J Ind Microbiol Biotechnol (2011) 38:1619–1634
DOI 10.1007/s10295-011-1020-x

REVIEW

Detection and identification of microorganisms in wine: a review of molecular techniques

Melissa L. Ivey · Trevor G. Phister

Received: 17 February 2011 / Accepted: 20 July 2011 / Published online: 7 August 2011
© Society for Industrial Microbiology 2011

Abstract The microbial ecology of wine is complex. Microbes can play both positive and negative roles in the quality of the final product. Due to this impact, the microbial ecology of wine has been well studied. Traditional indirect methods, such as plating, have largely been replaced by a number of molecular methods. These methods are typically either indirect methods used for identification of cultured organisms, or direct methods used to profile whole populations or identify specific microbes in a mixed population. These molecular methods offer a number of advantages over traditional methods including speed and precision. This review will examine both direct and indirect molecular methods, provide examples of their impact on the study of the microbial ecology of wine, and also discuss their strengths and limitations.

Keywords Wine · Molecular detection · Spoilage bacteria · Yeast · Microbial identification

Introduction

Molecular methods can be used to detect and identify microbes throughout the winemaking process in one of two ways (Fig. 1). First, if a sample has been analyzed using conventional methods such as plating or enrichment, which require growth of the microbe, molecular methods can be use to identify the organisms present in the sample at the genus or species level and, even to the specific strain. So we could not only tell you that a *lactobacilli* was growing in your wine, which could be determined through traditional plating and biochemical analysis, but we could also tell you that it was a specific strain of *Lactobacillus hilgardii* (this microbe is often misclassified using conventional biochemical identification as *L. brevis*). However, modern molecular methods such as a RAPD-PCR will correctly differentiate the two species [105]. Since the analysis of the microbial population in these cases is not conducted on the original population, these methods are considered to be indirect methods [72].

The second way of using molecular methods is to analyze the microbial population directly from the sample itself. A classic example of direct analysis, which has been used from the vineyard through bottling, would be denaturing gradient gel electrophoresis (DGGE) where DNA is isolated directly from the fermentation or the grape, amplified by PCR and analyzed by gradient gel electrophoresis without ever having to culture the microbes present in the sample (Fig. 2).

In general, direct methods have two major advantages over indirect methods. First, they can identify nonculturable microbes. These nonculturable microbes may be injured, in a viable nonculturable state, or unable to grow on the medium chosen for culturing [50, 71]. For example, a study by du Toit et al. [28] demonstrated a difference between *Acetobacter pasteurianus* populations observed by epifluorescence microscopy and plating. Microscopy, a direct method, demonstrated a higher microbial population than plating in the absence of oxygen, an indirect method. This difference, however, was dispelled by the addition of oxygen to the wine. These results suggest that a nonculturable population may survive in wine, which would not be noticed using conventional analyses. Additionally,

M. L. Ivey · T. G. Phister (✉)
Department of Food, Bioprocessing and Nutrition Sciences, North Carolina State University, 339 Schaub Hall, Campus Box 7624, Raleigh, NC 27695-7624, USA
e-mail: trevor_phister@ncsu.edu

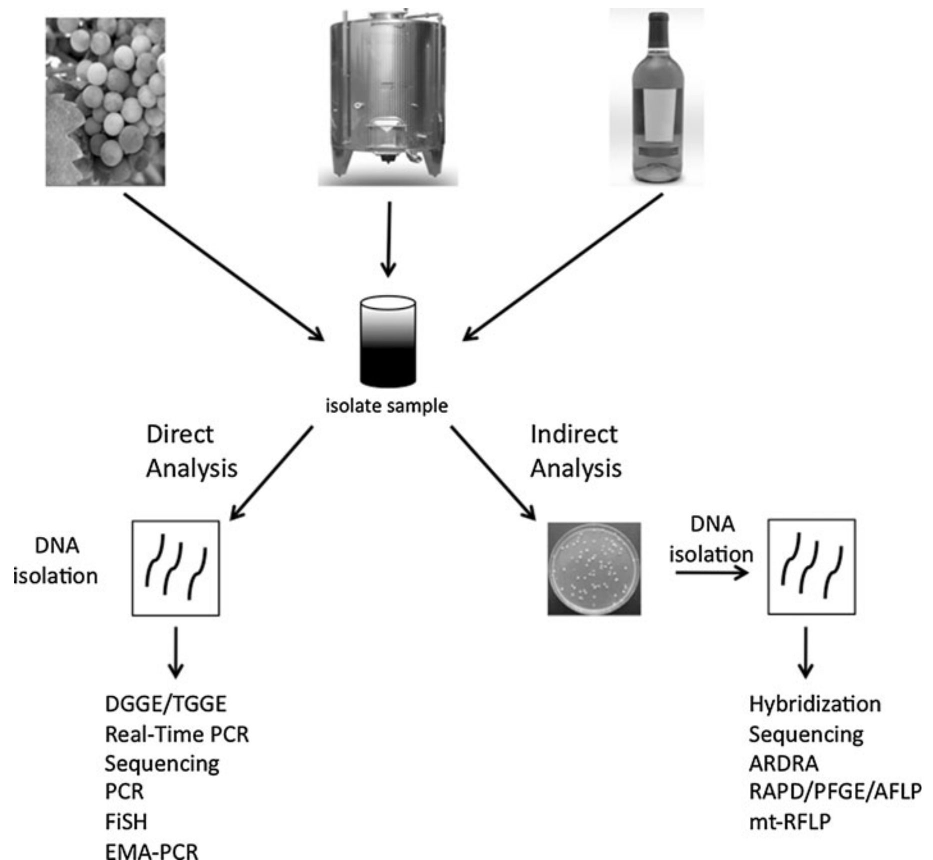

Fig. 1 Direct versus indirect microbial analysis. Molecular methods can be used to analyze samples taken anywhere from the vineyard to the bottle in two different ways. In indirect analysis the sample is plated, the microorganisms are allowed to grow, and then DNA is isolated from the sample and used most often for identification. In direct molecular analysis, either DNA or RNA is isolated directly from the sample and used for further analysis. Indirect analyses are typically more sensitive, being able to identify organisms to the strain level. While direct methods are often faster, they are typically less specific, being able to provide the genus and possibly species-level information for identification. They are however, useful for rapid identification and profiling of communities often being able to detect nonculturable organisms

Fugelsang and Zoecklein [38] found 4-ethyl phenol production by *D. bruxellensis* even in the absence of a culturable population.

The second advantage of direct analysis is speed. For instance, detection of *D. bruxellensis* by plating can take as long as 1–2 weeks to complete. Consequently, simple enological decisions, such as antimicrobial additions, are greatly delayed [89]. However, by using a direct analysis method such as quantitative real-time PCR (QPCR) (Fig. 3), *D. bruxellensis* contamination can be identified in less than 1 day, thus allowing the winemaker to prevent a potential problem [27, 89].

The major disadvantage of direct methods when compared to traditional indirect methods is the inability of many direct methods to differentiate viable from dead cells, as both may contain DNA or RNA. While DNA is very stable and will often persist long after the cell has died, RNA may have a short half-life and, in some cases, may be a useful marker for cell viability. However, this is very dependant on the gene transcribed and the sample environment. In fact, a study by Hierro et al. [47] examined the persistence of both ribosomal RNA and DNA by heating an *S. cerevisiae* culture for 20 min and following the 26S ribosomal DNA and RNA using QPCR. They found that both the rRNA and rDNA persisted for over

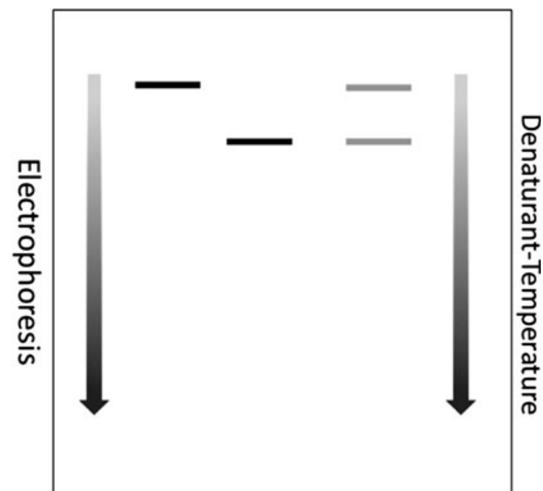

Fig. 2 Overview of denaturing gradient gel electrophoresis (DGGE). DNA from an environmental sample is amplified using a universal primer set containing a GC clamp. As the DNA fragment runs through the gel, the denaturant increases in concentration. At a certain concentration of denaturant, the double-stranded DNA fragment melts and stops migrating through the polyacrylamide gel. This melting is determined by the GC content of the fragment and complete melting or dissociation of the fragment is prevented by the GC clamp. Thus, fragments differing in just a single nucleotide will stop migrating at different concentrations of denaturant. The bands can be excised for further analysis, often DNA sequencing, or compared to a standard curve generated using PCR products from known organisms

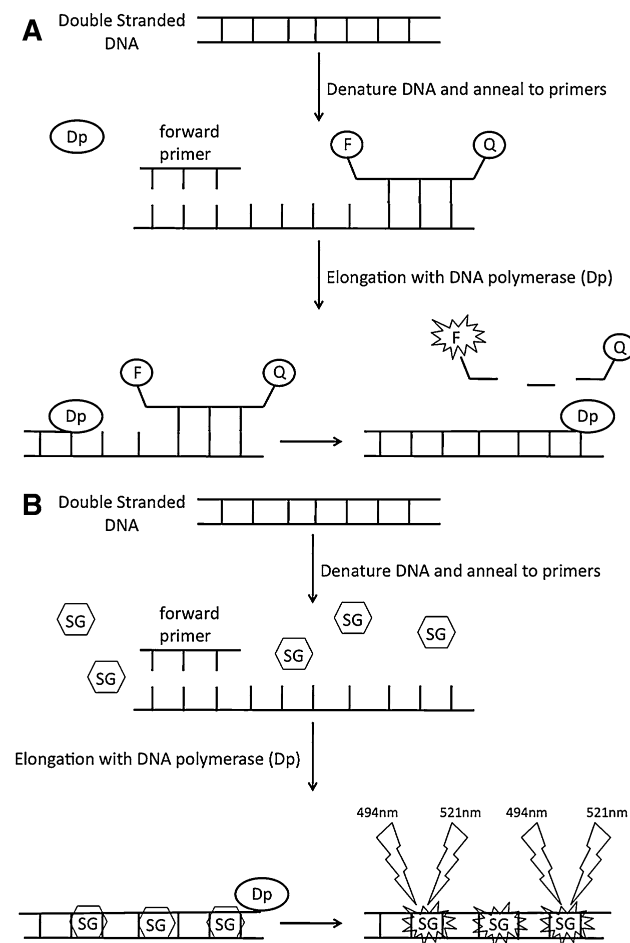

Fig. 3 Real-time PCR. **a** An example of probe-base detection systems such as Scorpion or Taqman. The target DNA is denatured into single-stranded DNA and is hybridized with specific primers. These primers have a fluorochrome (F) and a quencher (Q) attached to them. Once the primers are hybridized, the DNA is elongated using DNA polymerase. When the DNA elongates past the targeted DNA, the quencher and the fluorochrome are separated and thus the fluorochrome emits light. This light can be detected immediately and thus give a real-time quantification of genes present in the DNA. Different probes may be tagged with different fluorophore, thus enabling assays to be multiplexed. **b** SYBR Green assays. The double-stranded DNA is melted, which allows the primers to anneal to the single-stranded DNA. Double-stranded DNA is created by DNA polymerase, which allows the SYBR Green dye (SG) to bind the DNA strands and fluoresce. The excitation wavelength for the dye is 494 nm. Once SYBR Green is excited by light at 494 nm, it then emits light at 521 nm, which is known as the emission wavelength

1 day. Further, Cocolin and Mills [18] using DGGE (Fig. 2) found that *Hanseniaspora* rRNA persisted for 29 days in the absence of a culturable population.

