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PERSPECT

Expectable diversity patterns in wine yeast communities

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One sentence summary: Wine fermentations harbor a higher-than-expected yeast diversity that deserves to be explored.
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Abstract

Wine fermentations are dominated by *Saccharomyces* yeast. However, dozens of non-*Saccharomyces* yeast genera can be found in grape musts and in the early and intermediate stages of wine fermentation, where they co-exist with *S. cerevisiae*. The diversity of non-*Saccharomyces* species is determinant for the sensorial attributes of the resulting wines, both directly (by producing aroma impact compounds) and indirectly (modulating the performance of *Saccharomyces*). Many research groups worldwide are exploring the great diversity of wine yeasts to exploit their metabolic potential to improve wine flavor or to prevent wine spoilage. In this work, we share a new data set from a wide ITS amplicon survey of 272 wine samples, and we perform a preliminary exploration to build a catalogue of 242 fungal and yeast genera detectable in wine samples, estimating global figures of their prevalence and relative abundance patterns across wine samples. Thus, our mycobiome survey provides a broad measure of the yeast diversity potentially found in wine fermentations; we hope that the wine yeast research community finds it useful, and we also want to encourage further discussion on the advantages and limitations that meta-taxonomic studies may have in wine research and industry.

Keywords: Wine yeasts, *Saccharomyces*, non-*Saccharomyces* yeasts, Mycobiome survey, Yeast diversity patterns

Wine fermentations harbor a higher-than-expected yeast diversity that deserves to be explored

Fermented foods are becoming more and more popular as system models for research studies in ecology and evolution (Wolfe and Dutton 2015). In particular, wine fermentations host several dozens of yeast species, mainly coming from grapes where the abundance of non-*Saccharomyces* yeasts exceeds largely that of *Saccharomyces cerevisiae*. Initially, the low pH (between 3.2–4) and the osmolarity derived from the high concentration of sugars (200-250 g/L of glucose and fructose, in 1:1 proportion), determine the structure of yeast populations found in grape musts. As the fermentation process develops, it is the increasing concentration of ethanol released by fermentative yeasts which determines the population succession -characterized by a progressive reduction in yeast diversity-, in most cases, giving rise to the dominance of *S. cerevisiae*. However, some other inter-species interaction mechanisms can also shape the population dynamics and *S. cerevisiae* fermentation performance, such as competition for nitrogen nutrients, production of killer toxins or *quorum*-sensing mediated processes, among others (Mencher et al. 2021).

Biogeography determines the vineyard microbiome (Gobbi et al. 2022), but also the microbial populations established in the grapes' surface (Bokulich et al. 2014, Knight et al. 2015) which contribute to the flavor of wines produced in a particular region (Bokulich et al. 2016), with a special relevance of the vineyard and grape must fungal populations (Liu et al. 2020). These regional patterns exist at different spatial scales, and, at the local scale, apart from the contribution of the surrounding native ecosystems (Morrison-Whittle and Goddard 2018) and the role of physical forces and animal vectors (insects and birds) in yeast dispersal (Liu et al. 2019), the vineyard-winery cross connection is especially important in shaping the assembly of fermentative yeast populations. Several authors suggest that there is a progressive enrichment in those strains that are more adapted to the local environment and the viti-vinicultural practices applied in the vineyard and the winery, which, sometimes, end up establishing and dominating the local resident microbiota (Beltran et al. 2002, de Celis et al. 2019). In this context, there is a strong phylogenetic signal correlating the phylogenetic and the phenotypic distance between wine yeasts (unpublished data). Thus, understanding the diversity of yeast species potentially found in wine fermentations is of great interest to infer the range of expectable phenotypes across wine yeast communities.

The main aim of this brief perspective piece is to provide some reference values for the expectable diversity of yeasts to be found in wine samples. To do that, here we release and explore a wide dataset of wine fungal communities, analyzed through ITS-amplicon sequencing (raw sequences -fastq files- available at NCBI Bioproject: PRJNA814622). This dataset is composed of 272 wine samples collected at different stages of the winemaking process (45 from grape must; 144 from alcoholic fermentation and 83 from post-fermentative stages), in wineries from different countries (mainly Spain, USA and France, but also Italy, Denmark and Georgia) (metadata for the samples available in Table S1). Briefly, DNA was extracted from wine samples using the Dneasy Powerlyzer Powersoil Kit (Qiagen). Libraries were prepared following the two-step PCR protocol from Illumina and sequenced on an Illumina MiSeq using paired-end sequencing (2 × 300 bp). Libraries were prepared for the ITS1 region using custom primers (patent

