

REALTIME PCR SYSTEMS TO MONITOR YEASTS
IN GRAPE MUST AND WINE

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REALTIME PCR SYSTEMS TO MONITOR YEASTS IN GRAPE MUST AND WINE

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The quality of beverages, particularly wine, depends largely on their microflora. Yeast and bacterial fermentations shape the product flavor profile, and can cause spoilage. Current detection methods for microorganisms are usually either inaccurate, slow, or both. In this project, realtime PCR systems were designed to test wines for the presence of certain microorganisms.

The sampling method during wine production was optimized to ascertain that the microflora in the samples is representative of the whole tank. Four different sampling locations within the vertical axis of stainless steel tanks were tested during both the alcoholic and the malolactic fermentation. *Saccharomyces cerevisiae* and *Oenococcus oeni* were enumerated by plating. The results showed that a representative number of viable cells is obtained by taking a sample from the sampling valve.

Part of the actin gene (*Act1*) was sequenced as the basis for the realtime PCR.

Detection systems for the three undesirable wine yeasts *Brettanomyces bruxellensis*, *Hanseniaspora uvarum*, and *Pichia anomala* were designed and validated. The extraction methods were optimized to ensure that they were quantitative within a suitable range. The specificity of the detection systems as well as their threshold and range were very satisfactory.

The realtime PCR system for *B. bruxellensis* was used to determine whether there is a quantitative connection between *B. bruxellensis* contamination and the marker phenols

4-ethylphenol, 4-ethylguaiacol, and 4-ethylcatechol or the subjective sensory impression. The experiments show that although *B. bruxellensis* does produce the known marker phenols, there is no mathematical correlation between the volatile phenol concentration and cell counts.

The influence of sanitation procedures on the occurrence of *B. bruxellensis* in wineries was investigated using plating and realtime PCR. *B. bruxellensis* was found in most locations in wineries, including the wines.

The inheritance of the teinturier phenotype was studied in a cross segregating for this character. Segregation results suggest that the primary gene controlling berry skin color also controls berry flesh pigmentation.

This new detection method is faster, more accurate, and more sensitive than previous microbial and chemical detection methods. Routine testing of beverages would minimize the effects of spoilage microorganisms, resulting in decreased product losses for the beverage producers.

BIOGRAPHICAL SKETCH

Naomi Porret was born in Cotonou, Benin on April 23, 1971, and grew up in Zürich and Moutier, Switzerland. After completing the Scientific option (Type C) at the 'Deutsches Gymnasium Biel' Academic High School, she studied Veterinary Medicine at the University of Neuchâtel. She received a Diplom Naturwissenschaft ETHZ at the Swiss Federal Institute of Technology in Zürich, with a concentration in the field of systematic and ecological biology (option XAa). She presented her Diploma Thesis 'Impact of the slug *Arion lusitanicus* on early secondary succession' at the Geobotanical Institute with Prof. Dr. P. J. Edwards in the summer of 1997. She also received several teaching assistantships in different fields. After graduation, she was a scientific employee at the Agroscope FAW Wädenswil (Switzerland), where she worked in the field of molecular diagnostics (wine microbiology, microsatellite analyses for grape varieties and virology).

For my mother, Dr. Marianne Porret, who never gave up on me.

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CHAPTER 1: INTRODUCTION

Beverages, as other foods, are very susceptible to spoilage by microorganisms. A wide range of microorganisms can be found in foods and beverages. Some of them are essential as agents to process the foods. Others can render the food undesirable for consumption, either by producing off-flavors or allergenic substances. And some can be harmful to humans when consumed with the food. Therefore the ability to detect spoilage microorganisms before they accumulate in high numbers, and undesired substances are formed, is a very valuable and important tool in enhancing food quality.

Microorganisms in wine

Beverages with low pH, low sugar, or high ethanol content and few available energy sources limit opportunities for microbial growth. Since wine typically has a low pH and high alcohol content, it is a difficult environment for microorganisms to survive (Kalathenos et al., 1995). Potential spoilage microorganisms occur on grapes and in the winery environment, uncontrolled growth or production of off-flavors during fermentation makes their presence undesired. For this reason, winemakers usually inoculate their musts with the yeast *Saccharomyces* and with the bacteria *Oenococcus oeni* to obtain a more predictable alcoholic and malolactic fermentation, respectively (Krieger et al., 1993; Lallemand, 1998; Laurent et al., 1994).

