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Yeast-like fungi and yeasts in withered grape carposphere: Characterization of *Aureobasidium pullulans* population and species diversity

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ABSTRACT

Yeast-like fungi and yeasts residing on carposphere of withered grapes for Italian passito wine production have been scarcely investigated. In the present study, isolates from single berries, both sound and damaged, of Nosiola, Corvina and Garganega varieties were analyzed at the end of the withering process. Great variation of cell concentration among single berries was observed. In sound berries, yeast-like fungi were significantly more frequent than yeasts. Species identification of isolates was carried out by BLAST comparative analysis on gene databases and phylogenetic approach. All yeast-like fungi isolates belonged to *Aureobasidium pullulans*. They displayed different culture and physiological characteristics and inhibitory capacity against phytopathogenic fungi. Moreover, PCR profile analysis revealed high genotypic similarity among these strains. A total of 35 species were recognized among yeast isolates. Ascomycetes prevailed over basidiomycetes. To the best of our knowledge, *Naganishia onofrii* and *Rhodosporidiobolus odoratus* were identified for the first time among yeasts isolated from grapes, must or wine. *Hanseniaspora uvarum* and *Starmerella bacillaris* were the most frequent species. Most species were found only in one grape variety (nine in Nosiola, 10 in Corvina and five in Garganega). The sanitary state of withered grapes could have an important impact on the structure of these epiphytic populations.

1. Introduction

Wine grapes are inhabited by a complex consortium of microorganisms that affects their quality and the resulting wine. Climate, season, variety, vineyard practices and stage of ripening have been identified as the most important factors that have an impact on the structure of fungal and bacterial populations on the grape carposphere (Bokulich et al., 2014; Martins et al., 2014; Milanović et al., 2013; Renouf et al., 2005).

Yeast communities residing on grapes and involved in wine fermentations have been widely investigated due to their great importance in the winemaking. Several species of wine yeasts have been thoroughly analyzed about their role in improving or, conversely, depleting the quality of wine (Barata et al., 2012a; Bisson et al., 2017). Many grape varieties and types of wine, both international and local, have been the subject of yeast population analysis (Alessandria et al., 2013; Drumonde-Neves et al., 2017; Li et al., 2010; Nemcová et al., 2015).

The dehydrated grapes by off-vine withering for Italian passito wine production are a particular habitat for yeasts due to their distinctive environmental conditions (Domizio and Lencioni, 2011). Low water activity and exosmosis on berry surface are important factors that strongly affect the survival of epiphytic populations. Moreover, the presence of filamentous fungi, especially those more infective such as *Botrytis* and *Penicillium*, can strongly alter the structure of the yeast community (Nisiotou and Nychas, 2007). At grape crushing, the high sugar content juice and low fermentation temperature (must fermentation occurs in the winter) affect the composition and the succession of yeast species involved in the fermentation. Hence, this complex microbial consortium has a considerable impact on the final characteristics of passito wines (Domizio and Lencioni, 2011).

Few studies have been carried out on yeasts residing on withered berries for passito wine production (Rantsiou et al., 2013; Stefanini et al., 2017). In particular, Rantsiou et al. (2013) described the species evolution during the withering of Erbaluce grapes, where *Hanseniaspora uvarum* and *Metschnikowia fructicola* dominated the initial and middle phases and *Aureobasidium pullulans* the last phase of this post-harvest process. The presence of *A. pullulans* in withered grapes has been also documented in our previous investigations (Lorenzini et al., 2016; Lorenzini et al., 2018; Lorenzini and Zapparoli, 2015). This yeast-like fungus is very common in the phyllosphere and carposphere of fruit and vegetable crops and has a potential action against phytopathogenic fungi (Rathnayake et al., 2018). On the other hand, *A. pullulans* can

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cause rot on withered grapes (Lorenzini and Zapparoli, 2015).

Barata et al. (2012b) indicated that grape damage is the main driving force altering the berry microbiota. Deep changes in species diversity and population sizes are caused by the occurrence of the rotting process. These alterations can have a strong impact on the quality of grapes and resulting wine. Since withered grapes are easily subjected to damage during withering due to fungal contamination, insects and handling, investigations into the ecology of the grape carposphere are recommended.

In this study, epiphytic populations of yeast-like fungi and yeasts from withered grapes of the Nosiola, Corvina and Garganega varieties for passito wine production were analyzed. Enumeration and identification of isolates from sound and damaged single berries was carried out. The BLAST comparative analysis on gene databases and phylogenetic analysis were performed to have a reliable taxonomic resolution of isolates at species level. Cultural, physiological and molecular characterization of yeast-like fungi was also performed.

2. Materials and methods

2.1. Sampling

Sampling was carried out on three viticulture areas of Northeast Italy such as Valle dei Laghi (Trento), Valpolicella (Verona) and Soave-Gambellara (Verona and Vicenza) for the production of different types of passito wine. The main cultivated varieties are Nosiola, Corvina and Garganega, respectively. Grape samples of these three varieties were collected during the 2015–17 vintages from nine fruit-drying rooms, randomly selected within each winemaking area (three for each area). Samplings were carried out one-two weeks before the crushing when the grape weight loss was approximately from 35 to 45% of initial fresh weight (35–40% for Corvina and 40–45% for Nosiola and Garganega). Both sound and damaged berries were sampled. Damaged berries displayed symptoms (singular and mixed) as follows: i) sour-rotten, ii) partially or totally covered with mycelium, iii) with visible fractures or cracks, iv) largely shrivelled (mummy-like). Comparison among these different categories falls outside the confines of this study and, except for total cell counts, damage berries were considered as a single sampling for each variety. The incidence of these damaged berries, estimated by visible inspection of 80–100 bunches of grapes in each fruit-drying room, were variable from < 2 to 20% depending to sampling year, grape variety and fruit-drying room. Single berries were cut with ethanol-sterilized scissor from different grape bunches, that were randomly selected in plastic boxes taken from different parts of the fruit-drying room, and immediately put in sterile bag without hand contact. Hence, the berries were transferred in laboratory for the microbiological analysis carried out at the same day of the sampling. A total of 326 berries were singularly analyzed: 136 (71 sound and 65 damaged) of Nosiola, 100 (60 sound and 40 damaged) of Corvina and 90 (46 sound and 44 damaged) of Garganega.