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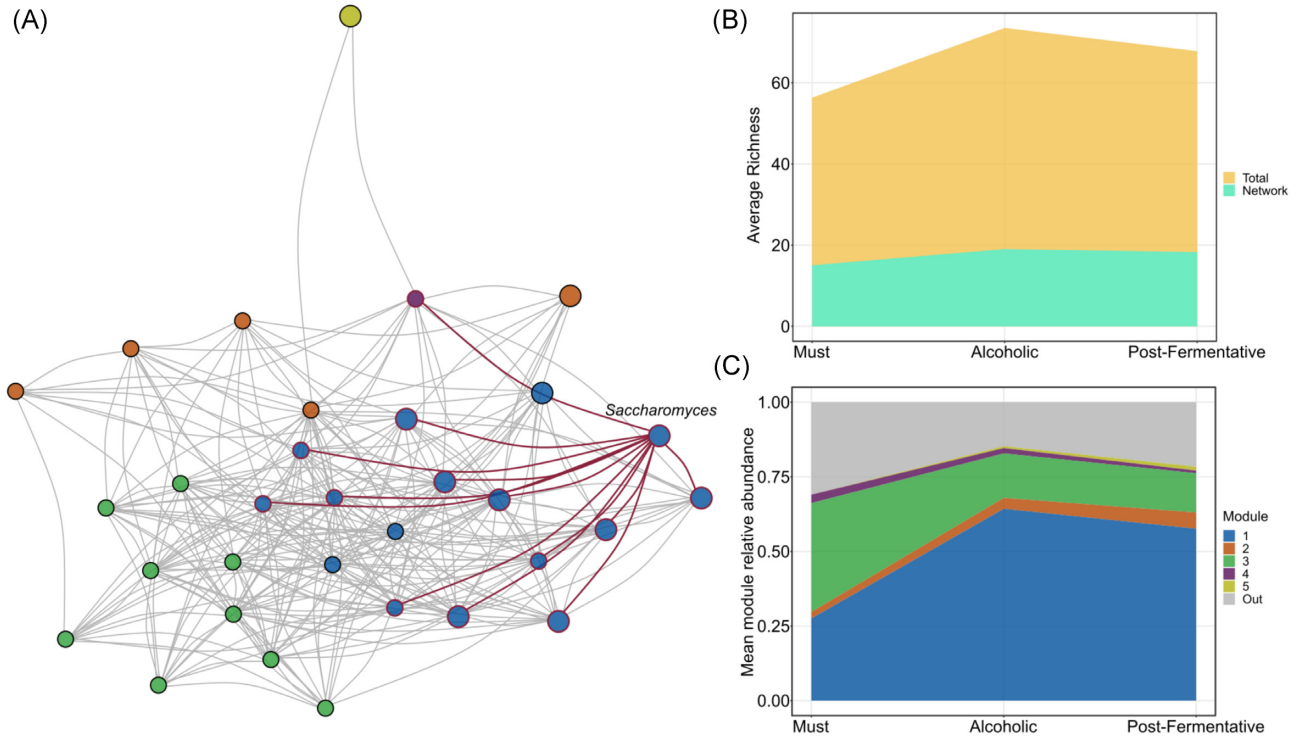

Figure 1. Structural patterns of yeast communities found across wine samples. **(A)** Co-occurrence network of the prevalent fraction of fungal genera found in wine samples (genera with a relative abundance higher than 0.05% in, at least, 5% of samples from alcoholic fermentation are shown) divided in modules according to their co-occurrence patterns. Significant associations with *Saccharomyces* are highlighted with red edges connecting nodes surrounded by a red ring. The node corresponding to *Saccharomyces* is labeled in the network, but the taxonomic diversity found across modules are detailed in Fig. 2. **(B)** Diversity (richness) of fungal genera identified at different stages of wine fermentation. The total diversity of genera identified is shown in dark yellow and the prevalent fraction of the diversity highlighted in the network is shown in turquoise blue. **(C)** Relative abundance of the total diversity of fungal genera detected at different stages of wine fermentation highlighting the abundance patterns of the modules defined in the network.

publication number: WO2017096385, Biome Makers). Then, the sequence analysis was performed using DADA2 algorithm (Callahan et al. 2016) implemented in R pipeline, including primer trimming, denoise, quality filter and chimera removal. A total of 33 583 710 good quality reads, corresponding to 14 233 Amplicon Sequence Variants (ASVs), were obtained, and their taxonomic assignment was performed using the naïve Bayesian classifier implemented in DADA2 using UNITE database (Nilsson et al. 2019) and a bootstrap cut-off of 80%.