Hanseniaspora uvarum is the most frequent yeast in grape musts, comprising 50 to 90% of the yeast flora (Gafner et al., 1996). *H. uvarum* was found in vineyards both in Israel (Zahavi et al., 2002) and South Africa (Pretorius et al., 1999). The spoilage yeast *H. uvarum*, also called *Kloeckera apiculata* in its asexual form, is capable of fully fermenting the sugar in grape must to ethanol, but can produce up to 2 g/l of

acetic acid during the process. Acetic acid undergoes esterification in the presence of alcohol, resulting in an agreeable flavor. However, if esterification goes too far, the wine will take on a nail polish remover type of odor, and is considered unfit for consumption. The formation of the different esters cannot be controlled by the winemaker, so it is much safer to prevent *H. uvarum* from taking over the fermentation. *H. uvarum* disturbs the alcoholic fermentation from *S. cerevisiae* in mixed cultures, lowering the final ethanol concentration from 11% to 6.4% (Velazquez et al., 1991). It was found 50 years ago that *H. uvarum* disturbs the fermentation when it is present at similar cell numbers as *S. cerevisiae*, lowering the final amount of ethanol and slowing the fermentation, but that the fermentation is activated when *H. uvarum* is present in small numbers (Schulle, 1953). This yeast is sensitive to ethanol: at 15°C, growth is inhibited with 9% ethanol, but the cells can survive in 10-12.5% of ethanol. The alcohol tolerance of *H. uvarum* is usually highest at a temperature of 15°C. Alcohol tolerance is only marginally influenced by the pH between pH 3 and pH 6 (Gao and Fleet, 1988). In mixed cultures of *S. cerevisiae* and *H. uvarum*, *S. cerevisiae* is highly favored at 20°C and 25°C, and *H. uvarum* dies off. But at temperatures below 20°C, the influence of *H. uvarum* increases, and the fermentation is carried out by both yeasts (Heard and Fleet, 1988). *H. uvarum* can produce increased amounts of volatile substances, like acetoin. Ciani and Ferraro found that up to 145 mg/l of acetoin, up to 763 mg/l ethylacetate and 68 mg/l acetaldehyde can be produced by this yeast (Ciani and Maccarelli, 1998). Mateo et al. report levels of 870 mg/l ethylacetate (Mateo et al., 1991), and Romano et al. up to 187 mg/l acetoin (Romano et al., 1993). *H. uvarum* is present in high cell numbers at the beginning of the alcoholic fermentation (Mortimer, 1995). Cold settling or bentonite treatments have little influence on the cell numbers of *H. uvarum* at the beginning of the fermentation (Mora and Mulet, 1991), but *H. uvarum* tends to

decrease sharply during the alcoholic fermentation (Lema et al., 1996).

The yeast *Pichia anomala* can occur in grape musts, during both fermentations and later, during wine maturation. Although not a very strong fermenter, *P. anomala* survives and can form up to 1200 mg/l of ethyl acetate in pure culture (Rojas et al., 2003). If *P. anomala* has some oxygen at its disposal, it can grow on the surface of the wine and produce unpleasant odors. In anaerobic culture, the ethyl acetate increases tenfold compared to aerobic growth for a short period, and is still higher after six hours (Fredlund et al., 2004). *P. anomala* can produce a fungicidal killer toxin which can kill *Brettanomyces* even if *P. anomala* cells are not present (Comitini et al., 2004).

