefore restricted ourselves to this nomenclature. The genus *Candida* has long been the taxonomic dustbin of yeasts,

resulting in many unclassified sequence deposits for this genus in GenBank (see Table S3). Nomenclatural problems with yeast species were already highlighted by Douglass et al. (2018) who assessed, based on genome sequencing, that *Candida krusei*, *Pichia kudriavzevii*, *Issatchenkia orientalis* and *Candida glycerinogenes* were in fact all the same species. As Saccharomycetales have small genomes, it has been suggested that genome sequencing is cheaper and that it will be easier to identify cryptic and closely related species in this order in comparison to obtain sequences for multi-locus phylogenetic analyses (Koufopanou et al. 2013). Genome sequencing has successfully be used for the large-spored *Metsnchikowia* clade (Lachance et al. 2016). At present, a multigene or genomic phylogenetic approach is however not applicable to the study of fungal communities that involve hundreds of isolates. A second limitation comes from the possibility of intra-genomic variability in the ribosomal tandem repeats as indicated by Colabella et al. (2018). In their study, authors obtained from single fungal isolates of the genus *Candida* several different ITS. Yet, such a variability in the ITS of a single strain has, to our knowledge, not been reported so far for other Saccharomycetales.

Due to these practical limitations, we refrained from directly adopting the species names associated with the BLAST top score for our ITS sequence genotypes for Saccharomycetales. This decision was made even though the results assigned several different ITS genotypes to the same species (i.e., *Hanseniaspora uvarum*, *Pichia terricola*, etc.). We therefore often adopted the suffixes cf. (probably that species) or aff. (close but could be another species) rather than taking the risk to misidentify our isolates (Table S1-S3; Hofstetter et al. 2019). The only Saccharomycetales for which we adopted a species name associated with BLAST top score was when a single ITS sequence obtained from a type specimen showed 100% of similarity to our isolate. To clarify the taxonomy of Saccharomycetales and delimit species within the genera of this fungal order, multi-locus or genomic phylogenetic analyses as well as ITS sequences for type collections are imperatively needed.

Microbial communities associated with sour rot and grey mould symptomatic grape clusters

Less than half a century ago, sour rot was considered to be the final stage of grey mould caused by *Botrytis cinerea* (Bisiach et al. 1982; Zerbetto 1987). However, grey mould is now considered to be a distinctly different disease, although the term sour rot is still used by several authors to refer in more general terms to grape rot (Haviland et al. 2017; McFadden-Smith and Gubler 2015). Comparing the microbial communities present in grape clusters symptomatic for sour rot or grey mould within the same vineyard, the microbial communities of net-protected clusters suffering from grey mould and unprotected clusters suffering from sour rot were found to be clearly distinct (Figure 1A). Although the two fungal genera *Penicillium* and *Pichia* were associated with both diseases, the remaining ten fungal genera were only isolated from clusters affected by one or the other disease. Sour rot symptomatic clusters predominantly hosted yeast genera from the order Saccharomycetales (i.e., *Candida*, *Geotrichum*, *Pichia*, and *Saccharomycopsis*) as well as the bacterial genus *Acetobacter*, confirming thereby the results of previous studies (Bisiach et al. 1982; Guerzoni and Marchetti 1987; Barata et al. 2012b; Hall et al. 2019a; Gao et al. 2020). However, Saccharomycetales were much less abundant in grey mould than in sour rot symptomatic clusters. Grey mould symptomatic clusters hosted principally non-yeast genera such as a *Botrytis* species of the *B. cinerea* clade and four other fungal genera absent in sour rot affected clusters. All the species in the *B. cinerea* clade are considered as grapevine grey mould agents. Overall, only the bacterial genus *Pantoea* was isolated from grey rot affected clusters, but no acetic acid bacteria (AAB). Despite our limited sampling efforts, the observed differences between the microorganisms associated with sour rot and grey mould confirm that they are two distinct diseases as suggested by Crandall et al. (2022).