There are several research studies exploring the diversity of yeasts found in grapes and grape musts in different geographical locations (Drumonde-Neves et al. 2017, Castrillo et al. 2019), however, there is very little information about the diversity patterns of fungal and yeast communities during the alcoholic fermentation at large spatial scales. A beta-diversity analysis of our subset of samples collected during the alcoholic fermentation showed that the country of origin has a significant effect on the variance between samples ($R^2 = 0.08$, P-value $= 0.001$). At the country scale (considering the 91 alcoholic fermentation samples analyzed from Spain), the province ($R^2 = 0.12$, P-value $= 0.001$), and the winery ($R^2 = 0.28$, P-value $= 0.001$) from where samples were collected have a significant impact in the beta-diversity patterns of wine fungal populations, while the grape variety did not show a significant effect ($R^2 = 0.03$, P-value $= 0.085$). These figures are similar to those found by some of us (Gobbi et al. 2022) in a global survey of the vineyard soil microbiota, where we also found an increasing importance of geographical distance in defining the similarity of fungal communities at lower scales (from intercontinental and country-level to province-level comparisons). The greater effect of the winery in defining the beta-diversity patterns of wine fermentations, highlights the importance of studying the filtering and selection processes happening from the vineyard to the winery-resident microbiota, which will be the one directly impacting in wine flavour.

A 70.33% of the 14233 ASVs detected were taxonomically assigned at the genus level, including a total of 896 genera, belonging to 330 families, and 14 phyla. Filtering out those genera with a prevalence, in the alcoholic fermentation stages, lower than 5% of the samples analyzed, we obtained a list of 242 genera whose population prevalence and abundance patterns (minimum, maximum and average) are detailed in the Supplementary Table S2. Among these 242 fungal genera, 42 yeast genera (indicated in Supplementary Table S2) have been already isolated from wine environments (Drumonde-Neves et al. 2021). At this point, we should highlight the surprising result of not detecting any ASV assigned to the *Metschnikowia* genus. *Metschnikowia* species are widespread in wine samples, and they have an active role in wine fermentations (Vicente et al. 2020). Thus, we should assume this as a weakness of our work, but we also draw attention to the problem for the correct detection and quantification of species from this genus through amplicon sequencing techniques. In fact, some previous works highlighted the problems associated with the taxonomic assignment of *Metschnikowia* species by metabarcoding (Sipiczki 2022) and their abundance estimation (Sternes et al. 2017), although in the case of Sternes et al., and contrary to what we observed here, they had a significant overabun-