During the maturation of red wines in barrels, the yeast *Dekkera bruxellensis* (also called *Brettanomyces bruxellensis* in its asexual form, treated as synonyms in this work) can lead to the 'bretty' off-flavor (Bravo-Plasencia and Bravo-Abad, 1996; Chatonnet et al., 1997; Llanos Company, 1997). Bretty wine aroma has been compared to horse stable, horse sweat, band-aid, dried cow manure, medicinal flavors and others (Licker et al., 1999). Additionally, *B. bruxellensis* can produce increased amounts of acetic acid. The production of acetic acid under anaerobic conditions is very low, but it increases dramatically in aerobiosis: up to 24 g/l (Freer, 2002) or 14 g/l (Ciani and Ferraro, 1997). Studies on the occurrence of *B. bruxellensis* in different wine musts and wines were carried out (Larue et al., 1991), and the influence of different treatments of wine in barrels were investigated. This yeast produces two marker substances: 4-ethylphenol and 4-ethylguaiacol. Recently, a third potential marker phenol was found: 4-ethylcatechol (Hesford et al., 2005). Small concentrations of bretty aroma compounds may increase the wine's aroma complexity if they are kept at concentrations near the odor threshold (Arvik and Henick-Kling, 2002; Fugelsang, 1998). Some winemakers are convinced that any amount of *Brettanomyces* is bad for a

winery, while others like the character that some *B. bruxellensis* strains may give an under-ripe, simple fruity wine. Because there is evidence for the global presence of “Brett” (Fugelsang, 1997), efforts at finding solutions were increased recently. *B. bruxellensis* can also produce some biogenic amines (Caruso et al., 2002).

Intensive work on this subject has been carried out by the Cornell Enology Research Group (Geneva, NY) and their collaborators. Sensory influence of *B. bruxellensis* described defects in red wines as 'phenolic', 'plastic', 'smoky', 'barnyard' or 'mousy' (Egli et al., 1998; Henick-Kling et al., 2000; Licker, 1998; Licker et al., 1999).

Moreover, several methods were evaluated to detect *B. bruxellensis* using advanced molecular techniques (Egli and Henick-Kling, 2001; Mitrakul et al., 1999). Indications that sub-types of *B. bruxellensis* exist with differing physiological habits were recently found (unpublished data, Cornell enology group). Furthermore, in a survey of Finger Lakes Cabernet Franc and Pinot Noir wines, the 4-ethylphenol and 4-ethylguaiacol concentrations were related to cultivation of *B. bruxellensis* isolates from the same wines and characterized using molecular methods (Conterno and Henick-Kling, 2003; Egli et al., 2000).

Three different lactic acid bacteria occur frequently on grapes and in musts. These bacteria are able to perform malolactic fermentation (MLF), transforming malic acid into lactic acid (Henick-Kling, 1993). The desired *Oenococcus oeni* must outgrow the undesired species *Pediococcus* spp. and *Lactobacillus* spp., which can produce unwanted compounds with sugars that remain in the wine. *L. brevis* strains can produce up to 5 g/l of acetic acid. The presence of *P. damnosus* and *L. brevis* can lead to a concentration of lactic acid up to three times higher than normal. *L. brevis* can result in an unpleasant ‘mousy’ flavor (Heresztyn, 1986) and *P. damnosus* can produce high concentrations of diacetyl and biogenic amines, as well as ropiness (polysaccharides).

Traditional yeast detection methods

Usually, wines are plated on solid agar media to detect and quantify the living yeasts that are present in the sample. This method yields colony forming units (CFU), which are counted and reported as the number of yeast cells capable of growing on a particular media and a given temperature. The colonies are then studied with molecular methods to determine either the yeast species or strain differentiation (Querol and Ramòn, 1996). To determine strain differences, a very reliable method is karyotyping by PFGE (pulsed field gel electrophoresis) (Schütz and Gafner, 1993; Schütz and Gafner, 1994) and microsatellite analysis of the yeast (Richard Gardner, personal communication). For species differentiation, the most common method is sequencing of ribosomal DNA, particularly to compare phylogenetic relationships (Boekhout et al., 1994; Cai et al., 1996; Kurtzmann, 1992; Yamada et al., 1994). If the aim is to identify species without differentiating the strains, the yeast rDNA, often the internal transcribed spacer (ITS) region, can be amplified by PCR and then digested with restriction enzymes. The resulting gel electrophoresis band show a distinctive pattern for each species, depending on the enzymes that are used (Esteve-Zarzoso et al., 1999; Guillamón et al., 1998; Molina et al., 1993; Wyder and Puhán, 1997). Other useful determination methods are DNA fingerprinting (Lavallée et al., 1994), nested PCR (Ibeas et al., 1996) or intron splice sites (Lopes et al., 1996; Lopes et al., 1998). Non-molecular methods include selective plating media (del Pozo et al., 1991; Rodrigues et al., 2001) or the analysis of secondary products (Romano et al., 1997). The two major problems with the traditional plating technique are the delay in getting results and the nondetection of viable but not culturable cells (Millet and Lonvaud-Funel, 2000).