Bisiach et al (1982) and Hall et al (2018) have shown that when grape clusters infected with grey mould are surrounded by sour rot infected clusters, the expression of grey mould is inhibited. Conversely, when sour rot suffering clusters are surrounded by grey mould symptoms, sour rot expression is impaired but not completely inhibited. From these findings, the authors of the two

691 studies deduced that the two diseases compete with each other and that sour rot takes over grey
692 mould when the two diseases coexist in the same vineyard. We observed in our Chasselat vineyard
693 plot that grey mould developed only on clusters protected by a net, while sour rot developed only
694 on clusters that remained unprotected. These results are consistent with previous studies and
695 emphasize that when insect vectors such as vinegar flies are present and have full access to the
696 grapes, sour rot predominates, but when potential vectors are lacking (as in the case of net-
697 protected clusters), grey mould is able to develop. Yet, in our third experiment we were nonetheless
698 able to identify the presence of a *Botrytis* sp. in relatively high abundance in sour rot affected grape
699 clusters, while this genus was absent on either the surface of healthy berries or on drosophilids
700 (Figure 8; Table S3). The ITS BLAST top score genotype for this *Botrytis* sp. was 100% similar to
701 the sequences of four different *Botrytis* species, all belonging to the *B. cinerea* clade. Although our
702 initial experience (Figure 1A, Table S1) suggests that sour rot supplants grey rot when both
703 diseases are present in a vineyard (Bisiach et al. 1982; Hall et al. 2018), the outcome of the
704 interaction could change, as *Botrytis* is also capable of developing in sour rot affected grapes
705 (Figure 8). This result suggests that grey rot may coexist with sour rot and that it is not being
706 systematically stopped by sour rot at an advanced stage of ripening as suggested by Bisiach et al.
707 (1982).
708
709 *Sour rot associated microorganisms*
710 Comparing the microorganisms isolated from sour rot symptomatic grape clusters (Figures
711 1B, 2, 6, 8; Tables S1-S3), we identified the same sour rot disease-associated fungi compared to
712 previous studies (Barata et al. 2008, 2012a; Hall et al. 2018, 2019a; Gao et al. 2020). The fungal
713 community of sour rot affected grapes is essentially composed of the yeast genera *Candida*,
714 *Hanseniaspora*, *Pichia* (previously *Issatchenkia*), *Saccharomycopsis*, and *Zygoascus* (all
715 Saccharomycetales). However, in this study we also report for the first time yeasts of the genus
716 *Geotrichum* (also Saccharomycetales) to be associated with grape sour rot.
717 Representatives of the bacterial genus *Acetobacter* were isolated only in our first experiment
718 and only at one location, in the canton of Vaud; Figure 1B). Hall et al. (2019a) found *Acetobacter*
719 *aceti* and *A. pasteurianis* in sour rot symptomatic berries, but not the two species isolated in the
720 present study (i.e., *A. fabarum* and *A. gahnensis*; Table S1). In the present study, clusters were
721 tested for their acetic acid content to determine if they were really affected by sour rot using an
722 acetic acid threshold above 0.83 g/L as defined by Hall et al. (2019). Although AAB were absent in
723 some symptomatic clusters from the Grisons (Figure 1B) and also in the second experiment (Figure
724 3-5), their juice exceeded the acidity threshold and grapes were thus considered to be affected by
725 sour rot. In our second experiment, only bacteria from the genus *Bacillus* (Bacillales) were present
726 in sour rot affected clusters, a genus also isolated from diseased grapes in previous studies but
727 commonly reported not to be implicated in the expression of sour rot (Gao et al. 2020, Hall et al.
728 2019a). In fact, we isolated AAB only in our first experiment where clusters were left for two
729 weeks at room temperature before culturing the microorganisms, which evidently accentuated the
730 ripening stage of the berries. We renounced from this procedure in the other two experiments but
731 should nonetheless have been able to isolate AAB, if present, from sour rot affected berries in the
732 second experiment, in particular because the sampling effort was considerably greater than in the
733 first experiment and our methodology previously proved to be suitable for isolating and sequencing
734 *Acetobacter* species (Figure 1). Our results therefore suggest that AAB may appear late in the
735 development of sour rot and that fungi might be able to initiate the catalyzation of acetic acid by
736 themselves, even in the absence of AAB. Consequently, the genera *Acetobacter* and
737 *Gluconobacter,* reported to be responsible for sour rot in Europe and North America (Barata et al.
738 2012b; Hall et al. 2019a), might not be mandatory for disease expression.
739 The yeast genus *Geotrichum* was isolated from sour rot symptomatic clusters in all three
740 experiments (Figures 2, 6, 8; Tables S1-S3). Species of that genus have recently been identified as
741 the principal agents for post-harvest sour rot in different fruits and vegetables such as jocote
742 (*Spondias purpurea*), tomato or potatoes in Brazil (Paes et al. 2021). Additionally, Zhu et al. (2016)