2.2. Enumeration of cells and isolation

At the laboratory, each berry was weighted and placed in sterile tube containing an isotonic peptone solution (1 g/L peptone with 0.1 g/L Tween 80). The tubes were incubated at 25 °C for 3 h with slow shaking to release microorganisms. The suspension was used undiluted and decimally diluted with physiological solution (0.9% w/v NaCl) for plating on GYP agar (20 g/L glucose, 5 g/L yeast extract, 5 g/L peptone, 20 g/L agar) supplemented with 0.025% w/v biphenyl (Sigma-Aldrich, Saint Louis, MO) and 0.01% w/v chloramphenicol (Sigma-Aldrich) to inhibit mould development and bacterial growth, respectively. GYP is a general purpose medium enables the recovery high number of species (Barata et al., 2008).

The enumeration of yeast-like fungi and yeasts was carried out by colony counting (CFU/g berry). Colonies of yeast-like fungi and yeasts were distinguished on their different morphology (presence and absence of visible mycelium around the colony center for yeast-like fungi and yeasts, respectively). Many plates did not show colonies. It is probable that in some plates colonies had been not visible since hidden by fungal mycelium grown despite the use of high concentration of biphenyl. For this reason, the lowest limit of enumeration was arbitrarily of 10 CFU/g. Representative colonies were picked up from each plate. A total of 48 isolates (11 of Nosiola, 16 of Corvina and 21 of Garganega) of yeast-like fungi and 266 of yeasts (93 of Nosiola, 101 of Corvina and 72 of Garganega) were isolated.

2.3. Identification of isolates

All isolates were identified at species level through molecular and physiological analysis. Genomic DNA was extracted from pure cultures as previously described by Cocolin et al. (2000). The PCR amplification of D1/D2 domain of the large subunit (LSU or 26S) rRNA gene and the internal transcribed spacer (ITS) gene region was carried out using primers NL1/NL4 and ITS1/ITS4, respectively (Kurtzman and Robnett, 1998; White et al., 1990). Amplicons were sequenced at the Eurofins Genomics (Eurofins Genomics, Edersberg, Germany) using the same primers used for amplification. Sequence similarity searches were performed using the BLASTn tool in NCBI-GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi), YeastIP (http://genome.jouy.inra.fr/yeastip/index.php) and UNITE (https://unite.ut.ee/analysis.php#) databases. The sequence similarity was estimated considering the 99–100% similarity for NCBI-GenBank and YeastIP, and the highest score obtained in each alignment for UNITE database. The partial DNA sequences of isolates and reference taxa of each genus were used to establish datasets for phylogenetic analysis. The sequences of reference strains closely related to isolates used in this study were recovered from the most recent literature. Phylogenetic analysis was conducted using sequences from the Clustal W multiple alignment output using neighbor joining (NJ) statistical methods and maximum likelihood (ML) substitution model on the MEGA 7.0 interface. The NJ trees were inferred with 1000 bootstrap replicates (BS) to ascertain the reliability of a given branch pattern in the trees.

The physiological characterization was performed according to Kurtzman et al. (2011).

2.4. Characterization of yeast-like fungi

Colony morphology of each isolate was observed on MEA (20 g/L malt extract, 1 g/L peptone, 20 g/L dextrose, 15 g/L agar) at 25 °C in darkness. Growth assay was performed on MEA at 4, 10, 25, 30 and 37 °C in darkness, and on MEA supplemented with 5, 10 and 15% NaCl (w/v) or 20 and 40% D-glucose (w/v) at 25 °C in darkness. The growth was determined by measuring the width of colony obtained by streaking on plate a 3-old days culture. For each condition the growth variation was calculated, and expressed as percentage, with respect to the growth on MEA at 25 °C (standard condition). Each assay was performed in triplicate.

The ability of yeast-like fungi to inhibit growth of phytopatogenic fungi was tested in vitro using dual cultures on MEA. Five strains (*Botrytis cinerea* ITEM 17199, *Alternaria alternata* ITEM 17196, *Cladosporium halotolerans* ITEM 17203, *Penicillum expansum* P34, *Aspergillus uvarum* AN3) isolated from withered grapes (Lorenzini et al., 2016) were used. These strains were cultivated on MEA at 25 °C for 7–14 days 12 h light/12 h dark. Briefly, a 50 mm line of the yeast-like fungal cells, collected from 3-day-old cultures on ME (MEA without agar), was streaked about 30 mm from a five mm fungal plug. Each yeast-like fungal strain was tested in triplicate. Antifungal activity was assessed by measuring the inhibition distance between the edge of yeast-like colony and mycelium as described by Raspor et al. (2010), after 7 days for *B. cinerea*, *As. uvarum* and *A. alternata* and 12 days for *P. expansum* and *C. halotolerans*.