The Actin gene in yeasts

The complete actin sequence of *S. cerevisiae* was published 25 years ago (Gallwitz and Sures, 1980). After cloning it into *E. coli*, it was determined that *S. cerevisiae* contains only one copy of the actin gene (Ng and Abelson, 1980) and that the intron has conserved splicing sites (Gallwitz and Seidel, 1980). This structure was published in 1980. The intron sequences were then studied in more detail, and compared to the sequence of *S. carlsbergiensis* (which is now reclassified as *S. cerevisiae*). There were only three point mutations found in the intron of the gene (Nellen et al., 1981). The actin gene has been studied extensively in a variety of yeasts; there is a large family of actin related proteins (Poch and Winsor, 1997). Since the gene is conserved within one species, it is a valuable phylogenetic marker (Daniel et al., 2001). Similar intron-encoded splicing sites have been found in the actin gene of the yeast *Kluyveromyces lactis* (Deshler et al., 1989). Two sequences seem to be responsible for the splicing signal to be effective and accurate in *S. cerevisiae* (Castanotto and Rossi, 1998). This intron contains a promoter of an antisense RNA of unknown function (Thompson-Jäger and Domdey, 1990). *Candida dubliniensis* can be identified rapidly using the actin intron sequence for a PCR (Donnelly et al., 1999), and the phylogenetic relationship between *C. dubliniensis* and *C. albicans* was determined. Their introns are 97.9% identical, but their exons share only 83.4% bases. The actin sequence of *Hansenula polymorpha* is split by two introns (Kang et al., 2001), with different splicing sites. In contrast, the actin gene of *Schizosaccharomyces pombe* does not have an intron (Mertins and Gallwitz, 1987).

Realtime PCR using actin

TaqMan Real Time PCR works like a standard PCR, except that a probe is designed to anneal between the primers. This probe is an oligonucleotide carrying a fluorescent marker on the 5' end and a quencher on the 3' end. The quencher suppresses the fluorescence of the marker because it is only a short distance from it (the probes are 20 to 30 bases long). During each PCR cycle, the probe anneals to the DNA between the primers, and is cleaved by the 5' -3' exonuclease activity of the DNA polymerase. The quencher and the fluorescent marker are no longer in close proximity, and so the fluorescence of the PCR augments with each cycle. The Real Time PCR instrument measures the increase in fluorescence. A threshold of fluorescence is chosen manually, taking care to lie in the exponential phase of the PCR. The Real Time instrument calculates at which cycle (Ct) this threshold is crossed. The Ct is then compared to the Ct values of a standard curve with known yeast quantities, and the cell numbers of the samples are calculated.

Actin was used to calculate cell numbers from viable yeasts and molds in yogurt and pasteurized food products (Bleve et al., 2003). SybrGreen dye was used for the realtime PCR assays, which intercalates with double stranded DNA and can be read by realtime PCR instruments in a similar way to TaqMan probes. It was shown that actin is a reliable gene for this type of work. However, the primers that were used were not specific for certain yeast species, they amplify many different yeasts to allow to measure a general contamination. Another group used the *rad4* gene for a specific realtime system to detect *B. bruxellensis* (Delaherche et al., 2004), using a SybrGreen detection system. The resulting detection threshold was high at 10^4 cells/ml. *S. cerevisiae* wine strains were tested for their polyploidy. It was found that these

enologically important strains were commonly di- to tetraploid. (Guijo et al., 1997), and sometimes aneuploid (Pretorius and van der Westhuizen, 1991). This is important because it gives a measure of the reliability of realtime PCR to quantify total cell numbers. If the difference in ploidy is too large, it will affect the results, causing overestimating the actual number of yeast cells present. The viability of yeasts can be assessed using actin, because it is a housekeeping gene. As such, actin is expressed at each phase of the cell cycle, even if it is quite low compared to other genes (Riou et al., 1997).

Project objective and rationale

The aim of this project is to design a detection system which allows fast and accurate differentiation between organisms that are used for wine fermentations and spoilage organisms.