743 showed that yeasts of the genus *Geotrichum* are able to metabolize sugars in acetic acid. Similarly,
744 it has been reported that the yeast *Hanseniaspora uvarum* can produce oxidation defects in wine by
745 increasing acetic acid, ethyl acetate and acetaldehyde content (Clemente-Jimenez et al. 2004;
746 Garde-Cerdan & Ancín-Azpilicueta 2006). Finally, results from a recent study (Lozano et al. 2024)
747 indicate that species of the genus *Candida* and *Hanseniaspora* are able to transform sugar into
748 acetic acid. Thus, our results, together with the aforementioned studies, support the hypothesis that
749 fungi can cause sour rot, even in the absence of bacteria. Further research is therefore needed to
750 confirm that yeasts of the genera *Geotrichum, Candida and Hanseniaspora* are able to initiate sour
751 rot expression in the absence of AAB.
752
753 *Determining the impact of wounds and insect vectors on the microbial community*
754 When comparing the microbial communities of adjacent healthy berries with those affected
755 by the disease, and using berries taken from disease-free grape clusters as controls (Figure 3), our
756 data suggest that sour rot disease is not directly spreading from symptomatic berries to adjacent
757 healthy berries. Sour rot affected grapes are characterized by a high relative abundance of yeasts
758 (Saccharomycetales, 70%) and the presence of bacteria (Bacillales), that characterize berries
759 affected by sour rot, while asymptomatic berries hosted only few Saccharomycetales and no
760 bacteria. This observation is consistent with previous studies (Barata et al. 2012b, Gao et al. 2020,
761 Hall et al. 2019a) and suggests that insect vectors such as drosophilids play an important role in the
762 dissemination of the disease.
763 Regardless of the fact whether or not insects had access to the grapes, the microbiomes on
764 the surface of intact berries were relatively similar three weeks after the start of the second
765 experiment, (Figure 4). None of the grape clusters sampled in this experiment reached the acetic
766 acid threshold associated with sour rot. This reflects probably the relatively low abundance of
767 Saccharomycetales (i.e., < 20%) on healthy grapes, whether fruit flies had access to the berries or
768 not. It therefore seems that the presence of insect vectors had not a major effect on the evolution of
769 the grape microbiome in the absence of skin wounds, an observation shared by Hall et al. (2018).
770 However, the presence of insect vectors accelerated the evolution of the microbiome in wounded
771 berries by approximately a week compared to unwounded berries (Figure 5). Cracks in the grape
772 skin enable microorganisms to access nutrients in the grape pulp, thereby facilitating the
773 development of Ascomycota and in particular yeasts (Barata et al. 2012b, Hall et al. 2018).
774 According to these authors, the role of *Drosophila* individuals in the expression of sour rot is
775 primarily mechanical as the presence of insects prevents the healing of wounded berries, this most
776 likely by activity of larvae. This was confirmed by the observation that the activity of hatching
777 larvae retards the healing of the skin in wounded berries (Rombaut et al. 2017). Our results are
778 consistent with these previous studies, as wounds heal more slowly when insects are attracted to the
779 wounds. This allows fungi and bacteria to access the berry pulp to initiate a microbial community
780 that leads to sour rot expression. Our results also support the observations of Hall et al. (2019a) that
781 sour rot associated yeasts are present on the surface of intact berries but unable to reach the pulp in
782 the absence of skin wounds. This observation is supported by the similar evolution of the microbial
783 communities in wounded berries at both order and genus ranks, independently of whether fruit flies
784 have access or not (Figure 6A and 6B; Table S2). Our data, however, emphasize that insect vectors
785 only started to influence the microbiome composition in wounded berries after three weeks,
786 particularly by boosting yeast development (Saccharomycetales, genera *Geotrichum*,
787 *Saccharomycopsis* and *Zygoascus* – see Table S2), and this while net-protected wounded berries
788 predominantly hosted Pleosporales species. Nevertheless, a few Saccharomycetales were also
789 isolated from net-protected wounded berries, suggesting that sour rot might develop later, even in
790 the absence of insect vectors such as drosophilids. To verify this hypothesis, future studies should
791 monitor the evolution of the microbiomes in wounded berries protected by a net over a longer
792 period.
793
794

Potential contribution of the mycobiomes present in surface sterilized berries, on the surface of healthy berries and on the surface of Drosophila spp. to the mycobiome isolated from sour rot symptomatic berries

It is assumed that the microbiome associated with sour rot symptomatic berries results from the penetration of fungi and bacteria present at the surface of the berries or from contact with the microbiome associated with visiting insects (Hall et al. 2019a). Here, we did not consider the transport of spores in the air because a recent study indicated that the airborne microbiome has a minor contribution to the grape microbiome (Abdelfattah et al. 2019).