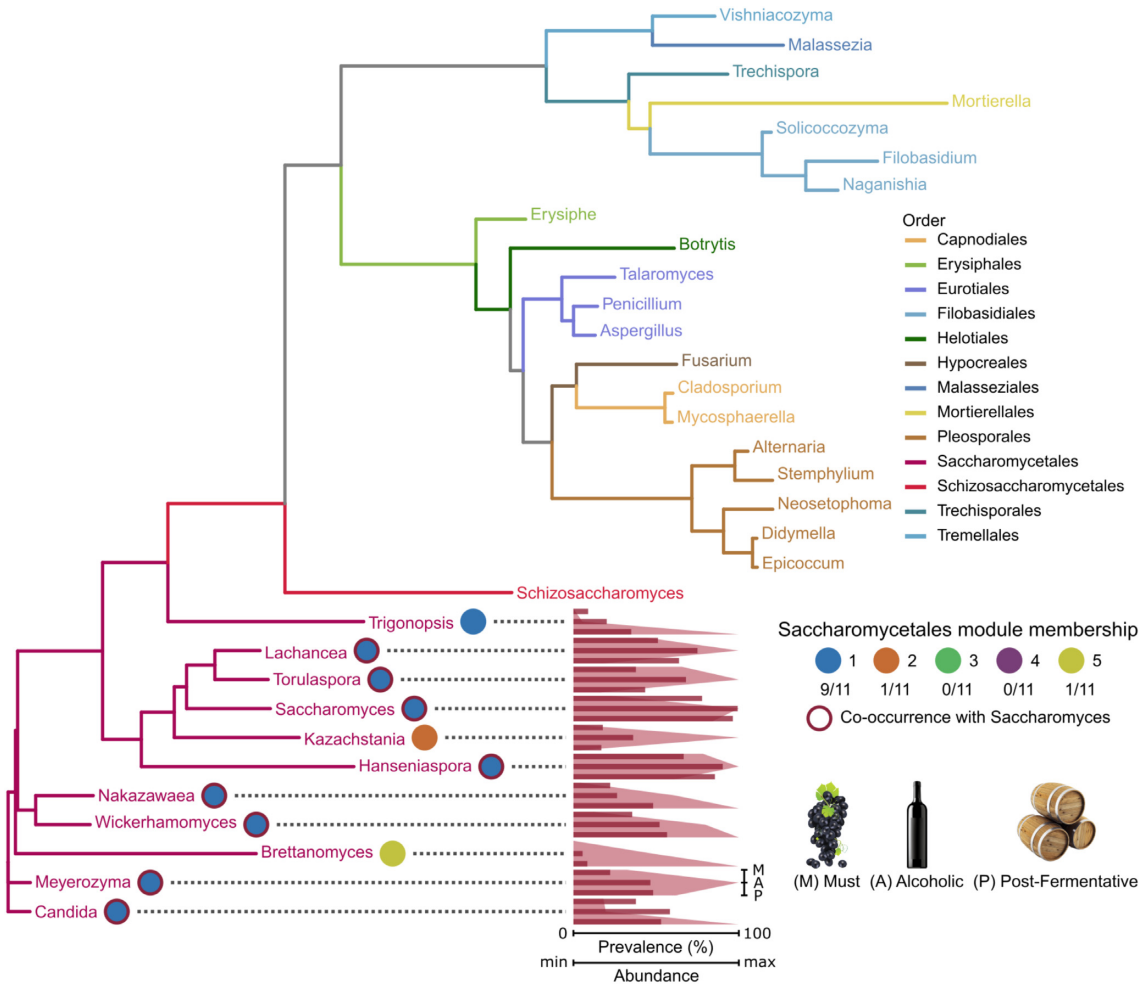

Figure 2. Dendrogram based on ITS sequences of the prevalent fraction of fungal and yeast diversity found across wine samples. The longer ITS sequences used to build the dendrogram, selected by BLAST searching with the dominant ASV detected for each genus, are referenced (genbank accession codes) in Supplementary Table S4. The colour of the branches indicate the order to which the detected genera belong. The colour of the circles at the tip of the *Saccharomycetales* branches indicates the module of the network presented in Fig. 1A to which the detected genera belong, and those circles surrounded by a red ring indicate those genera with significant co-occurrence with *Saccharomyces*. Finally, the prevalence (bars) and relative abundance (curve)—normalized to the minimum–maximum of each species patterns across wine fermentation stages (Must-M, Alcoholic fermentation-A, and Post-fermentative stages-P) of *Saccharomycetales* yeast genera are presented in maroon color (detailed numerical data available in Table S2). The diversity of yeast species detected among these *Saccharomycetales* genera, and their prevalence and relative abundance patterns across wine fermentation stages are reported in Table S3. It should be noted that the genus *Metschnikowia* is not included as part of this work, as the ITS amplicon sequencing and bioinformatics approaches used in here failed to detect it.

dance bias in the ITS phylotyping estimations for *Metschnikowia* yeasts.

Figure 1A shows the structure of wine yeast populations in a co-occurrence network composed by the 32 most widespread yeast genera (those with a relative abundance higher than 0.05% in, at least, 5% of samples from alcoholic fermentation stage). These 32 genera, which represent a minor fraction of the total diversity (richness) of yeast genera found across winemaking stages (Fig. 1B), are, however, the dominant taxa in terms of relative abundance, accounting, on average, for about an 80% of the total fungal populations during the alcoholic fermentation (Fig. 1C). In particular, the module 1 (blue) in the network appears as the most important fraction of this diversity which increases dramatically its relative abundance during the alcoholic fermentation stages, and it is composed by *Saccharomyces* and most of *Saccharomyces*-correlating phylotypes. As expected, the module 1 includes the great majority of fermentative yeast genera, encompassing most *Saccharomycetales* yeasts found (Fig. 2). The population prevalence and the mean abundance patterns of the *Saccharomycetales* yeasts detected as part of the conserved fraction of fungal taxonomic diversity found in the studied wine samples are summarized in Fig. 2. During the alcoholic fermentation, the highest intra-genus diversity was found in *Hanseniaspora* (80 ASVs detected and 9 species identified), *Saccharomyces* (77 ASVs detected and 6 species identified) and *Candida* (72 ASVs detected and 20 species identified). Thus, the prevalence and relative abundance trends showed in Fig. 2 should be checked with detailed data showed in Supplementary Table 3 (data at species level for *Saccharomycetales* yeasts), especially for those genera with a high intra-genus diversity, such as *Candida*, where we can find some species only found during the alcoholic fermentation (such as *C. stellata*) and some others mainly found in post-fermentative stages (such as *C. tropicalis* or *C. railenensis*).