The rest of this review will be a discussion of specific indirect and direct molecular methods, how they work, and also how they are used in the analysis of wine. Table 1 provides an outline of the methods discussed in this paper and the discrimination level.

Hybridization methods

DNA hybridization is most often used as an indirect method to identify various microorganisms and may include simple probes or microarrays to examine similarities in whole genomes. Hybridization is performed by adding a labeled probe of single-stranded DNA, specific to a gene or species in the case of total genomic DNA probes, to a sample of DNA isolated from an unknown organism. The mixture is then heated and the double helix of the DNA unravels or denatures to form single strands. After cooling, the helices will begin to reform and if there is similarity, the labeled probe will bind to the unknown DNA.

Early probes were labeled with radioactive material, however, these labels were rapidly replaced by various chemoluminescent systems, such as peroxidase enzymes, which react with a substrate to create a color change or even with fluorophores, which can be detected by monitoring for fluorescence.

Early hybridizations were often conducted using total DNA isolated from a reference strain as the probe. This technique was used by Lonvaud-Funel's group to identify *Leuconostoc oeni* (since renamed *Oenococcus oeni*) and other lactobacilli isolated from wine [104]. Newer methods have often relied on probes for specific genes instead of total genomic DNA, most often ribosomal RNA genes (rDNA), as this is the only sequence available for many organisms. These probes can, like the earlier total genomic DNA probes, be used to identify specific yeasts and bacteria or they can be used to detect the presence of a particular group, such as lactic acid bacteria.

The probes are often used for fluorescence in situ hybridization (FISH). This method uses fluorescently labeled oligonucleotide probes that typically target the rDNA of a species [11]. This technique uses the same principle as DNA hybridization, but is done in situ, meaning there is no DNA isolation before hybridization occurs. This allows for quick and easy detection of species in a mixed population sample without the need of plating. The procedure is outlined in Fig. 4. A probe labeled with a tag, usually digoxigenin (DIG), is added directly to cells fixed on a slide and hybridizes to the DNA inside the cell. Once hybridization is complete, the slide is covered with antibodies to DIG that contain a fluorophore. After the antibody treatment, the cells can be viewed under a microscope, which allows for direct visualization of the species present in the sample, as well as the analysis of functional cell parameters such as structure. The cells may also be used for flow cytometry, which is an optical system that allows the estimation of cell numbers, as well as shape, size, and cell viability through light measurements and scattering [66]. This method can either be used as

Table 1 Molecular methods used in detection and identification of wine-related microbes

Type of method	Identification method	Level of discrimination	References
Hybridization methods			
Probes	Indirect	From groups of lactic acid bacteria to genus and species	[86, 104]
Fluorescence in situ hybridization (FISH)	Indirect/direct	From groups of lactic acid bacteria to genus and species	[7, 10, 21, 48, 86, 97, 112]
Flow cytometry	Direct	Genus and species	[2, 42, 66, 103]
Complete genome hybridization	Indirect	From groups to specific strains	[9, 29, 100, 111]
Sequencing methods			
Ribosomal, *actin-1* or *rpoB* DNA sequencing	Indirect	Species	[25, 41, 51, 52, 91, 95]
Multilocus sequence typing	Indirect	Species and strain better discrimination for bacteria than *Saccharomyces*	[3, 6, 12, 26, 33, 57, 64, 75, 82]
Whole genome sequencing	Indirect	Strain	[9, 65]
Fingerprinting methods			
ITS-RFLP	Indirect	Species only used for yeasts	[1, 11, 14, 31, 32, 34, 44, 46, 58, 92]
26S rDNA-RFLP	Indirect	Species only used for yeasts	[23]
16S rDNA-RFLP	Indirect	Species only used for bacteria	[96, 98]
Karyotyping	Indirect	Strain only used for yeasts	[74, 101]
REA-PFGE	Indirect	Strain	[35, 41, 54, 73]
mt-RFLP	Indirect	Strain-yeast	[49, 70, 75, 90, 101]
AFLP	Indirect	Strain	[4, 13, 24, 30, 39, 43, 59, 84, 85, 100]
RAPD-PCR	Indirect	Strain	[6, 11, 26, 39, 99, 101, 106, 109, 114]
δ-sequence amplification	Indirect	Strain-yeast	[15, 56, 101]
Microsatellite	Indirect	Strain-yeast	[39, 101]
DGGE/TGGE	Direct	Typically species but may identify strains depending on targets for PCR	[17, 19, 36, 37, 53, 60, 67–69, 72, 76, 77, 91, 94, 95]
PCR detection			
Bacterial targets	Direct	Species to strain	[5, 22, 40, 55, 113]
Yeast targets	Direct	Species to strain	[18, 49, 61]
QPCR			
Bacterial targets	Direct	Currently species but depending on the target gene they may be strain specific	[45, 78, 106]
Yeast targets	Direct	Currently species	[27, 45, 47, 89, 93, 108]

described above to identify already cultured organisms, or it can be used to identify microbes directly from the sample without culturing.

Perry-O'Keefe et al. [86], developed a number of peptide nucleic acid-based probes for wine-related yeasts and bacteria. Peptide nucleic acid (PNA) molecules are pseudopeptides that can pair in accordance to Watson–Crick base pairing with DNA or RNA and have a polyamide backbone [79, 83, 107]. The uncharged nature of the PNA backbone gives high specificity, strong affinity, and rapid kinetics that improve hybridization to highly structured targets such as 26S rDNA, when compared to traditional DNA-based probes. Additionally, PNA probes allow hybridization under more stringent conditions than traditional DNA probes, enabling greater specificity [79].

Perry-O'Keefe et al. [86] used PNA probes, designed to rDNA, to detect both total bacteria and yeasts, as well as, the wine-related yeasts *D. bruxellensis*, *S. cerevisiae*, and *Zygosaccharomyces bailii*. They also designed probes to the bacterium *L. brevis* among other non-wine-related bacteria. Using these probes, in conjunction with membrane filter concentration of a wine sample, which was then enriched on appropriate media, they where able to detect, identify, and enumerate wine-related microbes in one-third of the time needed for traditional plating, as the probes could be used before visible colonies were present. However, there was not a good correlation between colony counts and the probe-based enumeration for *D. bruxellensis* and *Z. bailii* [86]. Connell et al. [22] adapted this same technique for the detection of *D. bruxellensis* in winery air.

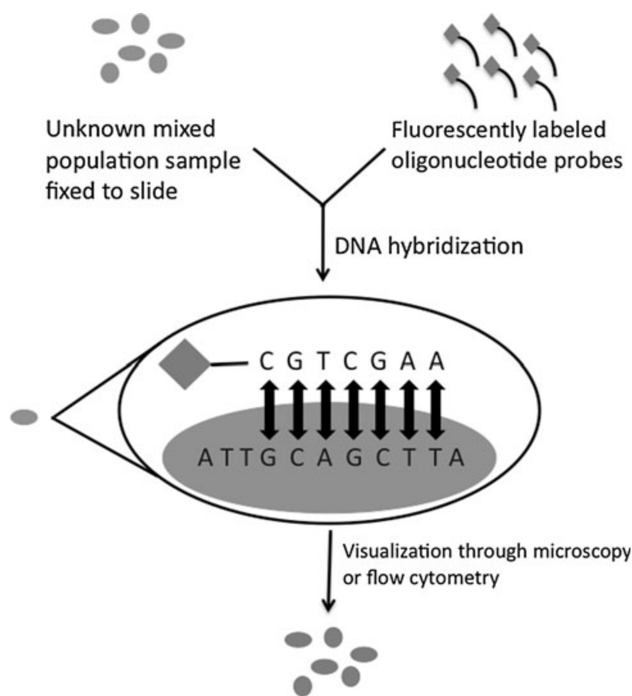

Fig. 4 Fluorescent in situ hybridization overview. An unknown population is fixed to a slide and hybridized with labeled DNA probes. A secondary antibody is applied to the sample and binds to the labeled probe. This antibody is attached to a fluorophore that will emit a specific wavelength of light upon excitation. The cells can then be viewed either through microscopy or flow cytometry and will fluoresce the color of the DNA probe that hybridized with it. This allows easy differentiation of species in a mixed population sample

Both papers consider this method to be a form of in situ hybridization, however, in this case it is not a direct detection method, as the probes are used on an enriched culture grown on media. Even though the technique takes less time than traditional indirect methods, growth of the organism is still needed. Xufre et al. [112] also extended the use of this technique. By using the D1/D2 domain of the 26S rRNA they were able to detect *S. cerevisiae*, *Candida stellata*, *Hanseniaspora uvarum*, *H. guilliermondii*, *Kluyveromyces thermotolerans*, *K. marxianus*, *Torulaspora delbrueckii*, *Pichia membranifaciens*, and *P. anomala* in both red and white wine must samples. When compared to many of the newer, PCR amplification-based techniques, hybridization methods are both cumbersome and time-consuming, as you need to wait for growth of the organism, hence the switch to the faster in situ hybridization techniques and a greater use of PCR-based methods in the more recent literature [41].

FISH has not only been used to identify already isolated microbes but also for the direct identification of microbes from fermentation samples, including wine. This method saves substantial time, as you do not need to wait for growth of the organism. Blasco et al. [8] designed oligonucleotide probes that target the 16S rDNA subunit to directly detect *L. brevis*, *L. collinoides*, *L. coryniformis*, *L. farciminis*, *L. mali*, *L. casei/paracasei*, *L. zeae*, *O. oeni*, *P. damnosus*, and *P. parvulus*, from wine samples. Hirschhäuser et al. [48] used the 5S rDNA as a target to distinguish *O. oeni* from closely related lactic acid bacteria species. The 5S rDNA subunit of *O. oeni* is highly conserved and has a different sequence than other phylogenetically related lactic acid bacteria. Thus, it makes an excellent target for FISH analysis. It was shown, however, that using only one probe with this method resulted in rapid bleaching of the fluorophore, while using multiple probes showed an increase in fluorescent yield. Overall, three probes for the 5S rDNA target were designed and utilized for detection of *O. oeni* [48].