The number of fungi isolated from surface-sterilized berries was ten to seventeen times lower than on unsterilized or symptomatic berries. Such differences may be partially explained by a larger number of berries sampled from sour rot affected clusters (64 berries/507 fungal isolates; Table S3) but this does explain the observed differences between surface-sterilized berries and unsterilized berries as the same numbers of berries were sampled (50 berries for both conditions and 29 versus 265 fungal isolates obtained respectively). The mycobiome of surfaced sterilized berries consisted mainly of species belonging to the order Xylariales (particularly the genera *Annulohypoxylon*, *Biscogniauxia*, *Daldinia* or *Nemania*; see Figure 7; Table S3). Many Xylariales species are known to be common plant endophytes (Becker and Stadler 2021; Ma et al. 2022, U'Ren et al. 2016). Yet, the surface-sterilized berries did not only host fungi from different systematic groups compared to those from the other three modalities, but no yeasts were isolated from these surfaced sterilized berries (Figure 7; Table S3). Our results thus contradict those reported by Hall et al. (2019b), who isolated mainly yeasts from surface-sterilized healthy berries. However, we think that this might be explained by the different condition of the analyzed grapes. Indeed, Hall et al. (2019b) sampled grapes in vineyards one to five days before harvest, but also table grapes obtained from supermarkets, where these were most likely kept in cold storage several days before being sold. One can imagine that microfissures could have developed in the grapes on sale allowing yeasts that were present on the surface of commercialized and late harvested berries to penetrate into the grapes. While the mycobiome present on the surface of healthy grapes has been widely studied, our study, as well as the study by Hall et al. (2019b), are to our knowledge, the only studies that investigated the fungi living within the pulp of healthy grape berries. Further research is needed to confirm whether Saccharomycetales can thrive as endophytes of healthy berries or if they are only present on the surface of grape berries as suggested by our data.

Our results have shown that, at the systematic rank of order, the fungal community associated with sour rot symptomatic berries was more similar to the one isolated from the surface of fruit flies than to the one isolated from the surface of healthy berries (Figure 7; Table S3). This was particularly evident for yeasts, which constituted the vast majority of fungi associated with symptomatic berries but also of those isolated from the surface of *Drosophila* adults. And this when yeasts represented less than a third of the fungal community isolated from the surface of healthy berries. Consistent with previous observations, fruit flies appear to boost the presence of yeasts in sour rot symptomatic berries, especially for the genera *Hanseniaspora* and *Pichia* (Table S3; Figure 8). This finding is reinforced by the pairwise comparisons of the relative abundance of these two genera being more frequent in the mycobiomes associated with sour rot affected berries and on the surface of vinegar flies compared to the surface of healthy berries (Figure 9). A high intraspecific diversity of *H. uvarum* has been documented in winery environments (Grangeteau et al. 2015), as well as an intraspecific nucleotide diversity based on multi-locus typing analyses (Saubin et al. 2020). We also retrieved many different ITS genotypes for *Hanseniaspora*, all highly similar to sequences deposited as *H. uvarum* in GenBank (Table S3; Figure 8-9), but with certain genotypes being more prevalent in some of the experimental setups. Given that *H. uvarum* related sequences corresponded to the most abundant fungi in the third experiment across all three modalities, our results align with previous studies (Barata et al. 2012b; Hall et al. 2018). This species has extensively been identified as one of the most common yeasts in grape musts across various countries (Albertin et al. 2016, Romano et al. 2019) as well as in sour rot symptomatic berries in Europe (Barata et al. 2012a). Instead, the most prevalent ITS genotypes of *Pichia* were shared

among the surface of healthy berries, surface of *Drosophila* spp. and sour rot symptomatic berries (Figure 8; Table S3). However, not all *Pichia* species appear to proliferate equally in diseased grapes. *Pichia kluyveri* et *P. terricola* (including closely related species cf. or aff.) accounted for over half of the *Pichia* strains isolated from symptomatic berries, with the most abundant genotypes being more prevalent in sour rot symptomatic berries than on the surface of *Drosophila* flies or on healthy grape berries (Table S3; Figure 8). These two *Pichia* species therefore seem to have a greater impact in the process leading to sour rot than the other *Pichia* spp. Also Hall et al. (2019a) observed that *P. kluyveri* was the most common species in sour rot symptomatic berries. On the other hand, half of the six ITS genotypes for *P. kudriazewii* were exclusively isolated from the surface of healthy berries and/or drosophilids, while two other genotypes were equally or less abundant in sour rot affected berries compared to the other two modalities (Table 3). It therefore seems that *P. kudriazewii* is less adapted than *P. kluyveri* and *P. terricola* to survive and proliferate in ripening grapes. *Geotrichum* and *Saccharomycopsis* were predominantly isolated from sour rot affected berries and from drosophilids. Vinegar flies thus seem to contribute more of these two genera to sour rot affected grapes than healthy berries, as well as of *Candida*, which was absent on the surface of healthy berries. On the other hand, the genus *Metschnikowia* was absent on the surface of *Drosophila* species but relatively abundant in symptomatic berries and on the surface of healthy berries. It might therefore be that this yeast genus is not well adapted to be transported by vinegar flies, as yeast adhesion has been demonstrated to be dependent on both the yeast strain as well as on the surface it interacts with (Pawlikowska et al. 2019; Whitehead et al. 2021), an aspect that is certainly worth to be investigated in more detail. Overall, yeasts present on the surface of vinegar flies and of healthy berries seem to contribute to the fungal community associated with sour rot in grapes. However, their contribution does not necessarily involve the same genera, species, genotypes and/or in the same proportions.