Sixteen out of the 32 genera found as widespread in our work (Figs 1 and 2) has been already isolated from wine environments (listed by prevalence in alcoholic fermentation sam-

ples): *Saccharomyces, Hanseniaspora, Solicoccozyma, Lachancea, Filobasidium, Naganishia, Torulaspora, Candida, Vishniacozyma, Wickerhamomyces Meyerozyma, Kazachstania, Schizosaccharomyces, Nakazawaea, Trigonopsis, Brettanomyces*. Figure 2 suggest the existence of different environmental affinities among *Saccharomycetales* yeasts; some closely related genera show their maximum relative abundance patterns during the alcoholic fermentation stages (mainly, the cluster formed by *Saccharomyces, Lachancea, Torulaspora, Hanseniaspora,* and *Kazachstania*), and some others (mainly, *Trigonopsis, Brettanomyces* and *Candida*) show their maximum relative abundance patterns at post-fermentative stages. At this point, it is interesting to mention that in the three post-fermentative samples where we did not detect *Saccharomyces* (3 out of 81; all coming from the same winery), the populations were dominated by *Kregervanrija* (a low fermenting yeast species pertaining to the *Pichiaceae* family; *Saccharomycetales* order). A *Kregervanrija* species (*K. fluxuum;* syn. *Candida vini*) has been previously isolated from wines (Drumonde-Neves et al. 2021), so its potential impact in wine quality during post-fermentative stages should be further studied.

The role in wine fermentations of several species from these genera is well documented (reviewed in Jolly et al. 2014 and Belda et al. 2017), but there is little to no information about the potential role of some others (e.g. *Solicoccozyma, Filobasidium, Vishniacozyma, Trigonopsis*), even though some of them have been found as part of the resident microbiota established in the surfaces and machinery of a winery (Abdo et al. 2020). Some previous works report how meta-taxonomics coupled by classic isolation procedures can help us to characterize the still under-explored spectrum of yeasts potentially found in wine samples (Ruiz et al. 2019). Although the impact of some rare and minority species in wine fermentation can be seemingly negligible, we argue that they should be isolated and characterized (fermentation capacity, environmental preferences, interaction with *S. cerevisiae*), to expand our knowledge on the importance of the biotic factors (i.e. inter-species interactions) in the fermentation performance of complex wine microbial communities and to anticipate potential kinetics problems or sensorial deviations in wine fermentations. In addition, in a current scenario where some chemical aspects of grape musts are changing as a consequence of climate change (pH, balance of organic acids, sugar concentration) (Mira de Orduña, 2010), added to the irruption of some trends in winemaking (lower doses of SO_2, prefermentative cold soak, low temperature fermentations, back to spontaneous fermentations, etc), we must be attentive to how some of these parameters can benefit and promote the growth of yeast species that, until now, have been very rare, and how this can change or hinder the dominance patterns of *Saccharomyces* in wine, which until now was taken for granted.

As mentioned before, our main objective here is to share a useful resource for all of us working in wine microbiology, by releasing an extensive data set of wine fungal communities and providing reference figures of population prevalence and abundance for most yeast genera and species potentially found in wines. Although the results provided in this work should be interpreted with the caution that any meta-taxonomic survey should arouse with regard to the viability of the species detected, we hope that our work will be of interest for those studying the ecology of wine fermentations and for those yeast hunters who continue exploring the role of new and rare yeast strains in wine fermentations.

Data and code availability statement

Raw sequences -fastq files- of the 272 wine samples surveyed in this work are available at NCBI Bioproject: PRJNA814622. The script necessary to reproduce the analysis showed in the main figures of this work (Figures 1 and 2) is available in the GitHub repository: https://github.com/Migueldc1/wineyeasts_pers.

Supplementary data

Supplementary data are available at *FEMSYR* online.