The new tests based on realtime PCR are meant to surpass the traditional methods of microorganism detection, like microscopy, enrichment with subsequent microscopy, or enrichment with PCR, in significance. Traditional microscopy does not allow the detection of microorganisms below 10^5 - 10^6 cells/ml. Realtime PCR is fast; samples can be processed in six hours.

Microbial monitoring is essential to produce high quality wines. Early detection of undesirable organisms can avoid product spoilage, including high concentrations of diacetyl, acetic or lactic acid; 'bretty' off-flavors, biogenic amines, mousy or ropy wines.

Timely knowledge of the presence of spoilage microorganisms in wine allows the the winemaker to take immediate action and prevent further deterioration. Testing with

realtime PCR would be appropriate if the winemaker notices off-flavors during processing, or wants to screen the barrels or fermenting tanks before bottling, or determine whether the wines need to be filtered. This would be especially important in wines with residual fermentable sugar.

With this new technique, spoilage organisms can be detected before major deterioration occurs. Each step of the fermentations, maturation, and aging in bottles can be monitored and appropriate actions can be taken in the case of the appearance of unwanted organisms. Massive quality and monetary losses could be prevented.

Teinturier character segregation

Grapevine (*Vitis vinifera* L.), one of the oldest domesticated crops, is mainly grown to produce different kinds of wine. Grapes are also used for grape juice, table grapes, dried or fermented as distilled beverages. Grape pigmentation shows a wide variety of colors: black, red, pink, white, green, gray and intermediate shades. For most grapevine cultivars, the pigments which define the color are located in the skin of the berry. This color is controlled by the gene *VvmybA1* (Kobayashi et al., 2004), with the red allele being dominant. Crosses between white berried varieties always yield white-berried offspring. The teinturier varieties, on the other hand, contain red pigments throughout the berry flesh. These plants also contain more anthocyanin pigments in their other tissues, especially the leaves. When the chlorophyll decomposes in the fall, the leaves of these varieties turn bright red. A well known example is the variety Rubired, which is grown in California and used mainly to blend with red wines to give them deeper color. The teinturier character of grapevines is known to be distinct. Crosses between a teinturier and a white berried variety can produce teinturier

offspring. Scarlet has colored berry flesh and is the result of a cross between Teinturier du Cher and Golden Muscat, making Scarlet heterozygous for the *VvmybA1* gene. The segregation of the color could be studied by crossing Scarlet with a white berried variety. If this gene alone is responsible for both the skin color and flesh color, the seedling population of the cross would be white: teinturier 1:1. If another gene is responsible for teinturier besides *VvmybA1*, and they are not linked, the distribution should be teinturier:red:white:white/teinturier 1:1:1:1.

The white allele of the gene *VvmybA1* contains a large insert in the promoter region, but is otherwise identical to the wild type. Sequencing of the gene of different teinturier varieties would also contribute understanding the color heredity. Differences in the transcribed part of the gene or in the promoter region might explain the teinturier phenomenon. Additionally, crosses could be tested genetically (marker assisted breeding) to help predict berry color without waiting for the grapevine to mature.

Preliminary work

Actin sequencing

The yeast strains that were used for this project were amplified with the ITS 4 primers to check their correct species affiliations (Guillamón et al., 1998). The ITS regions were then sequenced to compare them to prior sequences published in GenBank (www.ncbi.nlm.nih.gov). Several *Saccharomyces* yeasts were sequenced for the actin gene and compared to sequences on GenBank. The ITS region of five strains of *S. cerevisiae* and one strain of each *S. bayanus* and *S. uvarum* were sequenced. The intron sequences showed many differences, because this region is very variable and