A mutualistic relationship between *H. uvarum* and *D. suzukii* has been demonstrated in immature raspberries where this yeast seems to promote the development of insect larvae (Chakraborty et al. 2022). There is also evidence that *H. uvarum* is one of the most frequent yeasts in the gut of several *Drosophila* species, including *D. melanogaster* (Chandler et al. 2012). When comparing our fungal isolates obtained from the introduced *D. suzukii* compared to native *Drosophila* species (Figure 10), we conclude that fungal communities among *Drosophila* species are overall quite similar and that all vinegar flies carry a high and equal amount of yeasts with *Hanseniaspora* (*H. uvarum*) being the most abundant genus, followed by *Pichia* (several species). It was suggested that *Pichia kluyveri* and *P. terricola* maintain the same positive mutualistic association with *D. suzukii* on the basis of their abundance in the gut of this vinegar fly (Hamby et al. 2012). Stamps et al. (2012) showed that this relationship also exists for *D. melanogaster*, with the yeast species involved in this mutualistic association including not only *Pichia kluyveri*, but also *Candida californica* and *C. zemplinina*. Our results confirm previous studies, with *H. uvarum* and its allies, as well as *Pichia spp.* being also frequent on the surface of both endemic and introduced vinegar flies (Figure 10). As observed by Stamps et al. (2012), *Candida californica* appears to be associated only with *D. melanogaster*, this genus being absent on the surface of *D. suzukii*. All *Drosophila* species seem to play a similar role in the transport and transmission of yeast cells between grape berries and they may thus all contribute to the incidence of Saccharomycetales in sour rot affected berries as suggested by Entling & Hoffman (2020). Given that our study is the first that investigated the contribution of the surface of *Drosophila* species to the fungal community associated with sour rot in grapes, it will be interesting to see if future studies are able to confirm our findings.

In conclusion, skin wounds in grape berries were identified as the most important factor promoting the development of sour rot, confirming the results obtained by Hall et al. (2018). Rainy weather at an early ripening stage (Vogel et al. 2021), together with warm temperatures and a sugar content in grape berries above 15 ^{o}Brix (Hall et al. 2018), has been identified as the main factor leading to sour rot expression in North America. A possible explanation for the high incidence of sour rot in particular millesimes in Switzerland might be that high precipitation leads to an

excessive water uptake by the plant resulting in a rapid swelling of grape berries and, finally, in cracks in the berrie skin, especially in compact grape clusters. These cracks may thereafter allow fungi to penetrate the berries and allow both native and invasive *Drosophila* species to lay their eggs in injured grapes. Moreover, our data also suggest that grape clusters can be affected by sour rot in the absence of acetic acid bacteria. This suggests that AAB species of the genera *Acetobacter* and *Gluconobacter,* are not mandatory for the expression of sour rot in grapes. It might well be that yeast species of the genus *Geotrichum*, recently reported as a sour rot agent in other fruits and vegetable species, along with *Hanseniaspora* and/or *Candida* yeasts, are able to metabolize sugars and to convert these into acetic acid, thereby initiating sour rot in the absence of bacteria. Overall, this study sheds new light on the expression of sour rot and on the mutualistic relationship between yeasts and *Drosophila* spp.. It is a further step to entangle the complex interactions between the various actors that will ultimately lead to a better management of this common disease in commercial vineyards.

DECLARATION

Data availability

All sequence data have been deposited in GenBank with corresponding accession numbers and are accessible through the hyperlink https://github.com/agroscope-ch/sour-rot/ and can be downloaded from the Data folder (Tables S1-S3) as well as the scripts to reproduce figures 8 and 9.

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Authors contributions

SH, PK, KG, J-LW and VH conceived the study; SH and VH collected the data, SH, MW and VH analyzed the data; SH, PK, BB, and VH led the writing of the manuscript. All authors commented on the manuscript.

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