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***Conflict of interest statement.*.** Alberto Acedo is co-founder and currently employed by Biome Makers Inc, a company providing microbiome analysis for the agri-food sector. Ignacio Belda was an employee of Biome Makers, but he is now an independent researcher at the Complutense University of Madrid

References

Abdo H, Catacchio CR, Ventura M *et al.* The establishment of a fungal consortium in a new winery. *Sci Rep*. 2020;**10**:7962

Belda I, Ruiz J, Esteban-Fernández A *et al.* Microbial contribution to wine aroma and its intended use for wine quality improvement. *Molecules*. 2017;**22**:189

Beltran G, Torija MJ, Novo M *et al.* Analysis of yeast populations during alcoholic fermentation: a six year follow-up study. *Syst Appl Microbiol*. 2002;**25**:287–93

Bokulich NA, Collins TS, Masarweh C *et al.* Associations among wine grape microbiome, metabolome, and fermentation behavior suggest microbial contribution to regional wine characteristics. *Mbio*. 2016;**7**:e00631–16

Bokulich NA, Thorngate JH, Richardson PM *et al.* Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. *Proc Natl Acad Sci* 2014;**111**:139–48.

Callahan BJ, McMurdie PJ, Rosen MJ *et al.* DADA2: high-resolution sample inference from illumina amplicon data. *Nat Methods*. 2016;**13**:581–3.

Castrillo D, Rabuñal E, Neira N *et al.* Yeast diversity on grapes from galicia, NW spain: biogeographical patterns and the influence of the farming system. *OENO One*. 2019;**53**:573–87.

de Celis M, Ruiz J, Martin-Santamaria M *et al.* Diversity of *saccharomyces cerevisiae* yeasts associated to spontaneous and inoculated fermenting grapes from Spanish vineyards. *Lett Appl Microbiol* 2019;**68**:580–88.

Drumonde-Neves J, Franco-Duarte R, Lima T *et al.* Association between grape yeast communities and the vineyard ecosystems. *PLoS One* 2017;**12**:e0169883.

Gobbi A, Acedo A, Imam N *et al.* A global microbiome survey of vineyard soils highlights the microbial dimension of viticultural terroirs. *Commun Biol* 2022;**5**:1–9.

Drumonde-Neves J, Fernandes T, Lima T *et al.* Learning from 80 years of studies: a comprehensive catalogue of non-*Saccharomyces* yeasts associated with viticulture and winemaking, *FEMS Yeast Res* 2021;**21**:foab017.

Jolly NP, Varela C, Pretorius IS. Not your ordinary yeast: non-*saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Res* 2014;**14**:215–37.

Knight S, Klaere S, Fedrizzi B *et al.* Regional microbial signatures positively correlate with differential wine phenotypes: evidence for a microbial aspect to terroir. *Sci Rep* 2015;**5**:1–10.

Liu D, Chen Q, Zhang P *et al.* The fungal microbiome is an important component of vineyard ecosystems and correlates with regional distinctiveness of wine. *Msphere* 2020;**5**: e00534–20.

Liu D, Zhang P, Chen D *et al.* From the vineyard to the winery: how microbial ecology drives regional distinctiveness of wine. *Front Microbiol* 2019;**10**:2679.

Mencher A, Morales P, Tronchoni J *et al.* Mechanisms involved in interspecific communication between wine yeasts. *Foods* 2021;**10**:1734.

Mira de Orduña R. Climate change associated effects on grape and wine quality and production. *Food Res Int* 2010;**43**:1844–55.

Morrison-Whittle P, Goddard MR. From vineyard to winery: a source map of microbial diversity driving wine fermentation. *Environ Microbiol* 2018;**20**:75–84

Nilsson RH, Larsson KH, Taylor AFS *et al.* 2019. The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. *Nucleic Acids Res* 2019;**47**: 259–64.

Ruiz J, Ortega N, Martín-Santamaría M *et al.* Occurrence and enological properties of two new non-conventional yeasts (*Nakazawaea ishiwadae* and *lodderomyces elongisporus*) in wine fermentations. *Int J Food Microbiol* 2019;**305**:108255.

Sipiczki M. When barcoding fails: genome chimerization (admixing) and reticulation obscure phylogenetic and taxonomic relationships. *Mol Ecol Resour* 2022, In press, **22**:1–24. https://doi.org/10.1111/1755-0998.13586.

Sternes PR, Lee D, Kutyna DR *et al.* A combined meta-barcoding and shotgun metagenomic analysis of spontaneous wine fermentation. *GigaScience* 2017;**6**:gix040.

Vicente J, Ruiz J, Belda I *et al.* The genus *metschnikowia* in enology. *Microorganisms* 2020;**8**:1038.

Wolfe BE, Dutton RJ. Fermented foods as experimentally tractable microbial ecosystems. *Cell* 2015;**161**:49–55.