because the sequencing was rendered difficult by several poly-A stretches. The intron goes from base number 11 to 315. In the exon 2 (base number 316 to 1432) the sequences were identical for *S. cerevisiae* except for dual bases. These are points where two peaks on top of each other in the electropherogram prevent the base determination, when sequenced from both sides. *S. bayanus* showed clear differences compared to *S. cerevisiae* (29 %). When there were differences between *S. cerevisiae* and *S. bayanus*, *S. uvarum* almost always had the corresponding dual base. When it was clear that the wine yeast strains did not show any significant differences to the published sequences on GenBank, the sequencing for *Saccharomyces* was stopped. Partial actin sequences were obtained for different wine relevant yeast species. As a comparison, the mRNA sequence NCBI L00026 from *S. cerevisiae* was used. This mRNA was determined to be 1129 base pairs long. *S. cerevisiae* has an intron after 10 bases which is 304 bases long. For *H. uvarum* several hundred bases more were sequenced on the 5' side, but the start codon could not be determined. All the sequences stop shortly before the stop codon on the 3' end. The alignment of the actin sequences of the different species which were sequenced are shown in FIGURE 1.1. The alignments and the figure were made with the program Sequencher version 4.0.5. In addition to *Saccharomyces*, the actin gene of 9 species were sequenced: *Dekkera bruxellensis*, *Dekkera anomala*, *Hanseniaspora uvarum*, *Hanseniaspora guilliermondii*, *Brettanomyces abstinens*, *Metschnikowia pulcherrima*, *Pichia anomala*, *Zygosaccharomyces bailii* and *Zygosaccharomyces fermentati*.

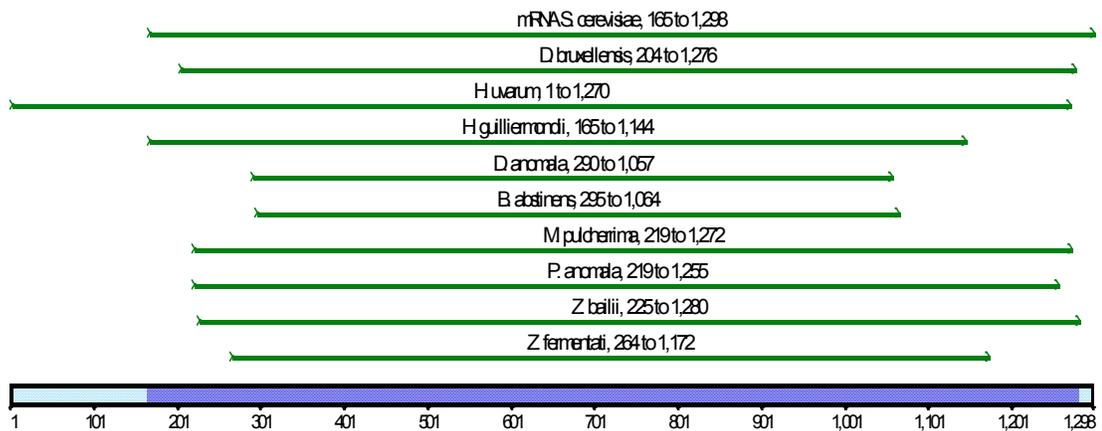


FIGURE 1.1 Alignment of the actin sequences from the different yeast species, shown against the mRNA of the actin gene of *S. cerevisiae* from GenBank (NCBI L00026)

The longest common sequence within the actin gene of all the yeast species (760 base pairs) was analyzed with the program BioEdit version 5.0.9. by comparing the sequence of each species against all of the other species. A sequence identity matrix was drawn to show the identity of each sequenced species against all the others. The results are shown in TABLE 1.1.

TABLE 1.1 Identity of the actin sequences over 760 base pairs for each sequenced species against all the others. The matrix was calculated with the program BioEdit version 5.0.9.

Species	<i>S. c.</i>	<i>H. u.</i>	<i>H. g.</i>	<i>D. b.</i>	<i>B. a.</i>	<i>D. a.</i>	<i>Z. f.</i>	<i>M. p.</i>	<i>P. a.</i>	<i>Z. b.</i>
<i>S cerevisiae</i>	1.00	0.86	0.85	0.84	0.83	0.83	0.88	0.84	0.84	0.85
<i>H uvarum</i>	---	1.00	0.95	0.83	0.82	0.81	0.82	0.81	0.85	0.81
<i>H guilliermondii</i>	---	---	1.00	0.82	0.81	0.82	0.84	0.81	0.84	0.82
<i>D bruxellensis</i>	---	---	---	1.00	0.99	0.93	0.82	0.81	0.79	0.82
<i>B abstinens</i>	---	---	---	---	1.00	0.93	0.82	0.81	0.78	0.82
<i>D anomala</i>	---	---	---	---	---	1.00	0.83	0.82	0.78	0.82
<i>Z fermentati</i>	---	---	---	---	---	---	1.00	0.85	0.76	0.88
<i>M pulcherrima</i>	---	---	---	---	---	---	---	1.00	0.78	0.85
<i>P anomala</i>	---	---	---	---	---	---	---	---	1.00	0.76
<i>Z bailii</i>	---	---	---	---	---	---	---	---	---	1.00