Other organisms examined with this technique include the *Dekkera* and *Brettanomyces* species, which are known for giving off-flavors to wine and are usually undetected until spoilage has already occurred. Röder et al. [97] developed probes for all five known species: *D. anomala*, *D. bruxellensis*, *B. custersianus*, *B. nanus*, and *B. naardenensis*. These probes target the D1/D2 domain of the large subunit (26S) rDNA. To improve probe hybridization, 'side' probes, which are partially complementary or directly adjacent to the probe, were used to enhance the accessibility of highly structured RNA, a common problem with rDNA-targeted probes [48, 97].

While accurate and rapid, the use of FISH for the direct detection of microbes from wine is not common in the industry. It requires an expensive fluorescent microscope and has the limits of any microscopy-based method. Typically, microbes present at levels below 10^3 cfu ml^{-1} will not be observed and the procedure is prone to operator fatigue [62].

Flow cytometry

Flow cytometry is a technique using instrumentation that examines particles such as a yeast or bacterial cells, suspended in a fluid stream. The fluid is passed across a focused beam of light so that a single cell can be measured. The systems may detect a combination of light scattering and fluorescent measurements depending on the types of detectors available. Typical instruments may detect up to 100,00 cells per second and have been used in the wine industry to examine cell viability and physiology. For example, Graca da Silveira et al. [42] used flow cytometry and the fluorescent dyes carboxyfluorescein and propidium iodide to examine the membrane integrity of ethanol adapted and non-adapted *O. oeni* cells. They found that the population with intact membranes (staining only with carboxyfluorescein) was larger in the ethanol-adapted cells. This example illustrates what may be the best use of this technology for the wine industry, checking the viability of

starter cultures before inoculation. The method is rapid and with the dyes used, able to distinguish viable, dead, and injured cells in minutes, allowing the winemaker better control of the fermentation process [2].

The primary use of flow cytometry in the wine industry, however, has been to detect spoilage organisms [66, 103]. Most often, methods use the 16S or 26S rDNA genes as a target for FISH analysis, as discussed above. However, in a few cases, antibodies to specific organisms are available and these can be used in place of DNA or PNA probes. The method is rapid and takes less technical skill than methods such as QPCR, discussed below. However, the lack of commercial antibodies for many of the yeasts and bacteria present in wine means that probes need to be designed for detection of most spoilage microbes. Serpaggi et al. [103] were able to detect *Dekkera* grown in Pinot noir at a concentration of 10^2 cfu ml^{-1} using FISH-based flow cytometry. However, phenolic compounds interfered with initial methods for permeabilizing the cells and the method developed took over 24 h to complete. Malacrino et al. [66] used fluorescent dyes to estimate the populations of yeasts and bacteria in wine. They were able to detect viable yeasts at 10^3 cfu ml^{-1} and bacteria at 10^4 cfu ml^{-1}. However, when Pinot noir samples were tested directly without any pretreatment, they had difficulty distinguishing the bacteria from other debris present in the wine. For a more comprehensive review of flow cytometry in the food industry, see Comas-Riu and Rius [21].

Complete genome hybridization

Another hybridization method is complete genome hybridization (CGH). It is useful for comparing strains of bacteria or yeasts using a whole or partial genomic array. In essence, the genome of one microbe is hybridized to probes representing a whole or partial genome of the same organism. The method is sensitive and can detect both small nucleotide polymorphisms and gene-deletion events [72].

The discriminatory power of CGH is very high. For example, Salinas et al. [100] were able to differentiate between *S. cerevisiae* strains LV CB, L-957 and the commercial strain EC118 using CGH, while both AFLP and RAPD-PCR (discussed below) considered them to be the same strain. To date, two other studies have used CGH to study *S. cerevisiae* strains. Dunn et al. [29] used the method to compare four commercial *S. cerevisiae* strains to a laboratory strain. Winzeler et al. [111] compared 14 different strains isolated from Tuscany and found that there were geographical differences among the species, and that the wild strains and laboratory strains formed distinct, separate clusters.

Currently, only one study of wine bacteria has employed CGH, which is surprising given the far greater number of relevant bacterial genomes that have been sequenced [65]. The study by Borneman et al. [10] compared ten commercial strains of *O. oeni* using an *O. oeni* PSU-1-based array. They found that each strain lost up to 7% of the open reading frames present in PSU-1. This demonstrates one of the issues with CGH. If a strain contains a large deletion, it is impossible to determine if it is truly a deletion or a divergent orthologous sequence, which does not hybridize to the probes designed to the reference sequence. Although in this case, the authors did find that the CGH produced accurate data for characterizing the genomic profile of bacterial strains when compared to whole genome sequencing [10]. Additionally, while the method is rapid and, thanks to ever-improving bioinformatics, the analysis is fairly straightforward, it is expensive and limited by the availability of a genome sequence. However, as more genome sequences become available, it is likely that CGH will be used more often in wine-related ecological studies.

Sequencing methods

Ribosomal DNA (rDNA) sequencing

The most commonly used method to identify wine-related microbes is rDNA sequencing. It is a powerful tool for rapid and accurate microbial identification and is even used in conjunction with many direct analysis techniques such as DGGE. However, it is still common to isolate DNA from a colony on a plate and sequence a gene from that DNA. The resulting gene sequence is then compared to other genes in a database to identify that colony. Sequencing is more accurate than traditional biochemical identification methods as it is not dependant on the growth state or previous environment of the microbe. Because we are examining the genome of the organism directly, the technique is not dependent on an enzyme being expressed to metabolize certain sugars or other compounds.

Currently, the most-sequenced genes are the variable rDNA regions typically, the 16S rDNA in bacteria and the 26S rDNA in yeasts [41, 52]. These genes have been chosen as they have a long history in the identification of microbes, and for large numbers of yeast and bacteria these are the only sequences present in a database. In fact, sequences are even available for microbes we are unable to culture [81]. This region, however, is not always the best choice, as it can provide limited resolution. Using the D1/D2 domain of the 26S rDNA in yeast, typically a 600-bp fragment, provides species-level information, with different strains of a species having no more than three different nucleotides, while a separate species will have no more than six differences [51]. Because of this limit, other genes have also been used in the identification of both bacteria

and yeasts. These genes may, in some cases, provide greater resolution, such as the *actin-1* gene or internal transcribed spacer (ITS) regions in yeast, or the *rpoB* gene in bacteria [25, 51, 91, 95].

While these genes may provide greater resolution between taxa, for example sequencing of the actin genes provides better resolution than sequencing of the D1/D2 region, they do have a number of limitations when compared to rDNA sequencing [25]. First, it is more difficult, if not impossible, to develop universal primers for many genes, while the rDNA has well-defined, highly conserved regions that allow a single primer pair to work for all yeasts [51] or all bacteria [60]. Second, the available databases for alternative genes, while growing, remain much smaller than those available for the rDNA genes, thus making identification of unknown organisms more difficult.

Multilocus sequence typing (MLST)

While rDNA sequencing is useful for genus and species identification, it does not have enough resolution to differentiate strains of an organism. MLST, however, may be used to differentiate strains. MLST compares the sequence of 6–8 genes in an organism. The approach was originally devised by Maiden et al. [64] and is used extensively in the typing of pathogenic bacteria. The method is highly discriminative, reproducible, and little subjectivity is needed for analysis of organisms, such as *Candida albicans*, which have well-established procedures. In fact, standardized sequencing targets and online databases (http://pubmlst.org and http://www.mlst.net) have allowed comparison of many strains of pathogenic bacteria and yeasts between laboratories [82].

Typically, the target genes used for bacterial typing are housekeeping genes, while the genes used in yeast typing vary [63, 82]. Ayoub et al. [3] tested 26 loci before settling on seven genes, including a number of housekeeping genes and unknown loci, to type 84 strains of *S. cerevisiae* including both commercial starter cultures strains and strains found in wineries from Lebanon and Asia. They found that MLST was less discriminatory than microsatellite or δ-sequence typing (discussed below). Munoz et al. [75] analyzed only five loci and also found the method to be less discriminatory than restriction analysis of mitochondrial DNA (mt-RFLP) with MLST showing 13 different genotypes, while mt-RFLP gave 17 different restriction patterns for the 18 yeasts analyzed. This suggests that the proper MLST scheme for analysis of *Saccharomyces* remains to be developed, as the method can discriminate 99.9% of *C. albicans* strains [82]. Of note, Fay and Benavides [33] and Legras et al. [57] used MLST and multilocus microsatellite typing, respectively, to examine the domestication of *Saccharomyces* with Legras et al. [57], demonstrating that 95% of the world's wine yeast clustered together.

The same limited discriminatory powers are not observed when MLST is used to type wine-related bacteria. In fact, a study by Bilhere et al. [7] suggested that the use of eight housekeeping genes provided better discriminatory power than pulsed-field gel electrophoreses (PFGE) strain typing for 43 strains of *O. oeni*. Similarly, Calmin et al. [13] were able to differentiate 19 strains of *P. parvulus* and *P. damnosus* using only five different loci for comparison. de Las Rivas et al. [26] also found better discrimination by MLST than RFLP analysis when examining *O. oeni*.

Fingerprinting methods

Fingerprinting, in general, examines the whole genome of an organism, often creating a banding pattern by digesting or amplifying regions of the genome, which can be compared between organisms. The fingerprinting methods, such as amplified ribosomal DNA restriction analysis, can be used to differentiate species. However, most fingerprinting methods are only able to differentiate between strains of a particular organism. Although new methods such as MLST, whole genome arrays, and genome sequencing are available, many studies comparing strains still rely on some type of fingerprinting, as they can provide rapid and less-expensive alternatives.

For instance, the difference between *S. pastorianus* and *S. bayanus* cannot be determined by sequencing the 26 S rDNA gene as the sequences are identical [88]. It would also be difficult to determine differences in the strains of *O. oeni* found in a winery using rDNA sequencing. Thus, to differentiate microbes at the strain level, a variety of different techniques are used. These include: AFLP and RAPD-PCR in both yeast and bacteria and, of course, pulse-field gel electrophoreses (PFGE), also known as karyotyping, in yeasts. PFGE has long been considered the gold standard for strain identification and is extensively used for strain typing of bacteria responsible for food-borne illness outbreaks [87].

Amplified ribosomal DNA restriction analysis (ARDRA)

One less-expensive and equally effective alternative to 26S rDNA sequencing for the rapid identification of yeasts is restriction fragment length polymorphism analysis of the internal transcribed spacer region (ITS-RFLP). It has been successfully applied for the identification at the species level to almost all yeasts found in wine, and used in conjunction with other techniques to identify yeasts from various regions around the world, ranging from vineyards

in China and Slovenia to Jura fermentations in France [15, 58, 92]. It has also been used to examine the succession of yeasts during wine fermentations as well as the effects of various winemaking practices, such as cold maceration, have on the yeast population [46].

In this technique, the region in between the 18S and 26S rRNA genes, which contains the internal transcribed spacer regions flanking the 5.8S rRNA gene, is amplified by PCR. This PCR product is then digested with three restriction enzymes to produce a banding pattern, which when run on an agarose gel, typically gives between two and four bands. This banding pattern is compared to the banding patterns for the type strains of the suspected yeast species, a number of patterns are available in the literature [1, 31].