Data analysis of RAPD-PCR fingerprinting of yeast-like fungi was carried out in two separate reactions using the primers M13 and OPD-02 according to Lorenzini and Zapparoli (2014). Rep-PCR fingerprinting was performed in three separate reactions using the primers ERIC1R/ERIC2 for ERIC-PCR, REP1R/REP2-1 for REP-PCR and primer BOXA1R for BOX-PCR according to Loncaric et al. (2009). Amplified products were visualized on a 1.5% w/v agarose gel after staining with EuroSafe Nucleic Acid Stain (Euroclone spa, Milan, Italy). All PCR reactions were performed in triplicate to evaluate band reproducibility. Conversion, normalization and numerical analysis of the patterns were performed by GelCompar 4.0 software (Applied Maths, Kortrijk, Belgium). The clustering analysis of PCR profiles was carried out by manually selecting the reproducible and clearly visible bands excluding those less intensely stained. The band-based dendrogram was produced by using a Dice coefficient and UPGMA (unweighted pair group method with arithmetic average).

2.5. Statistical treatments of data

Data of fungal populations were treated using statistical package XLSTAT 2017 (Addinsoft SARL, Paris, France). Comparison of total counts of yeast-like fungi and yeasts on each type of berries was statistically analyzed by Kruskal-Wallis test and multiple pairwise comparison was carried out using Dunn's procedure (two-tailed test). Individual rarefaction curves, diversity indexes (Simpson 1-D and Shannon H) and species evenness were calculated by PAST 3 (Paleontological statistic software, Øyvind Hammer, Natural History Museum, University of Oslo, Norway).

3. Results

3.1. Enumeration of yeast-like fungi and yeasts

Wide variation of concentration of yeast-like fungi and yeasts was observed among single berries (Fig. 1). High cell counts ($> 10^6$ CFU/g) were observed on damaged grapes and the maximum value (7.4 $\log_{10}$ CFU/g) was measured on a damaged Corvina berry. In all sound berries the average concentration of yeast-like fungi was at least 10-fold greater than that of yeasts. The yeast-like fungal population was significantly larger than that of yeasts only on damaged Garganega berries. The size of yeast-like fungi and yeast populations on different categories of damaged berries was extremely variable (Fig. 2). In particular, the concentration of yeasts detected on mummy-like berries was particularly low with respect to that on sour-rotten berries.

3.2. Identification and characterization of yeast-like fungi

All 48 yeast-like fungi, randomly selected from yeast-like fungal populations found on surface of berries of the three grape varieties, were identified as *Aureobasidium pullulans* by BLAST comparative and phylogenetical analyses of ITS gene sequence (data not shown). These 48 isolates were phenotypically and genotypically analyzed.

Strains displayed different colony morphology (colour, texture and margin) (Fig. S1). Twenty isolates showed a light olive-brown to brown mycelium, while the remaining had pinkish mycelium. Colonies appeared to be smooth, matt or shiny. Most isolates showed feathery mycelium, while few of them had arachnoid mycelium.

Strains displayed a different ability to grow in the presence of various concentrations of NaCl and D-glucose, and at different temperatures with respect to the standard condition (on MEA at 25 °C) (Fig. 3). At 10 and 15% NaCl almost all strains were strongly inhibited (> 50% of reduction), while at 5% NaCl most strains (27 out of 48) displayed a growth reduction of < 50%. The presence of high D-glucose concentrations did not affect negatively the growth in most isolates, and at 20% D-glucose an increase of mycelial growth was observed in 27 isolates. At 10 °C, 17 out of 48 strains showed no growth variation with respect to the control, while four strains increased their growth. Strong growth inhibition was observed in all strains at 37 °C and in most of them at 4 °C. Interestingly, some strains (e.g. C-vp10, N-t2, G-ls4, C-n12, N-p15 and G-mv23) displayed a good ability to grow in most of the tested conditions (Fig. 3).

Based on dual culture in-vitro assay, carried out as a preliminary study on the interaction between *A. pullulans* and some of the most common pathogenic fungi of withered grapes, strains showed very different behaviors depending to the fungus (Fig. S2). The mycelium growth of *B. cinerea* was partially inhibited (> 6%) only by six isolates. *Aspergillus uvarum* and *P. expansum* were partially inhibited by 13 and 16 isolates, respectively. Antagonistic effects were recorded in most of the isolates for *A. alternata*, while all isolates for *C. halotolerans* had antagonistic effect. No *A. pullulans* isolates exhibited antagonist activity against all five fungi, but five of them (N-l15, N-l13, N-p15, N-p3 and C-n8) caused growth reduction in four fungi (e.g. C-n8 in *B. cinerea*, *A. alternata*, *P. expansum* and *C. halotolerans*, or N-l13 in *B. cinerea*, *A. alternata*, *As. uvarum* and *C. halotolerans*).

The primers used for PCR reactions generated a reproducible DNA fingerprinting in all 48 yeast-like fungi. Rep- and RAPD-PCR profiles were characterized by a variable number of bands ranging from 150 to 1800 bp. Cluster analysis of combined data using UPGMA method generated a dendrogram that displayed two principal clusters with a