The complete sequences were aligned with the program ProSeq version 2.9. A neighbor-joining tree calculated with the program Mega version 2.1 can be found in FIGURE 1.2. The sequenced species can be structured clearly using the actin gene. The bootstrap numbers are especially high for the *Dekkera* and *Brettanomyces* species, indicating a close relationship between these organisms. Additionally, they are quite different from the other genera.

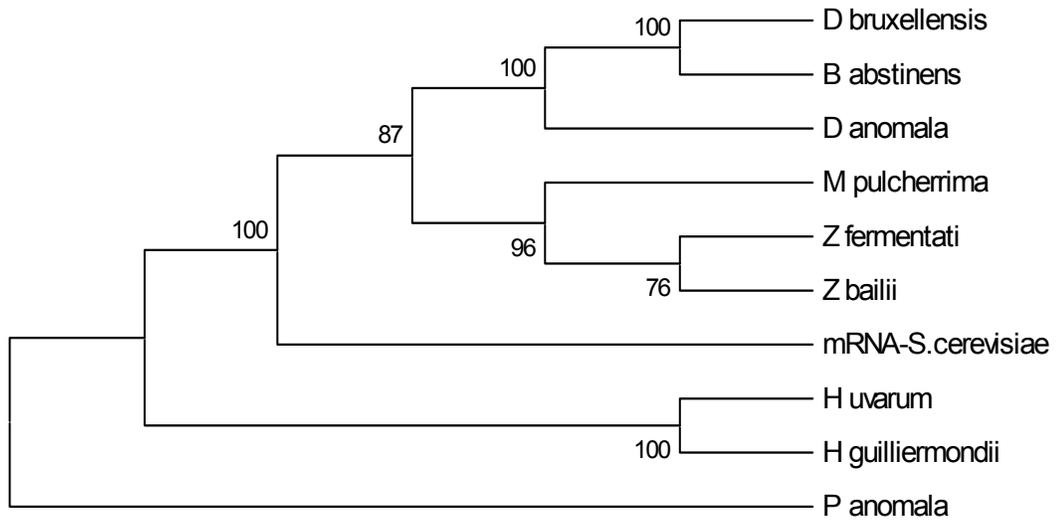


FIGURE 1.2 Neighbor-joining bootstrap consensus tree of the partial actin sequences of different wine relevant yeasts. The tree was calculated with the program Mega version 2.1, using the substitution model Kamura-2 parameters. The bootstrap values that are shown are for 500 replications.

Nine strains of *B. bruxellensis* and 10 strains of *H. uvarum* were partially sequenced for the actin gene. No actin sequences are deposited on GenBank for those species. Between the 9 sequenced *B. bruxellensis* strains, there were 12 loci with base differences; eleven of them were dual bases. The mutations were spread out over the gene. Between the 10 sequenced *H. uvarum* strains, there were 16 loci with base differences. The differences were usually changes between C/T/Y (cytosine/thymine/cytosine or thymine), and the mutations were spread out over the gene.

The differences in the actin gene sequences allow a clear differentiation of the three species *S. cerevisiae*, *B. bruxellensis* and *H. uvarum*. This difference is the basis for a quantitative species specific PCR analysis with DNA and RNA.

L. brevis in musts and wines

In the fall of 2003, 129 wines and musts were sampled and tested for *L. brevis* using realtime PCR system designed by Christina Uermösi from the Hochschule Wädenswil (Wädenswil, Switzerland). 82 of them came from the winery of the Forschungsanstalt Wädenswil (Wädenswil, Switzerland). The results of the realtime PCR fell between 0 and 10^4 cells/ml. Only 29 samples were negative. The wine-year 2003 in Europe was especially warm, and the musts often contained less acid, and therefore higher pH values, than usual, favoring the growth of *L. brevis* and other undesired lactic acid bacteria.