Yeasts can also be speciated by comparing the total size of the PCR product. For instance, Guillamon et al. [44] found the PCR amplification of this region in *Metschnikowia pulcherrima* generated a 390-bp product while *Hanseniaspora uvarum* generated a 760-bp product and *S. cerevisiae*, *S. bayanus*, and *S. pastorianus* generated 880-bp fragments. Interestingly, when this technique was applied to 'flor' yeasts in sherry, a 24-bp deletion in the region was found when compared to other *S. cerevisiae* strains [15, 32, 34]. This is unusual, as this technique typically does not differentiate yeast strains but only species. However, this 24-bp deletion and the resulting banding patterns, after restriction enzyme digests, would only differentiate *S. cerevisiae* flor strains from non-flor strains [15, 32, 34]. The results obtained by comparing product length are, however, normally confirmed through comparison of the restriction enzyme banding patterns.

As stated above, the PCR product is typically digested with three restriction enzymes [44]. Depending on the species being examined, a number of the enzymes may be used for digestion of the fragment in order to better differentiate species in specific yeasts genera. For example, Cadez et al. [12] found digestion with only *DdeI* and *HinfI* allowed for differentiation between all *Hanseniaspora* and *Kloeckera* species.

While ARDRA typing has typically been performed to identify yeast species using the ITS regions discussed above, Balerias Couto et al. [5] used restriction enzyme analysis of the 26S rDNA to characterize non-*Saccharomyces* yeasts in wine. Additionally, restriction analysis of the 16S rDNA region in bacteria has also been used to identify both *Lactobacilli* and acetic acid bacteria associated with wine [96, 98].

In comparison to the sequencing methods described above, this method performs well and a large number of restriction patterns are available for wine yeasts [1, 31]. However, if the microbe of interest is not in the database, the larger available databases for rDNA sequencing will be needed.

Finally, while the sequencing of a single gene and ARDRA remain widely used techniques due to their speed and available infrastructure, they are often used in conjunction with other techniques that allow greater resolution to the subspecies and strain level. Thus, when ARDRA is used in conjunction with RAPD-PCR a complete picture of not only the yeast species involved in the fermentation, but also the strains of the species involved, becomes clear.

Pulse-field gel electrophoresis (PFGE)

PFGE is a technique that examines the whole chromosome of a microbe. For yeast, this method is called karyotyping. A single yeast colony is grown in liquid media and combined with melted agarose to form an agar plug. The yeast cells are lysed in the agar matrix and the plugs are inserted into an agarose gel and subjected to the alternating application of two electric fields. This allows for the separation of large DNA fragments. The only difference when this technique is applied to bacterial strain identification, is the chromosome in the agar plug is subjected to digestion with restriction enzymes. This variation is referred to as restriction endonuclease analysis PFGE (REA-PFGE).

Both REA-PFGE and karyotyping have been extensively used to identify yeast and bacteria isolated from wine. The method provides a high level of discrimination. For example, Schuller et al. [101] compared a number of different typing methods for identification of *S. cerevisiae* strains, and karyotyping had the highest resolution, being able to discriminate 22 of the 23 strains analyzed. The authors suspected that the two commercial strains analyzed, which could not be differentiated, were in fact the same *S. cerevisiae* strain, as they were originally isolated from the same geographical region [101]. Karyotyping alone may not always be sufficient to differentiate strains of certain yeast species. *Dekkera/Brettanomyces bruxellensis* strains typically have a small number of chromosomes thus creating ambiguity when undertaking traditional karyotyping [74]. To overcome this, Miot-Sertier and Lonvaud-Funel [73] developed an REA-PFGE method for strain typing *D. bruxellensis* that provided better resolution than the PCR-based methods used in the past [74]. Selection of appropriate restriction enzymes is important in the study of bacterial strains. Larisika et al. [54] found that the commonly recommended restriction enzyme, *ApaI*, gave poor resolution of their 65 *O. oeni* strains, while another enzyme, *SfiI*, provided better discrimination.

PFGE has been used to identify strains of both yeast and bacteria in many different wine and grape-related studies, and has been found to be reproducible, easy to interpret, and highly discriminative [35, 41]. The technique, however, is also laborious, expensive, and requires a high level

of training. These deficiencies have lead to the development of a number of faster, simpler, and less labor-intensive methods as those discussed below.

Restriction analysis of mitochondrial DNA (mt-RFLP)

This method is used extensively in the analysis of wine-related *S. cerevisiae* strains. In a simplified and widely used version of the method, developed by Querol et al. [90], the total DNA is isolated from yeast and digested with a restriction enzyme typically, *Hin*FI or *Hae*III. These enzymes degrade the chromosomal DNA into small fragments, while the mitochondrial DNA is cleaved less often, thus resulting in the banding pattern used to differentiate the *S. cerevisiae* strains. The method is rapid, easy to use, and inexpensive. Schuller et al. [101] found the method to be comparable in its discriminatory power to microsatellite and δ-sequence analysis, being able to differentiate 21 of 23 strains of *Saccharomyces* and Munoz et al. [75] found the method to be superior to MLST typing.

Restriction analysis of mitochondrial DNA has rarely been used to follow strains of non-*Saccharomyces* yeasts. Martorell et al. [70] used the method to differentiate strains of *D. bruxellensis* and *Pichia guilliermondii*. With 63 *D. bruxellensis* strains exhibiting only three mt-RFLP patterns, the authors further differentiated the largest strain pattern using RAPD-PCR. This low variability and limited usefulness for mt-RFLP with regard to *Dekkera* was also noted by Ibeas et al. [49]. The *P. guilliermondii* strains, however, were broken into seven patterns, with these patterns correlating with the level of 4-ethylphenol production [70].

Amplified fragment length polymorphism (AFLP)

AFLP is a technique most often used in relation to wine for the genotyping of grapes and molds [30, 43, 84, 85]. It has seldom been applied for the strain typing of wine-related bacteria, although Cappello et al. [14] found it to be a reliable method for the strain typing of *O. oeni*. With regard to strain typing of yeasts, few studies have been conducted. Azumi et al. [4] used the method to study laboratory and industrial strains of *Saccharomyces* sensu stricto and found that *S. cerevisiae*, *S. bayanus*, *S. carlsbergensis*, and *S. paradoxus* all had species-specific banding patterns with some strain variation. Curtin et al. [24] used the method to identify eight different genotypes of *D. bruxellensis* from 31 winemaking regions in Australia. Other studies have examined *Saccharomyces* strains from different geographical origins [59, 100].

While useful, this technique is laborious, and typically requires automated DNA sequencers, making it expensive. However, older versions of the technique, which relied on other less-expensive detection methods, usually involving radiation, may also be used. DNA from the microbe of interest is digested with a restriction enzyme and adaptors are bound to the fragments. The fragments are then PCR amplified using primers, which target the adaptors and the restriction sites. The fragments are then separated using an automated DNA sequencer to detect a pattern.

PCR-based finger printing methods

A number of different PCR-based techniques are used to identify strains of both yeast and bacteria from wine. The most commonly used of these methods is random amplified polymorphic DNA-PCR or RAPD-PCR. This technique has been used in many instances to follow the succession of specific yeast and microbial strains in wine. For example, work by Urso et al. [109] used RAPD-PCR to follow *Saccharomyces* strains during the alcoholic fermentation of Picolit, an Italian sweet wine, and found that the inoculated *S. cerevisiae* starter culture actually perform the alcoholic fermentation in only one of the two fermentations studied.

RAPD-PCR is fairly simple to perform, hence its extensive use in strain identification. It uses a single small primer to conduct PCR at a low annealing temperature. The small primer and low annealing temperature allow for random hybridization to the genome. When two primers land close to each other, a band will form. This allows for a survey of the polymorphisms found around a given genome, which are typically specific to a given strain of yeast or bacteria. Cadez et al. [12] noted that the method was not useful for discrimination of species, in their case *Hanseniaspora* and *Kloeckera* species, but it was useful for revealing relationships between strains of a species.

The method has also been used to study *O. oeni* strains in wine. Zavaleta et al. [114] were the first to use RAPD-PCR to study *O. oeni* and found that the strains studied formed two main groups. This result was also seen by Bihere et al. [7] using MLST. Ruiz et al. [99] used RAPD-PCR to determine which of 22 strains isolated from a Spanish winery were best able to implant and conduct malolactic fermentation (MLF). Finally, Solieri et al. [106] isolated and sequenced a band created via RAPD-PCR to create a QPCR method to follow a specific *O. oeni* strain during the course of the fermentation. The method works well for bacteria, but as mentioned earlier, MLST appears to be more discriminatory [26].

While the technique is rapid, amenable to high-throughput analysis, and has the advantage of not needing any previous sequence information to compare strains, it does have a number of drawbacks. The most important of these is the difficulty in reproducing the banding patterns for a specific strain among laboratories. The procedures must be carefully standardized, as small variations in the

DNA isolation or even the type of thermocycler can change the banding pattern. We have even seen differences in our laboratory in banding patterns generated for the same organism by different researchers.

Similar methods have been developed for yeasts, which target specific repetitive chromosomal regions. For example, δ-sequence amplification, which targets the δ regions flanking the 100 or so Ty1 retrotransposons in *S. cerevisiae*, has been used extensively to type *S. cerevisiae* strains. An optimized protocol was developed by Legras and Karst [56] with the δ_2 and δ_{12} primers being the most discriminatory. However, the technique has similar inter-laboratory reproducibility issues to those of RAPD-PCR, although current work has helped, including standardizing both the DNA concentration and increasing the annealing temperature to remove ghost bands [16, 101].

Other targeted regions for yeasts include the mini- and micro-satellites, which are repetitive regions found throughout the *S. cerevisiae* genome. Using this method, Gallego et al. [39] was able to differentiate 20 of 27 *S. cerevisiae* strains, while AFLP typing was only able to differentiate 19 of 27 and RAPD-PCR 12 of 27 strains. Schuller et al. [101] found that microsatellite typing, δ-sequence analysis, and mt-RFLP analysis were able to differentiate 21 of 23 *S. cerevisiae* strains examined, which was only one less than karyotyping, suggesting that these methods may be used interchangeably. In practice, however, researchers often use a number of methods in order to confirm their findings.

Denaturing gradient gel electrophoresis (DGGE)

While the methods described above may be used to compare species and specific strains, DGGE is typically used to compare microbial communities directly from the environment. According to Cocolin et al. [20], the method is relatively sensitive, being able to detect yeast populations in wine, which represent at least 0.01% of the dominant *Saccharomyces* population. This, however, will vary with the DGGE assay used in analysis.

DGGE is based on separation of DNA fragments of the same length by polyacrylamide gels containing a linearly increasing gradient of denaturants such as urea and formaldehyde [77, 94]. Small sequence variations in the DNA fragments change each fragment's electrophoretic mobility. The chemical gradient causes them to denature and stop at a different point along the gradient [76]. Complete denaturation of the fragments is prevented by using primers with a GC-clamp. Figure 2 outlines the DGGE procedure.

For identification, the separated fragments are typically compared to a DNA ladder made of PCR products from already identified control strains suspected to be present in the environment. This identification method can, however, be a problem if a new or novel organism is present in the sample [37]. To overcome this limitation, researchers may also excise the fragments from the gel, sequence these fragments, and compare them to sequences in a database, a method particularly useful if rDNA sequences are the targets [18].