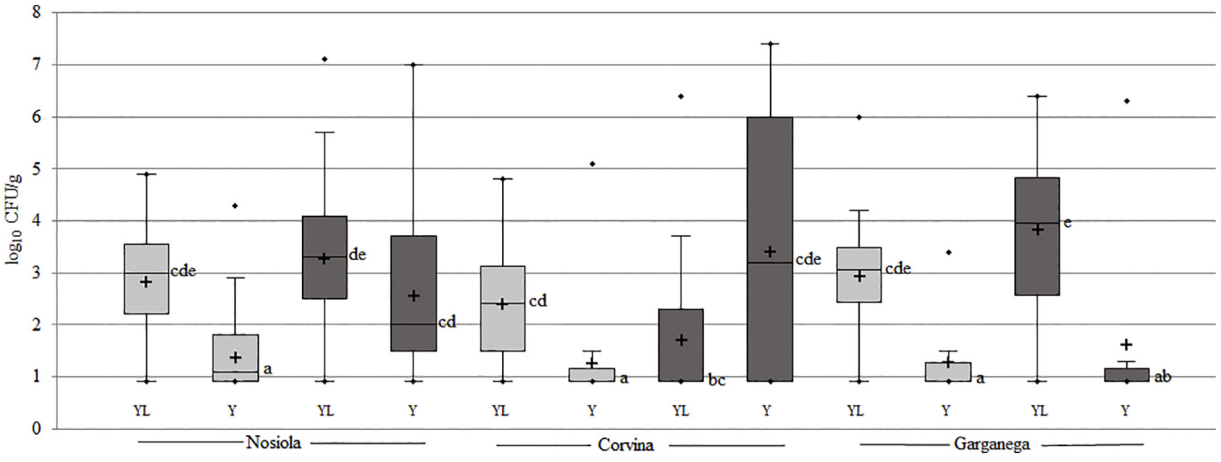

Fig. 1. Box plot of concentration ($\log_{10}$ CFU/g) of epiphytic yeast-like fungi (YL) and yeasts (L) on sound (light gray) and damaged (dark gray) withered grape berries of Nosiola, Corvina and Garganega variety. The lower and upper limits of the box are the first and third quartiles respectively, median (central horizontal bar), mean (cross), whiskers' upper and lower bounds (vertical lines) and outliers (point) are values that fall outside of the adjacent value region. Different letters, positioned at the median of each box plot, indicate that populations are significant for $p < 0.05$ (Kruskal-Wallis test, Dunn multiple comparison test).

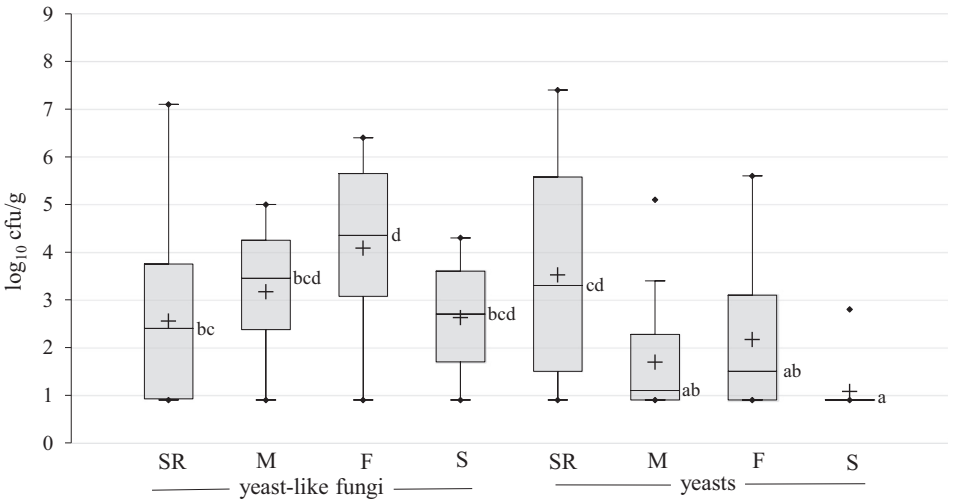

Fig. 2. Box plot of concentration (log_{10} CFU/g) of epiphytic yeast-like fungi and yeasts on different categories of damaged berries (SR, sour rotten; M, partially or totally covered with mycelium; F with visible fractures or cracks; S, largely shrivelled or mummy-like). The lower and upper limits of the box as the first and third quartiles respectively, median (central horizontal bar), mean (cross), whiskers' upper and lower bounds (vertical lines) and outliers (point) as values that fall outside of the adjacent value region. Different letters, positioned at the median of each box plot, indicate that populations are significant for $p < 0.05$ (Kruskal-Wallis test, Dunn multiple comparison test).