It is therefore important to select an appropriate gene for analysis. The DNA fragments must be relatively small, up to around 500 bp, and should possess well-conserved regions that can be used in primer design [76]. For most bacteria, the gene encoding the beta subunit RNA polymerase (*rpo*B) has become the target of choice, although the 16S rDNA gene is still used [91, 94, 95]. For yeasts, the target gene is almost always the D1/D2 loop of the 26S rDNA gene [72].

One aspect of primer design that is important to consider in analysis of the DGGE gels is the bias introduced by PCR. For example, Laforgue et al. [53] used DGGE of the β-tubulin gene to examine the fungal and yeast populations present on grapes. The assay was unable to detect some yeast species identified through traditional methods. This disparity was attributed to a PCR bias due to either a lack of accessibility of the β-tubulin region or differential lysis of fungi and yeast.

Another issue is a possible masking effect of non-specific targets with certain DGGE primer sets. For example, several common primer sets used for bacterial analysis were found to amplify yeast, fungal, or plant 18S rDNA. In fact, *L. plantarum* was only detectable in Chardonnay samples when *Vitis vinifera* DNA was not present [60]. Thus, several authors have identified new primer sets that eliminate this problem, and many researchers now use the *rpo*B primers for bacterial identification [91, 95].

In the most comprehensive use of DGGE to date, Renouf et al. [94] monitored the microbial population through the entire winemaking process from berry to wine. Fifty-two different yeast species were found on the surface of grapes sampled from eight different vineyards. DGGE analysis demonstrated that the yeast population declined significantly through the process in three distinct phases. Phase 1. The total population increased during initial fermentation to about 10^8 cfu ml^{-1} with *S. cerevisiae* being dominant. Phase 2. After the first racking, the population declined. Phase 3. During aging, the population rose again to around 10^{3-4} cfu ml^{-1} and stabilized with *D. bruxellensis* dominating the population [94].

A similar technique, known as temperature gradient gel electrophoresis (TGGE), can also be used to distinguish mixed populations. It employs the same basic principle as DGGE, except a temperature gradient is employed instead of increasing concentrations of a denaturant. Fernandez-Gonzalez et al. [36] used TGGE to characterize *Saccharomyces* and non-*Saccharomyces* species from wine must

during fermentation. Of the isolates used, 58% were shown to be the genus *Saccharomyces* using restriction enzyme analysis. TGGE was used to distinguish the genus of the non-*Saccharomyces* isolates: *Candida, Kluyveromyces*, and *Hanseniaspora* [36]. Manzano et al. [68] were able to study the ecology of the genus *Saccharomyces* directly in wine at the strain level. Again using the ITS1 and ITS2 regions as PCR targets, TGGE analysis showed seven different strains of *S. paradoxus*, all with similar migration patterns [69]. This technique was also able to distinguish those strains from samples of *S. cerevisiae*. TGGE patterns were identical for samples drawn directly from musts and those obtained from plated colonies, demonstrating the effectiveness of the technique for direct identification. This method has also been shown to be effective at verification of dry yeast strains before they are used for fermentation [67].

PCR detection

Traditional PCR has also been used to detect target populations. The targeted gene for the assay can differ greatly among species, but is generally chosen because it is unique to that species. Zapparoli et al. [113] used the gene encoding the malolactic enzyme, *mle*, of *O. oeni* as the PCR target. This method was able to detect *O. oeni* at 10^7 cfu ml^{-1} in grape must and at 10^4 cfu ml^{-1} in wine. Bartowsky and Henschke [6] also detected *O. oeni* using the *mle* gene and found similar results in wine samples with $10^{3–4}$ cfu ml^{-1} being detectable.

A targeted gene can help ensure specificity, but PCR can also be used with just a small random DNA sequence that is unique to the species. Ibeas et al. [49] was able to design a two-step PCR protocol to detect low levels of *Dekkera* in sherry, using an isolated DNA fragment to develop primers. This sequence amplified *Dekkera* strain OSB101 very well, but was also closely related to the DNA repair protein RAD4 found in *S. cerevisiae*. Therefore, a second set of primers was designed for the same region. This nested primer protocol proved successful at detecting only *D. bruxellensis* and synonymous strains, with no detection of *S. cerevisiae*. After the first amplification, a detection limit of 1,000 copies was reached and the second amplification allowed detection of a single organism.

Cocolin et al. [19] developed a method similar to the one of Ibeas et al. [49] for the detection of *B. bruxellensis* and *B. anomalus* using primers to the 26S rDNA gene. In this study it was determined that RNA and DNA persisted in the samples for both wine species, even though little to no growth was detected on plating media. Lopez et al. [61] used oligonucleotide primers homologous to the flanking region of *S. cerevisiae COX*1 introns to differentiate starter strains. In order to assure implantation of a dominant starter strain, this method was employed directly on wine samples. In testing the primers on 13 commonly used dry yeast strains of *S. cerevisiae*, it was determined that in a single PCR reaction, all strains were easily differentiated, not only from each other but also from three reference strains. Small-scale fermentations combining four strains at different ratios were also performed to test the sensitivity of the assay. It was determined that the primers were able to detect strains comprising 30% or more of the population. The same results were shown for several samples of must.

PCR methods have been used to determine the cause of many types of taint. Gindreau et al. [40] developed primers to detect exopolysaccharide-producing strains of *P. damnosus*, and were able to detect as few as 100 cfu ml^{-1}. Le Jeune et al. [55] developed primers for the histidine decarboxylase (HDC) gene present in many lactic acid bacteria. HDC produces the biogenic amine histamine. This assay was then used by Coton et al. [23] to survey wines from southwestern France. Of 118 samples screened, it was determined that almost half of them contained the HDC allele.

The two major limitations of using PCR for detection are that it is qualitative not quantitative (it only provides presence or absence information) and it may not differentiate between viable and nonviable microbes.

Real-time PCR

Real-time polymerase chain reaction (QPCR) (Fig. 3) is similar to traditional PCR but it incorporates a fluorescent dye, and after each PCR cycle, the fluorescence increases. The PCR cycle at which the fluorescence crosses a predetermined threshold value is counted as positive and is known as a cross threshold or Ct value. DNA amplification is linked to fluorescence in one of two ways, either through the addition of a DNA binding dye such as SYBR Green or by the addition of a probe labeled with a fluorophore.

SYBR Green-based QPCR assays work very simply (Fig. 3b). The SYBR Green dye binds to double-stranded DNA. During the course of PCR, a double-stranded product is created after each elongation step and the dye binds to this product and fluoresces. As the gene is amplified during each PCR cycle, the fluorescence increases.

A number of probe-based QPCR assays (Fig. 3a) such as Scorpions or Taqman probes exist and each works in a slightly different manner (for review see Hanna et al. [45]). In general, a probe is designed to a region between the two QPCR primers, giving the assay greater specificity than a SYBR Green assay, as a third region of DNA (the probe) will be specific to the organism in question. The probe contains both a fluorophore and a quencher, and because it binds between the two primers, it will be associated with the

single-stranded DNA during the PCR elongation step. In its simplest form, as the DNA polymerase elongates the DNA fragment, the probe is degraded causing the fluorophore to separate from the quencher, thus creating fluorescence.

In both the SYBR Green and probe-based assays, as DNA amplification continues, the amount of light emitted from the fluorophore and the sample will be considered positive once it passes the Ct. By comparing the Ct values for each sample to a standard curve, the sample can be quantified. Samples with small amounts of DNA will have higher Ct values than those with high amounts of DNA. Thus if a large number of microbes are present in a sample, they will have a large amount of DNA, and hence a lower Ct value.

This application is useful for the detection and enumeration of spoilage organisms. In fact, the first application of QPCR to wine was by Phister and Mills [89] for the detection *D. bruxellensis*. Other assays by Delaherche et al. [27] were developed not only to *D. bruxellensis* but also to 'ropy' *P. damnosus* in samples of spoiled wine. The *P. damnosus* assay targeted the *dps* gene, which is specific for exopolysaccharide production. Neeley et al. [78] used this technique to quantify wine-related LAB as a group. A primer set, WLAB1-2, was created to detect *L. plantarum* and *O. oeni* at cell densities as low as approximately 100 cfu ml^{-1}, even in the presence of *S. cerevisiae*, *G. oxydans*, or *A. aceti*. The method has since been used to follow just about every microbe related to wine. Care must be taken, however, as a number of compounds present in wine may interfere with the assay [110]. Tessonniere et al. [108] examined six different DNA isolation methods in developing a QPCR assay for *D. bruxellensis* and found that polyvinyl polypyrrolidone was able to eliminate most of the PCR inhibitors.

QPCR is rapid, taking a few hours, and is also sensitive. In some cases it can detect as few as ten organisms per milliliter, where other methods such as DGGE or microscopy generally require at least 1,000 organisms per milliliter [18, 62]. QPCR can even be multiplexed to detect a number of organisms in one assay [102]. The major disadvantage outside of cost and personnel training is centered around the method's inability to differentiate viable and nonviable microbes.

One of the few studies to address this issue was conducted by Hierro et al. [47]. They designed a real-time PCR assay to detect and quantify the total yeast population of a wine sample. While the assay itself was useful, the limit of detection for yeast grown in YEPD media was 10^2 and 10^3 cfu ml^{-1} in wine. The most informative part of the study used reverse transcriptase PCR (RT-PCR) to measure the viability of the cells.

In theory, RNA is often considered an indicator of viability, as it is thought that RNA degrades more quickly than DNA, and thus would only be found in metabolically active cells [72]. This, however, depends upon the gene used for the assay, with some RNA molecules persisting for long periods. Heirro et al. [47] examined the stability of rRNA by heating an *S. cerevisiae* culture for 20 min and following the 26S ribosomal DNA and RNA using their QPCR assay. They found that both the RNA and DNA persisted for over 1 day. In comparison, a study by Bleve et al. [9], examining the stability of *actin* mRNA, found complete degradation after 20 min using end-point PCR.

These studies suggest that mRNA may make a better target for differentiating viable and nonviable cells than rRNA or DNA. It should be pointed out, however, that the persistence of DNA and RNA in wine remains to be studied, as both previous studies examined persistence of RNA after heating in media, which is a very different environment. The only published study examining persistence of RNA and DNA in wine was conducted by Cocolin et al. [18], using DGGE. They found persistence of *Hanseniaspora* 26S rRNA for over 29 days in a wine fermentation in the absence of a culturable population. So it is important that any target, whether DNA, rRNA, or mRNA, be evaluated for its persistence in wine.

Ethidium monoazide (EMA) PCR

One possible solution to this issue may be found in the use of EMA, which is a fluorescent photoaffinity label that covalently couples to nucleic acids upon exposure to light. Further, EMA can only enter cells with compromised cell walls and cell membranes [80]. Therefore, it is believed to be a good indicator of cell viability, as only viable cells will have intact membranes, thus keeping the dye out. Once a sample is treated with EMA, real time-PCR can then be performed and only the viable cells will be quantified. Rawsthorne and Phister [93] used an EMA assay to distinguish viable cells of *Z. bailii* in different fruit juices. It was determined that the assay could detect as few as 12.5 viable cells in the presence of 10^5 cfu ml^{-1} of heat-killed *Z. bailii* cells. It was also shown that the EMA protocol was more consistent at determining viable cell number than conventional plating and fluorescent microscopy. While the application of EMA in wine is rather new, it holds much promise for examining questions such as those posed by Fugelsang and Zoecklein's [38] work on *D. bruxellensis* production of 4-ethyl phenol in the absence of a culturable population.

Future directions

A number of advances in molecular detection techniques hold promise for applications in the identification of wine-related microbial communities. Microarrays, for example,

could be applied to enological studies to provide simultaneous detection of multiple microorganisms. Microarrays have been developed that contain 16S rDNA gene sequences that can discriminate between multiple microorganisms with only one hybridization event [72]. However, as discussed above, microarrays, even for detection of multiple organisms, rely on existing knowledge about an organism, making it difficult to use when working with a new sequence.

Another emerging method, which could find application in studying the ecology of wine and would overcome this deficiency, is known as deep sequencing or pyrosequencing. Additionally, it is thought this method will also identify organisms present at lower levels in an environment when compared to traditional methods, thus giving a more complete picture of the ecology of any niche. Currently, this method is similar to rDNA sequencing. The rDNA from an environmental sample is amplified using universal primers and these are then sequenced using one of the new technologies. The method compares well with microarrays and little previous sequence knowledge is needed, only the rDNA sequence [17].

Acknowledgments We would like to thank the United States Department of Agriculture CREES National Research Initiative for funding this research through project number 2007-35504-18249.

References

1. Arias C, Burns JK, Friedrich LM, Goodrich RM, Parish ME (2002) Yeast species associated with orange juice: evaluation of different identification methods. Appl Environ Microbiol 68:1955–1961. doi:10.1128/AEM.68.4.1955-1961.2002
2. Attfield PV, Kletsas S, Veal DA, van Rooijen R, Bell PJ (2000) Use of flow cytometry to monitor cell damage and predict fermentation activity of dried yeasts. J Appl Microbiol 89:207–214. doi:10.1046/j.1365-2672.2000.01100.x
3. Ayoub MJ, Legras JL, Saliba R, Gaillardin C (2006) Application of multi locus sequence typing to the analysis of the biodiversity of indigenous *Saccharomyces cerevisiae* wine yeasts from Lebanon. J Appl Microbiol 100:699–711. doi:10.1111/j.1365-2672.2006.02817.x
4. Azumi M, Goto-Yamamoto N (2001) AFLP analysis of type strains and laboratory and industrial strains of *Saccharomyces sensu stricto* and its application to phenetic clustering. Yeast 18:1145–1154. doi:10.1002/yea.767
5. Baleiras Couto MM, Reizinho RG, Duarte FL (2005) Partial 26S rDNA restriction analysis as a tool to characterize non-*Saccharomyces* yeasts present during red wine fermentations. Int J Food Microbiol 102:49–56. doi:10.1016/j.ijfoodmicro.2005.01.005
6. Bartowsky EJ, Henschke PA (1999) Use of a polymerase chain reaction for specific detection of the malolactic fermentation bacterium *Oenococcus oeni* (formerly *Leuconostoc oenos*) in grape juice and wine sample. Aust J Grape Wine Res 5:39–44. doi:10.1111/j.1755-0238.1999.tb00150.x
7. Bilhere E, Lucas PM, Claisse O, Lonvaud-Funel A (2009) Multilocus sequence typing of *Oenococcus oeni*: detection of two subpopulations shaped by intergenic recombination. Appl Environ Microbiol 75:1291–1300. doi:10.1128/AEM.02563-08
8. Blasco L, Ferrer S, Pardo I (2003) Development of specific fluorescent oligonucleotide probes for in situ identification of wine lactic acid bacteria. FEMS Microbiol Lett 225:115–123. doi:10.1016/S0378-1097(03)00501-9
9. Bleve G, Rizzotti L, Dellaglio F, Torriani S (2003) Development of reverse transcription (RT)-PCR and real-time RT-PCR assays for rapid detection and quantification of viable yeasts and molds contaminating yogurts and pasteurized food products. Appl Environ Microbiol 69:4116–4122. doi:10.1128/AEM.69.7.4116-4122.2003
10. Borneman AR, Bartowsky EJ, McCarthy J, Chambers PJ (2010) Genotypic diversity in *Oenococcus oeni* by high-density microarray comparative genome hybridization and whole genome sequencing. Appl Microbiol Biotechnol 86:681–691. doi:10.1007/s00253-009-2425-6
11. Bottari B, Ercolini D, Gatti M, Neviani E (2009) FISH in food microbiology. In: Liehr T (ed) Fluorescence in situ hybridization (FISH)-application guide. Springer, Berlin Heidelberg New York, pp 395–408
12. Cadez N, Raspor P, deCock AWAM, Boekhout T, Smith MT (2002) Molecular identification and genetic diversity within species of the genera *Hanseniaspora* and *Kloeckera*. FEMS Yeast Res 1:279–289. doi:10.1111/j.1567-1364.2002.tb00046.x
13. Calmin G, Lefort F, Belbahri L (2008) Multi-loci sequence typing (MLST) for two lacto-acid bacteria (LAB) species: *Pediococcus parvulus* and *P. damnosus*. Mol Biotechnol 40:170–179. doi:10.1007/s12033-008-9073-4
14. Cappello MS, Stefani D, Grieco F, Logrieco A, Zapparoli G (2008) Genotyping by amplified fragment length polymorphism and malate metabolism performances of indigenous *Oenococcus oeni* strains isolated from *Primitivo* wine. Int J Food Microbiol 127:241–245. doi:10.1016/j.ifoodmicro.2008.07.009
15. Charpentier C, Colin A, Alais A, Legras J-L (2009) French Jura flor yeasts: genotype and technological diversity. Antione Van Leeuwenhoek 95:263–273. doi:10.1007/s10482-009-9309-8
16. Ciani M, Mannazzu I, Marinangeli P, Clementi F, Martini A (2004) Contribution of winery-resident *Saccharomyces cerevisiae* strains to spontaneous grape must fermentation. Antonie Van Leeuwenhoek 85:159–164. doi:10.1023/B:ANTO.0000020284.05802.d7
17. Claesson MJ, O'Sullivan O, Wang Q, Nikkila J, Marchesi JR, Smidt H, de Vos WM, Ross RP, O'Toole PW (2009) Comparative analysis of pyrosequencing and a phylogenetic picroarray for exploring microbial community structures in the human distal intestine. PLoS ONE 4(8):e6669. doi:10.1371/journal.pone.0006669
18. Cocolin L, Mills DA (2003) Wine yeast inhibition by sulfur dioxide: a comparison of culture-dependent and independent methods. Am J Enol Vitic 54:125–130
19. Cocolin L, Rantsiou K, Iacumin L, Zironi R, Comi G (2004) Molecular detection and identification of *Brettanomyces/Dekkera bruxellensis* and *Brettanomyces/Dekkera anomalus* in spoiled wines. Appl Environ Microbiol 70:1347–1355. doi:10.1128/AEM.70.3.1347-1355.2004
20. Cocolin L, Bisson LF, Mills DA (2000) Direct profiling of the yeast dynamics in wine fermentations. FEMS Microbiol Lett 189(1):81–87. doi:10.1111/j.1574-6968.2000.tb09210.x
21. Comas-Riu J, Rius N (2009) Flow cytometry applications in the food industry. J Ind Microbiol Biotechnol 36(8):999–1011. doi:10.1007/s10295-009-0608-x
22. Connell L, Stender H, Edwards CG (2002) Rapid detection and identification of *Brettanomyces* from winery air samples based on peptide nucleic acid analysis. Am J Enol Vitic 53(4):322–324
23. Coton E, Rollan G, Bertrand A, Lonvaud-Funel A (1998) Histamine-producing lactic acid bacteria in wines: early detection, frequency and distribution. Am J Enol Vitic 49(2):199–204