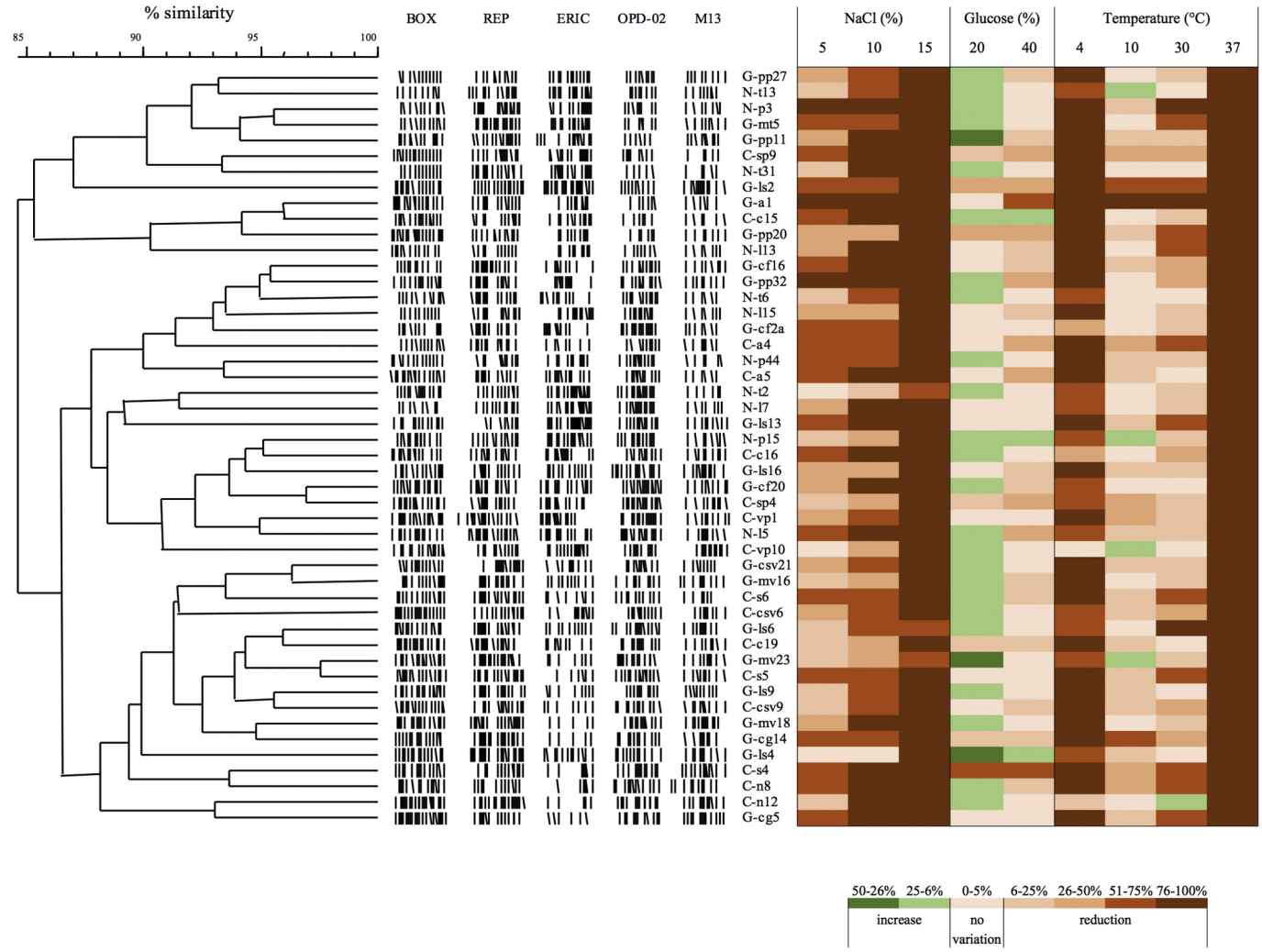

Fig. 3. Genotypic and physiological characterization of 48 *Auerobasidium pullulans* isolates from withered grapes. Dendrogram obtained analyzing RAPD-PCR profiles using M13 and OPD-02 primers, and ERIC-, REP-, and BOX-PCR profiles. Growth ability on MEA at different temperatures and NaCl or glucose concentrations.

similarity level of 84%. Moreover, the biggest cluster included two clusters at 87% of similarity level (Fig. 3). No clear correlation between genotypes of isolates and their isolation source was found since the principal clusters contained strains isolated from the three grape varieties.

3.3. Identification of yeasts

A total of 35 taxa (24 ascomycota and 11 basidiomycota) were identified by the analysis of 266 yeasts (Table 1). The delimitation of species based on LSU and ITS sequence analyzed on gene sequence

Table 1
Species attribution and frequency (percentage in brackets) of 266 yeasts isolated from sound (S) and damaged (D) withered grape berries of Nosiola, Corvina and Garganega variety.

Species	Nosiola		Corvina		Garganega		S	D
	S	D	S	D	S	D		
Candida apicola	0	0	1 (1.9)	2 (4.2)	0	0	1 (0.9)	2 (1.3)
Candida californica	0	0	2 (3.8)	3 (6.3)	1 (3.1)	3 (7.5)	3 (2.6)	6 (4.0)
Candida oleophila	0	0	1 (1.9)	0	0	0	1 (0.9)	0
Candida railenensis	0	0	0	1 (2.1)	0	0	0	1 (0.7)
Candida sorbosivorans	1 (3.2)	0	0	0	0	0	1 (0.9)	0
Candida bentonensis	0	0	1 (1.9)	0	0	0	1 (0.9)	0
Citeromyces matritensis	1 (3.2)	0	0	0	0	0	1 (0.9)	0
Hanseniaspora osmophila	2 (6.5)	0	0	0	4 (12.5)	1 (2.5)	6 (5.2)	1 (0.7)
Hanseniaspora uvarum	6 (19.4)	19 (30.6)	5 (9.4)	7 (14.6)	3 (9.4)	6 (15.0)	14 (12.1)	32 (21.3)
Hanseniaspora vinae	3 (9.7)	0	2 (3.8)	0	0	3 (7.5)	5 (4.3)	3 (2.0)
Issatchenkia terricola	0	0	0	3 (6.3)	0	0	0	3 (2.0)
Kregervanrija fluxum	0	0	0	1 (2.1)	0	0	0	1 (0.7)
Lachancea lanzarotiensis	0	0	0	0	2 (6.3)	1 (2.5)	2 (1.7)	1 (0.7)
Metschnikowia aduanensis/zizyphicola	2 (6.5)	6 (9.7)	7 (13.2)	1 (2.1)	5 (15.6)	6 (15.0)	14 (12.1)	13 (8.7)
Metschnikowia sinensis/shanxiensis	5 (16.1)	5 (8.1)	5 (9.4)	4 (8.3)	2 (6.3)	3 (7.5)	12 (10.3)	12 (8.0)
Pichia membranifaciens	2 (6.5)	1 (1.6)	7 (13.2)	5 (10.4)	0	0	9 (7.8)	6 (4.0)
Saccharomyces bayanus	0	0	3 (5.7)	0	0	0	3 (2.6)	0
Saccharomyces cerevisiae	0	1 (1.6)	0	0	0	0	0	1 (0.7)
Starmerella bacillaris	3 (9.7)	18 (29.0)	7 (13.2)	11 (22.9)	4 (12.5)	4 (10.0)	14 (12.1)	33 (22.0)
Torulaspora delbruekii	0	0	0	0	0	1 (2.5)	0	1 (0.7)
Zygoascus meyerae	0	0	0	2 (4.2)	0	0	0	2 (1.3)
Zygosaccharomyces bailii	0	3 (4.8)	0	2 (4.2)	2 (6.3)	2 (5.0)	2 (1.9)	7 (4.7)
Zygosaccharomyces bisporus	0	0	1 (1.9)	0	0	0	1 (1.7)	0
Zygosaccharomyces rouxii	1 (3.2)	0	0	0	0	0	1 (1.7)	0
Total ascomycetes	26 (83.9)	53 (85.5)	42 (79.2)	42 (87.5)	23 (71.9)	31 (75.0)	91 (78.4)	125 (83.3)
Curvibasidium pallidicorallinum	0	1 (1.6)	0	0	0	0	0	1 (0.7)
Filobasidium magnum	2 (6.5)	1 (1.6)	4 (7.5)	4 (8.3)	3 (9.4)	1 (2.5)	9 (7.8)	6 (4.0)
Hannaella luteola	0	0	0	0	1 (3.1)	0	1 (0.9)	0
Naganishia onofrii	0	1 (1.6)	0	0	0	0	0	1 (0.7)
Papiliotrema terrestris	0	0	2 (3.8)	1 (2.1)	0	0	2 (1.7)	1 (0.7)
Rhodosporidiobolus odoratus	0	1 (1.6)	0	0	0	0	0	1 (0.7)
Rhodotorula graminis	0	2 (3.2)	5 (9.4)	1 (2.1)	2 (6.3)	6 (15.0)	7 (6.0)	9 (6.0)
Rhodotorula mucillaginosa	0	3 (4.8)	0	0	0	0	0	3 (2.0)
Sporobolomyces pararoseus	0	0	0	0	2 (6.3)	1 (2.5)	2 (1.7)	1 (0.7)
Sporidiobolus metaroseus	0	0	0	0	1 (3.1)	2 (5.0)	1 (0.9)	2 (1.3)
Vishniacozyma carnescens	3 (9.7)	0	0	0	0	0	3 (2.6)	
Total basidiomycetes	5 (16.1)	9 (14.5)	11 (20.8)	6 (12.5)	9 (28.1)	10 (25.0)	25 (21.6)	25 (16.7)
Total isolates	**31 (100)**	**62 (100)**	**53 (100)**	**48 (100)**	**32 (100)**	**40 (100)**	**116 (100)**	**150 (100)**