24. Curtin CD, Bellon JR, Henschke PA, Goddon PW, de Barros Lopes MA (2007) Genetic diversity of *Dekkera bruxellensis* yeasts isolated from Australian wineries. FEMS Yeast Res 7:471–481. doi:10.1111/j.1567-1364.2006.00183.x
25. Daniel H-M, Meyer W (2003) Evaluation of ribosomal RNA and actin gene sequences for the identification of ascomycetous yeasts. Int J Food Microbiol 86:71–78. doi:10.1016/S0168-1605(03)00248-4
26. de las Rivas B, Marcobal A, Munoz R (2004) Allelic diversity and population structure in *Oenococcus oeni* as determined from sequence analysis of housekeeping genes. Appl Environ Microbiol 70(12):7210–7219. doi:10.1128/AEM.70.12.7210-7219.2004
27. Delaherche A, Claisse O, Lonvaud-Funel A (2004) Detection and quantification of *Brettanomyces bruxellensis* and 'ropy' *Pediococcus damnosus* strains in wine by real-time polymerase chain reaction. J Appl Microbiol 97(5):910–915. doi:10.1111/j.1365-2672.2004.02334.x
28. du Toit WJ, Pretorius IS, Lonvaud-Funel A (2005) The effect of sulphur dioxide and oxygen on the viability and culturability of a strain of *Acetobacter pasteurianus* and a strain of *Brettanomyces bruxellensis* isolated from wine. J Appl Microbiol 98(4):862–871. doi:10.1111/j.1365-2672.2004.02549.x
29. Dunn B, Levine RP, Sherlock G (2005) Microarray karyotyping of commercial wine yeast strains reveals shared, as well as unique, genomic signatures. BMC Genom 6:53. doi:10.1186/1471-2164-6-53
30. Ergul A, Kazan K, Aras S, Cevik V, Celik H, Soylemezoglu G (2006) AFLP analysis of genetic variation within the two economically important Anatolian grapevine (*Vitis vinifera* L.) varietal groups. Genome 49(5):467–475
31. Esteve-Zarzoso B, Belloch C, Uruburu F, Querol A (1999) Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. Int J Syst Bacteriol 49(1):329–337. doi:10.1099/00207713-49-1-329
32. Esteve-Zarzoso B, Fernandez-Espinar MT, Querol A (2004) Authentication and identification of *Saccharomyces cerevisiae* 'flor' yeast races involved in sherry ageing. Antonie van Leeuwenhoek 85(2):151–158. doi:10.1023/B:ANTO.0000020282.83717.bd
33. Fay JC, Benavides JA (2005) Evidence for domesticated and wild populations of *Saccharomyces cerevisiae*. PLoS Gent 1(1):e5. doi:10.1371/journal.pgen.0010005
34. Fernandez-Espinar MT, Esteve-Zarzoso B, Querol A, Barrio E (2000) RFLP analysis of the ribosomal internal transcribed spacers and the 5.8S rRNA gene region of the genus *Saccharomyces*: a fast method for species identification and the differentiation of flor yeasts. Antonie van Leeuwenhoek 78(1):87–97. doi:10.1023/A:1002741800609
35. Fernandez-Espinar MT, Martorell P, de Llanos R, Querol A (2006) Molecular methods to identify and characterize yeasts in foods and beverages. In: Querol A, Fleet GM (eds) The yeast handbook-yeasts in food and beverages. Springer, Berlin Heidelberg New York, pp 55–82. doi:10.1007/978-3-540-28398-0_3
36. Fernandez-Gonzalez M, Espinosa JC, Ubeda JF, Briones AI (2001) Yeasts present during wine fermentation: comparative analysis of conventional plating and PCR-TTGE. Syst Appl Microbiol 24(4):634–638. doi:10.1078/0723-2020-00072
37. Fröhlich J, König H, Claus H (2009) Rapid detection and identification with molecular methods. In: König H, Unden G, Fröhlich J (eds) Biology of microorganisms on grapes, in must and in wine. Springer, Berlin Heidelberg New York, pp 429–449. doi:10.1007/978-3-540-85463-0
38. Fugelsang KC, Zoecklein BW (2003) Population dynamics and effects of *Brettanomyces bruxellensis* strains on pinot noir (*Vitis vinifera* L.) wines. Am J Enol Vitic 54:294–300
39. Gallego FJ, Perez MA, Nunez Y, Hidalgo P (2005) Comparison of RAPDs, AFLPs and SSR markers for the genetic analysis of yeast strains of *Saccharomyces cerevisiae*. Food Microbiol 22(6):561–568. doi:10.1016/j.fm.2004.11.019
40. Gindreau E, Walling E, Lonvaud-Funel A (2001) Direct polymerase chain reaction detection of ropy *Pediococcus damnosus* strains in wine. J Appl Microbiol 90:535–542. doi:10.1046/j.1365-2672.2001.01277.x
41. Giraffa G, D Carminati (2008) Molecular techniques in food fermentation: principals and applications. In: Cocolin L, Ercolini D (eds) Molecular techniques in the microbial ecology of fermented foods. Springer, Berlin Heidelberg New York, pp 162–192. doi:10.1007/978-0-387-74520-6_1
42. Graca da Silveira M, Vitoria San Romao M, Loureiro-Dias MC, Rombouts FM, Abee T (2002) Flow cytometric assessment of membrane integrity of ethanol-stressed *Oenococcus oeni* cells. Appl Environ Microbiol 68(12):6087–6093. doi:10.1128/AEM.68.12.6087-6093.2002
43. Grando MS, Bellin D, Edwards KJ, Pozzi C, Stefanini M, Velasco R (2003) Molecular linkage maps of *Vitis vinifera* L. and *Vitis riparia* Mchx. Theor Appl Genet 106(7):1213–1324. doi:10.1007/s00122-002-1170-3
44. Guillamon JM, Sabate J, Barrio E, Cano J, Querol A (1998) Rapid identification of wine yeast species based on RFLP analysis of the ribosomal internal transcribed spacer (ITS) region. Arch Microbiol 169(5):387–392. doi:10.1007/s002030050587
45. Hanna SE, Connor CJ, Wang HH (2005) Real-time polymerase chain reaction for the food microbiologist: technologies, applications, and limitations. J Food Sci 70(3):R49–R53. doi:10.1111/j.1365-2621.2005.tb07149.x
46. Hierro N, Gonzalez A, Mas A, Guillamon JM (2006) Diversity and evolution of non-*Saccharomyces* yeasts populations during wine fermentation: effect of grape ripeness and cold maceration. FEMS Yeast Res 6(1):102–111. doi:10.1111/j.1567-1364.2005.00014.x
47. Hierro N, Esteve-Zarzoso B, Gonzalez A, Mas A, Guillamon JM (2006) Real-time quantitative PCR (QPCR) and reverse transcription-QPCR for detection and enumeration of total yeasts in wine. Appl Environ Microbiol 72(11):7148–7155. doi:10.1128/AEM.00388-06
48. Hirschhäuser S, Fröhlich J, Gneipel A, Schönig I, König H (2005) Fast protocols for the 5S rDNA and ITS-2-based identification of *Oenococcus oeni*. FEMS Microbiol Lett 244(1):165–171. doi:10.1016/j.femsle.2005.01.033
49. Ibeas JI, Lozano I, Perdigones F, Jimenez J (1996) Detection of Dekkera-Brettanomyces strains in sherry by a nested PCR method. Appl Environ Microbiol 62(3):998–1003
50. Kell DB, Kaprelyants AS, Weichart DH, Harwood CR, Barer MR (1998) Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. Antonie van Leeuwenhoek 73(2):169–187. doi:10.1023/A:1000664013047
51. Kurtzman CP (2006) Yeast species recognition from gene sequence analyses and other molecular methods. Mycoscience 47(2):65–71. doi:10.1007/s10267-006-0280-1
52. Kurtzman CP, Robnett CJ (1998) Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Antonie Van Leeuwenhoek 73(4):331–371. doi:10.1023/A:1001761008817
53. Laforgue R, Guérin L, Pernelle JJ, Monnet C, Dupont J, Bouix M (2009) Evaluation of PCR-DGGE methodology to monitor fungal communities on grapes. J Appl Microbiol 107(4):1208–1218. doi:10.1111/j.1365-2672.2009.04309.x
54. Larisika M, Claus H, König H (2008) Pulsed-field gel electrophoresis for the discrimination of *Oenococcus oeni* isolates from different wine-growing regions in Germany. Int J Food Microbiol 123:171–176. doi:10.1016/j.ijfoodmicro.2007.11.081

55. Le Jeune C, Lonvaud-Funel A, ten Brink B, Hofstra H, van der Vossen JMBM (1995) Development of a detection system for histidine decarboxylating lactic acid bacteria based on DNA probes, PCR and activity test. J Appl Microbiol 78(3):316–326. doi:10.1111/j.1365-2672.1995.tb05032.x
56. Legras J-L, Karst F (2003) Optimisation of interdelta analysis for *Saccharomyces cerevisiae* strain characterization. FEMS Microbiol Lett 221:249–255. doi:10.1016/S0378-1097(03)00205-2
57. Legras J-L, Merdinoglu D, Cornuet J-M, Karst F (2007) Bread, beer and wine: *Saccharomyces cerevisiae* diversity reflects human history. Mol Ecol 16(10):2091–2102. doi:10.1111/j.1365-294X.2007.03266.x
58. Li SS, Cheng C, Li Z, Chen JY, Yan B, Han BZ, Reeves M (2010) Yeast species associated with wine grapes in China. Int J Food Microbiol 138:85–90. doi:10.1016/j.ijfoodmicro.2010.01.009
59. Lopandic K, Gangl H, Wallner E, Tscheik G, Leitner G, Querol A, Borth N, Breitenbach M, Prillinger H, Tiefenbrunner W (2007) Genetically different wine yeast isolated from Austrian vine-growing regions influence wine aroma differently and contain putative hybrids between *Saccharomyces cerevisiae* and *Saccharomyces kudriavzevii*. FEMS Yeast Res 7(6):953–965. doi:10.1111/j.1567-1364.2007.00240.x
60. Lopez I, Ruiz-Larrea F, Cocolin L, Orr E, Phister T, Marshall M, VanderGheynst J, Mills DA (2003) Design and evaluation of PCR primers for analysis of bacterial populations in wine by denaturing gradient gel electrophoresis. Appl Environ Microbiol 69(11):6801–6807. doi:10.1128/AEM.69.11.6801-6807.2003
61. Lopez V, Fernández-Espinar MT, Barrio E, Ramón D, Querol A (2003) A new PCR-based method for monitoring inoculated wine fermentations. Int J Food Microbiol 81(1):63–71. doi:10.1016/S0168-1605(02)00194-0
62. Madigan MT, Martinko JM (2006) Brock biology of microorganisms, 11th edn. Person Prentice Hall, Upper Saddle River, NJ
63. Maiden MC (2006) Multilocus sequence typing of bacteria. Annu Rev Microbiol 60:561–588. doi:10.1146/annurev.micro.59.030804.121325
64. Maiden MCJ, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, Zhang Q, Zhou J, Zurth K, Caugant DA, Feavers IM, Achtman M, Spratt BG (1998) Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci USA 95(6):3140–3145
65. Makarova K, Slesarev A, Wolf Y, Sorokin A, Mirkin B, Koonin E, Pavlov A, Pavlova N, Karamychev V, Polouchine N, Shakhova V, Grigoriev I, Lou Y, Rohksar D, Lucas S, Huang K, Goodstein DM, Hawkins T, Plengvidhya V, Welker D, Hugues J, Goh Y, Benson A, Baldwin K, Lee J-H, Diaz-Muñiz I, Dosti B, Smeianov V, Wechter W, Barabote R, Lorca G, Altermann E, Barrangou R, Ganesan B, Xie Y, Rawsthorne H, Tamir D, Parker C, Breidt F, Broadbent J, Hutkins R, O'Sullivan DJ, Unlu G, Saier M, Klaenhammer T, Richardson P, Kozyavkin S, Weimer B, Mills D (2006) Comparative genomics of the lactic acid bacteria. Proc Natl Acad Sci USA 103(42):15611–15616. doi:10.1073/pnas.0607117103
66. Malacrino P, Zapparoli G, Torriani S, Dellaglio F (2001) Rapid detection of viable yeasts and bacteria in wine by flow cytometry. J Microbiol Methods 45(2):127–134. doi:10.1016/S0167-7012(01)00243-3
67. Manzano M, Medrala D, Giusto C, Bartolomeoli I, Urso R, Comi G (2006) Classical and molecular analyses to characterize commercial dry yeasts used in wine fermentations. J Appl Microbiol 100(3):599–607. doi:10.1111/j.1365-2672.2005.02807.x
68. Manzano M, Cocolin L, Longo B, Comi G (2004) PCR-DGGE differentiation of strains of *Saccharomyces* sensu stricto. Antonie van Leeuwenhoek 85(1):23–27. doi:10.1023/B:ANTO.0000020270.44019.39
69. Manzano M, Cocolin L, Iacumin L, Cantoni C, Comi G (2005) A PCR-TGGE (Temperature Gradient Gel Electrophoresis) technique to assess differentiation among enological *Saccharomyces cerevisiae* strains. Int J Food Microbiol 101(3):333–339. doi:10.1016/j.ijfoodmicro.2004.10.049
70. Martorell P, Barata A, Malfeito-Ferreira M, Fernández-Espinar MT, Loureiro V, Querol A (2006) Molecular typing of the yeast species *Dekkera bruxellensis* and *Pichia guilliermondii* recovered from wine-related sources. Int J Food Microbiol 106(1):79–84. doi:10.1016/j.ijfoodmicro.2005.05.014
71. Millet V, Lonvaud-Funel A (2000) The viable but nonculturable state of wine micro-organisms during storage. Lett Appl Microbiol 30(2):136–141. doi:10.1046/j.1472-765x.2000.00684.x
72. Mills DA, Phister T, Neeley E, Johannsen E (2008) Wine Fermentation. In: Cocolin L, Ercolini D (eds) Molecular techniques in the microbial ecology of fermented foods. Springer, Berlin Heidelberg New York, pp 162–192. doi:10.1007/978-0-387-74520-6_6
73. Miot-Sertier C, Lonvaud-Funel A (2007) Development of a molecular method for the typing of *Brettanomyces bruxellensis* (*Dekkera bruxellensis*) at the strain level. J Appl Microbiol 102(2):555–562. doi:10.1111/j.1365-2672.2006.03069.x
74. Mitrakul CM, Henick-Kling T, Egli CM (1999) Discrimination of *Brettanomyces/Dekkera* yeast isolates from wine by using various DNA fingerprinting methods. Food Microbiol 16(1):3–14. doi:10.1006/fmic.1998.0217
75. Muñoz R, Gómez A, Robles V, Rodriguez P, Cebollero E, Tabera L, Carrascosa AV, Gonzalez R (2009) Multilocus sequence typing of oenological *Saccharomyces cerevisiae* strains. Food Microbiol 26(8):841–846. doi:10.1016/j.fm.2009.05.009
76. Muyzer G, Smalia K (1998) Application of denaturing gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie van Leeuwenhoek 73(1):127–141. doi:10.1023/A:1000669317571
77. Muyzer G, deWaal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59(3):695–700
78. Neeley ET, Phister TG, Mills DA (2005) Differential real-time PCR assay for enumeration of lactic acid bacteria in wine. Appl Environ Microbiol 71(12):8954–8957. doi:10.1128/AEM.71.12.8954-8857.2005
79. Nielsen PE, Egholm M, Buchardt O (1994) Peptide nuclei acid (PNA) A DNA mimic with a peptide backbone. Bioconjug Chem 5(1):3–7. doi:10.1021/bc00025a001
80. Nocker A, Camper AK (2006) Selective removal of DNA from dead cells of mixed bacterial communities by use of ethidium monoazide. Appl Environ Microbiol 72(3):1997–2004. doi:10.1128/AEM.72.3.1997-2004.2006
81. Nocker A, Burr M, Camper AK (2007) Genotypic microbial community profiling: a critical technical review. Microb Ecol 54(2):276–289. doi:10.1007/s00248-006-9199-5
82. Odds FC, Jacobsen MD (2008) Multilocus sequence typing of pathogenic *Candida* species. Eukaryot Cell 7(7):1075–1084. doi:10.1128/EC.00062-08
83. Oliveira K, Hasse G, Kurtzman C, Hyldig-Nielsen JJ, Stender H (2001) Differentiation of *Candida albicans* and *Candida dubliniensis* by fluorescent in situ hybridization with peptide nucleic acid probes. J Clin Microbiol 39(11):4138–4141. doi:10.1128/JCM.39.11.4138-4141.2001
84. Perrone G, Susca A, Epifani F, Mulè G (2006) AFLP characterization of Southern Europe population of *Aspergillus* Section