databases and by phylogenetical approach was clear for most of the isolates. In particular, LSU gene sequence was informative to identify all species, except to *Curvibasidium pallidocorallinum*, *Filobasidium magnum*, *Naganishia onofrii* and *Rhodotorula graminis*. The resolution of these last species was possible through phylogenetical analysis of ITS sequence (Fig. 4A–D). In fact, the identification of *Cu. pallidicorallinum* by comparative analysis of LSU and ITS showed high similarity to *Cu. cygneicollum*, *Cu. pallidocorallinum*, *R. nothofagi* and *Curvibasidium* sp.. The LSU gene sequence of *Filobasidium* sp. isolates displayed high similarity in GenBank and UNITE databases to *F. magnum*, *F. floriforme*, *F. oeirense*, *F. elegans* and *Filobasidium* sp., while ITS gene sequence showed high similarity to *F. magnum*, *Filobasidium* sp. and many sequences of uncultured fungus. The BLAST comparative analysis had not been informative for the identification of isolates N-Y6 since gene sequences showed high similarity (GenBank and UNITE databases) to *N. globose*, *N. friedmannii*, *Cryptococcus saitoi*, *Cryptococcus* sp., *N. vaughanmartiniae* and *N. onofrii* for LSU, and *N. onofrii*, *Cryptococcus* sp., *N. friedmannii*, *N. globose* and Tremellomycetes for ITS. All isolates were ambiguously classified as *Rhodotorula graminis*, *Rhodosporidiobolus babjevae*, *Rhodotorula glutinis* or *Rhodotorula* sp. by BLASTn tool in GenBank and UNITE databases for LSU and ITS gene sequence analysis.

Phylogenetical analysis did not allow a reliable resolution at species level of several isolates, most of them belonging to *Metschnikowia pulcherrima* clade. These yeasts were distinguished in *M. aduanensis/zizyphicola* and *M. sinensis/shanxiensis* based on tests of fermentation of D-glucose and assimilation of citrate and D-xylose (Table S1).

Hanseniaspora uvarum, *S. bacillaris*, *M. aduanensis/zizyphicola* and *M. sinensis/shanxiensis* were the most frequent species. These yeasts, including *F. magnum* found at lower frequency, were recovered from both sound and damaged berries of the three grape varieties. Interestingly, most of species (24 out of 35) were found only in one grape variety (nine in Nosiola, 10 in Corvina and five in Garganega) (Table 1).

The rarefaction curves obtained by plotting species found on each variety or sound and damaged berries against the isolates did not converge and only the slope of curve of Garganega tended to flatten out with the increase in sample size (Fig. S3). The species diversity was not particular different among the three varieties as well as between all sound and damaged berries (Table S2). Shannon index, that is more sensitive to evenness than Simpson index, calculated among sound and damaged berries of each variety, ranged from 1.95 (damaged Nosiola berries) to 2.50 (sound Corvina berries).

4. Discussion

The sanitary state of withered grapes seems to be relevant in the distribution of epiphytic populations of yeast-like fungi and yeasts among berries, as revealed by enumeration data. The increase in yeast populations associated with damaged berries of fresh grapes has been well documented (Barata et al., 2008; Barata et al., 2012a). The wide concentration range of cells observed among damaged withered berries is probably linked to the type and level of disease. The massive presence of yeasts observed in most withered berries affected by sour rot disease

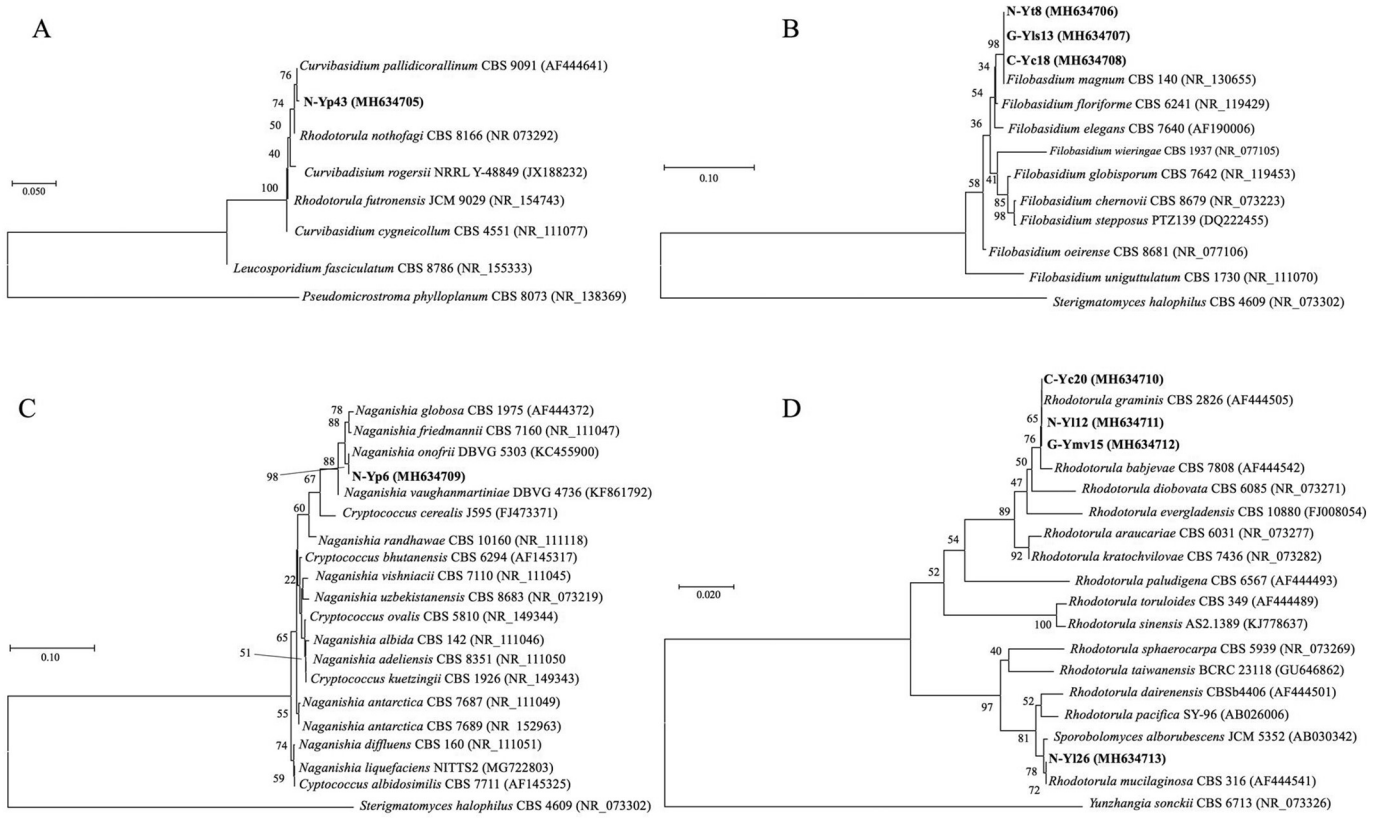

Fig. 4. The neighbor joining (NJ) trees inferred from the dataset containing the sequences of ITS gene sequences of basidiomycetous yeasts. The numbers labelled at each node indicate the bootstrap (BS) percentage (N = 1000). The GenBank accession numbers were reported in the brackets. Branch lengths are proportional to the numbers of nucleotide substitutions and are measured by the scale bars of sequence divergence. A) NJ tree of six taxa of *Curvibasidium* genus and the isolate N-Yp43 (in bold) from withered grapes; *Pseudomicrostroma phylloplanum* CBS 8073 was used as outgroup. Bar, 0.05 substitutions per nucleotide position. B) NJ tree of nine taxa of *Filobasidium* genus and the isolates N-Yt8, G-Yls13 and C-Yc18 (in bold) from withered grape; *Sterigmatomyces halophilus* CBS 4609 was used as outgroup. Bar, 0.1 substitutions per nucleotide position. C) NJ tree of 18 taxa of *Naganisha* genus and the isolate N-Yp6 (in bold) from withered grapes; *Sterigmatomyces halophilus* CBS 4609 was used as outgroup. Bar, 0.1 substitutions per nucleotide position. D) NJ tree of 15 taxa of *Rhodotorula* genus and the isolates C-Yc20, N-Yl2, G-Ymv15 and N-Yl26 (in bold) from withered grapes; *Yunzhangia sonckii* CBS 6713 was used as outgroup. Bar, 0.02 substitutions per nucleotide position.

is in accordance with Barata et al. (2008). Nevertheless, low yeast concentrations recorded in several damaged berries (in particular those partially or totally shrivelled berries) may be associated with altered properties of berry skin due to the high rate of water loss (Rolle et al., 2011). The skin of the latter berries may be a more adverse habitat for yeasts than that of sound berries. Their higher frequency on Nosiola and Garganega (data not published) may be due to longer withering with respect to that of Corvina. Despite these mummy-like berries harbor smaller populations, they can be a reservoir of yeasts and contribute to the colonization of withered grapes over years as supposed for on-vine mummified grapes in the Tokaj wine region (Sipiczki, 2016). This preliminary information on the distribution of yeast-like fungi and yeasts populations among the different categories of damaged berries suggests the importance of carrying out more in-depth investigations.