Nigri from grapes. Int J Food Microbiol 111:S22–S27. doi:10.1016/j.ijfoodmicro.2006.03.009

85. Perrone G, Mulè G, Susca A, Battilani P, Pietri A, Logrieco A (2006) Ochratoxin A production and amplified fragment length polymorphism analysis of *Aspergillus carbonarius*, *Aspergillus tubingensis*, and *Aspergillus niger* strains isolated from grapes in Italy. Appl Environ Microbiol 72:680–685. doi:10.1128/AEM.72.1.680-685.2006
86. Perry-O'Keefe H, Stender H, Broomer A, Oliveira K, Coull J, Hyldig-Nielsen JJ (2001) Filter-based PNA in situ hybridization for rapid detection, identification and enumeration of specific micro-organisms. J Appl Microbiol 90(2):180–189. doi:10.1046/j.1365-2672.2001.01230.x
87. Peters TM (2009) Pulsed-field gel electrophoresis for molecular epidemiology of food pathogens. Methods Mol Biol 551:59–70. doi:10.1007/978-1-60327-999-4_6
88. Peterson SW, Kurtzman CP (1991) Ribosomal RNA sequence divergence among sibling species of yeasts. Syst Appl Microbiol 14(2):124–129
89. Phister TG, Mills DA (2003) Real-time PCR assay for detection and enumeration of *Dekkera bruxellensis* in wine. Appl Environ Microbiol 69:7430–7434. doi:10.1128/AEM.69.12.7430-7434.2003
90. Querol A, Barrio E, Ramon D (1992) A comparative study of different methods of yeast strain characterization. Syst Appl Microbiol 15(3):439–446
91. Rantsiou K, Comi G, Cocolin L (2004) The *rpo*B gene as a target for PCR-DGGE analysis to follow lactic acid bacterial population dynamics during food fermentations. Food Microbiol 21(4):481–487. doi:10.1016/j.fm.2003.10.002
92. Raspor P, Milek DM, Polanc J, Možina SS, Čadež N (2006) Yeasts isolated from three varieties of grapes cultivated in different locations of the Dolenjska vine-growing region, Slovenia. Int J Food Microbiol 109(1–2):97–102. doi:10.1016/j.ijfoodmicro.2006.01.017
93. Rawsthorne H, Phister TG (2009) Detection of viable *Zygosaccharomyces bailii* in fruit juices using ethidium monoazide bromide and real-time PCR. Int J Food Microbiol 131(2–3):246–250. doi:10.1016/j.ijfoodmicro.2009.01.031
94. Renouf V, Claisse O, Lonvaud-Funel A (2007) Inventory and monitoring of wine microbial consortia. Appl Microbiol Biotechnol 75(1):149–164. doi:10.1007/s00253-006-0798-3
95. Renouf V, Claisse O, Miot-Sertier C, Lonvaud-Funel A (2006) Lactic acid bacteria evolution during winemaking: use of *rpo*B gene as a target for PCR-DGGE analysis. Food Microbiol 23:136–145. doi:10.1016/j.fm.2005.01.019
96. Rodas AM, Ferrer S, Pardo I (2005) Polyphasic study of wine *Lactobacillus* strains: taxonomic implications. Int J Syst Evol Microbiol 55(1):197–207. doi:10.1099/ijs.0.63249-0
97. Röder C, König H, Fröhlich J (2007) Species-specific identification of *Dekkera/Brettanomyces* yeasts by fluorescently labeled DNA probes targeting the 26S rRNA. FEMS Yeast Res 7(6):1013–1026. doi:10.1111/j.1567-1364.2007.00267.x
98. Ruiz A, Poblet M, Mas A, Guillamon JM (2000) Identification of acetic acid bacteria by RFLP of PCR-amplified 16S rDNA and 16S–23S rDNA intergenic spacer. Int J Syst Evol Microbiol 50(6):1981–1987
99. Ruiz P, Izquierdo PM, Seseña S, Palop ML (2010) Selection of autochthonous *Oenococcus oeni* strains according to their oenological properties and vinification results. Int J Food Microbiol 137(2–3):230–235. doi:10.1016/j.ijfoodmicro.2009.11.027
100. Salinas F, Mandaković D, Urzua U, Massera A, Miras S, Combina M, Ganga MA, Martinez C (2010) Genomic and phenotypic comparison between similar wine yeast strains of *Saccharomyces cerevisiae* from different geographic origins. J Appl Microbiol 108(5):1850–1858. doi:10.1111/j.1365-2672.2010.04689.x
101. Schuller D, Valero E, Dequin S, Casel M (2004) Survey of molecular methods for the typing of wine yeast strains. FEMS Micro Lett 231(1):19–26. doi:10.1016/S0378-1097(03)00928-5
102. Selma MV, Martinez-Culebras PV, Elizaquivel P, Aznar R (2009) Simultaneous detection of the main black aspergilli responsible for ochratoxin A (OTA) contamination in grapes by multiplex real-time polymerase chain reaction. Food Addit Contam 26(2):180–188. doi:10.1080/02652030802345623
103. Serpaggi V, Remize F, Grand AS, Alexandre H (2010) Specific identification and quantification of the spoilage microorganism *Brettanomyces* in wine by flow cytometry: a useful tool for winemakers. Cytometry A 77A(6):497–499. doi:10.1002/cyto.a.20861
104. Sohier D, Lonvaud-Funel A (1998) Rapid and sensitive in situ hybridization method for detecting and identifying lactic acid bacteria in wine. Food Microbiol 15(4):391–397. doi:10.1006/fmic.1998.0189
105. Sohier D, Coulon J, Lonvaud-Funel A (1999) Molecular identification of *Lactobacillus hilgardii* and genetic relatedness with *Lactobacillus brevis*. Int J Syst Bacteriol 49(3):1075–1081. doi:10.1099/00207713-49-3-1075
106. Solieri L, Giudici P (2010) Development of SCAR marker-targeted quantitative PCR assay for strain-specific detection of *Oenococcus oeni* during wine malolactic fermentation. Appl Environ Microbiol 76:7765–7774. doi:10.1128/AEM.00929-10
107. Stender H, Kurtzman C, Hyldig-Nielsen JJ, Sørensen D, Broomer A, Oliveira K, Perry-O'Keefe H, Sage A, Young B, Coull J (2001) Identification of *Dekerra bruxellensis* (*Brettanomyces*) from wine by fluorescence in situ hybridization using peptide nucleic acid probes. Appl Environ Microbiol 67(2):938–941. doi:10.1128/AEM.67.2.938-941.2001
108. Tessonnière H, Vidal S, Barnavon L, Alexandre H, Remize F (2009) Design and performance testing of a real-time PCR assay for sensitive and reliable direct quantification of *Brettanomyces* in wine. Int J Food Microbiol 129(3):237–243. doi:10.1016/j.ijfoodmicro.2008.11.027
109. Urso R, Rantsiou K, Dolci P, Rolle L, Comi G, Cocolin L (2008) Yeast biodiversity and dynamics during sweet wine production as determined by molecular methods. FEMS Yeast Res 8(7):1053–1062. doi:10.1111/j.1567-1364.2008.00364.x
110. Wilson IG (1997) Inhibition and facilitation of nucleic acid amplification. Appl Environ Microbiol 63(10):3741–3751
111. Winzeler EA, Castillo-Davis CI, Oshiro G, Liang D, Richards DR, Zhou Y, Hartl DL (2003) Genetic diversity in yeast assessed with whole-genome oligonucleotide arrays. Genetics 163(1):79–89
112. Xufre A, Albergaria H, Inácio J, Spencer-Martins I, Gírio F (2006) Application of fluorescence in situ hybridization (FISH) to the analysis of yeast population dynamics in winery and laboratory grape must fermentations. Int J Food Microbiol 108(3):376–384. doi:10.1016/j.ijfoodmicro.2006.01.025
113. Zapparoli G, Torriani S, Pesente P, Dellaglio F (1998) Design and evaluation of malolactic enzyme gene targeted primers for rapid identification and detection of *Oenococcus oeni* in wine. Lett Appl Microbiol 27(5):243–246. doi:10.1046/j.1472-765X.1998.00448.x
114. Zavaleta AI, Martinez-Murcia AJ, Rodriguez-Valera F (1997) Intraspecific genetic diversity of *Oenococcus oeni* as derived from DNA fingerprinting and sequence analyses. Appl Environ Microbiol 63(4):1261–1267