The sole presence of *Aureobasidium pullulans* among all yeast-like fungal isolates confirms the dominance of this species on withered grapes (Alessandria et al., 2013; Lorenzini et al., 2016; Lorenzini et al., 2018; Rantsiou et al., 2013). This yeast-like fungus is also predominant in fresh grapes (Rathnayake et al., 2018). The adaptive ability of *A. pullulans* in osmotically very stressed environments (Humphries et al., 2017) may explain high frequency of this yeast-like fungus in withered grapes. Interestingly, the presence of this fungus in both sound and damaged berries at similar average concentrations contrast with the observation in fresh grapes (Barata et al., 2012a; Renouf et al., 2005).

Physiological and in-vitro inhibition assays showed that the *A. pullulans* population from withered grapes was characterized by substantial strain variability. On the other hand, all strains displayed high genotypic similarity confirming our previous study carried out on few strains isolated from the same source (Lorenzini and Zapparoli, 2015). It has been demonstrated that phenotypic heterogeneity among genetically uniform population of fungi could be the major determinant of their survival in adverse conditions (Hewitt et al., 2016). It can be argued that the phenotypic variability of the *A. pullulans* population may reflect the adaptive response of this species to the changing environment such as the grape carphoshere during withering (Mencarelli and Tonutti, 2013).

Based on the result of the preliminary assay of antagonistic activity against the most common phytopathogenic fungi of withered grapes, it appears plausible that *A. pullulans* may have a potential role on affecting their colonization. The ability of this species to inhibit the growth of *Botrytis*, *Aspergillus* and *Penicillium* is well documented (Tsitsigiannis et al., 2012). The low frequency of strains with significant inhibitory effects on *B. cinerea*, the most detrimental fungus to withered grapes, indicates that wild yeast-like fungi can be scarcely effective against this pathogen. Conversely, the high effectiveness of most strains against *C. halotolerans* and *A. alternata* may be consistent with the lower incidence of their diseases than gray rot in grapes occurring during withering (data not published). Nevertheless, in-depth investigations are necessary to understand the effective role of indigenous *A. pullulans* strains in the occurrence of these pathogenic fungi in withered grapes.

The identification of 35 yeast species among 266 isolates indicates that the yeast community associated with withered grapes is characterized by high heterogeneity. This level of species diversity was similar or higher than that found in fresh grapes by analyzing culturable

epiphytic yeasts (Garofalo et al., 2016; Nemcová et al., 2015). The rarefaction curves indicate that further species could be recovered, especially on Nosiola and Corvina, through greater sampling and the use of selective/differential media for the isolation of minority or rare species on grapes. The use of general purpose culture media, like GYP, enables the recovery of the most frequent and faster growing species (Barata et al., 2012a). The isolation of *Zygosaccharomyces* and *Zygoascus* species on GYP suggests that their occurrence on withered grapes may be more frequent if selective and/or enrichment media (e.g., *Zygosaccharomyces* differential medium) are used (Barata et al., 2008).

Most of the species identified in this study were previously found in epiphytic populations from grapevines (leaves and grapes) (Barata et al., 2012a). *Hanseniaspora uvarum*, *C. californica*, *I. terricola*, *S. cerevisiae* and *S. bacillaris* were also recovered from off-vine withered grapes (Rantsiou et al., 2013). To the best of our knowledge, *N. onofrii* (formerly *Cryptococcus onofrii*) and *Rh. odoratus* (formerly *Sporobolomyces odoratus*) were identified for the first time among yeasts isolated from grapes, must or wine. The isolation of the former could be linked to its psychrophilic nature (Turchetti et al., 2015), as in the last phase of withering the temperatures in fruit-drying rooms are generally quite low (6–12 °C). *Rhodosporidiobolus odoratus* was previously isolated from the phylloplane of different trees (maple, lemon, plane and corn) (Valério et al., 2002). Therefore, its recovery on grapes may be consistent with its ability to inhabit the tree phyllosphere.

Based on the balance between ascomycota and basidiomycota, the yeast community of withered grapes does not substantially seem to differ from that inhabiting mature grapes analyzed in other wine-making areas (Garofalo et al., 2016; Li et al., 2010; Nemcová et al., 2015). Oxidative and fermentative ascomycetous species are mostly prevalent on the carposhere of ripe berries, while oxidative basidiomycetous species are prevalent on the phylloshere (Barata et al., 2012a). As regards the sanitary state of grapes, data on species diversity are not fully in line with previous investigations (Barata et al., 2012a), that reported wider species diversity on damaged grapes than sound grapes. It is possible that the recovery of minority species on damaged withered berries could be prevented due to the predominance of copiotrophyc ascomycetous yeasts (e.g. *Hanseniaspora*, *Starmerella* and *Metschnikowina*). Moreover, their colonization on damaged berries, at the expense of oligotrophic species, may be promoted by the exudation and leakage of berry content (sugars, acids and salts), increased by the post-harvest dehydration process (Mencarelli and Tonutti, 2013).

In conclusion, this study provided a comprehensive view of culturable yeast-like fungi and yeast communities on the carposphere of withered grapes for passito wine production. Analysis of single berries of three grape varieties, distinguished between sound and damaged, showed that withered grapes are a very heterogeneous habitat for these fungi. It has been possible to speculate on the occurrence of *A. pullulans* and its intra-specific phenotypic variability. It is reasonable to consider that the prevalence of this fungus over the yeasts can be a characteristic of the mycobiota associated with the withered grapes. Conversely, species recovery has not revealed strong evidence that the epiphytic yeast community of withered grapes is distinct and specific compared with that of other grape ecosystems. Moreover, the correlation between species composition and grape variety does not seem to be well supported. Further investigations are necessary to corroborate or to deny these assumptions. Culture-independent methods will be useful to give a broad picture of the microbiome of withered grapes. Nevertheless, these tools could limit the knowledge of yeast communities at a genus level, as they are not always reliable at species level.

Conflict of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2018.10.023.

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