For six of the musts, the corresponding wines were tested again after the alcoholic fermentation to find out how the cell numbers of *L. brevis* were evolving FIGURE 1.3. For the musts 1, 4, 5 and 6, the fermentations were carried out in two separate tanks.

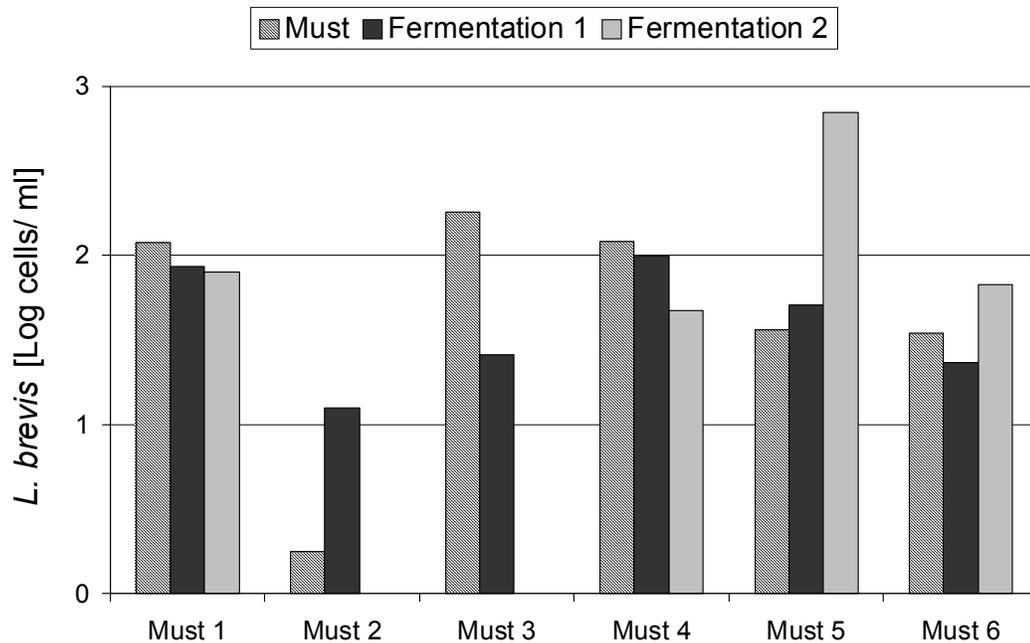


FIGURE 1.3 Comparison between the cell numbers of *L. brevis* in musts and the corresponding wines after the alcoholic fermentation.

The cell numbers clearly increased only in one case (must 5, especially in the second fermentation tank). The presence of *L. brevis* in musts does not necessarily lead to off-flavors in the final product, but wines with these bacteria should be closely monitored to avoid potential poor quality or un-salable wine.

Comparison between cell numbers and realtime PCR values from *H. uvarum* and *S. cerevisiae*

Wine samples were taken after the alcoholic fermentation and after the malolactic fermentation from a white wine (Müller-Thurgau grapes) and a red wine (Pinot Noir grapes). The cell numbers in these four samples were counted microscopically with accounting chamber and also enumerated with realtime PCR (*S. cerevisiae* realtime PCR system designed by Anne Terrettaz, HEVSS, Sion, Switzerland). Additionally, the samples taken before the malolactic fermentation were plated on Phyton Yeast Agar and the colony forming units counted. The realtime PCR results for *S. cerevisiae* and *H. uvarum* were compared to the total cell number counted with the microscope for both sampling times, and to the colony forming units number for the samples taken before the malolactic fermentation.

The difference between the measured values by PCR and the counted values is variable for the different stages of the fermentations. After alcoholic fermentation, the realtime PCR values are clearly smaller than the cell numbers counted by microscopy. After the MLF, the difference between these values gets much smaller (FIGURES 1.4-1.7). The plate count result usually lies below the realtime PCR values in the white wines, and above for the red wines (FIGURE 1.4-1.5). The reason for this could be a lower efficiency of the extractions from the red wine samples, although this was not found to be the case in other comparisons. It is also possible that after the fermentation red wine develops compounds that disturb the extraction, like polyphenols.

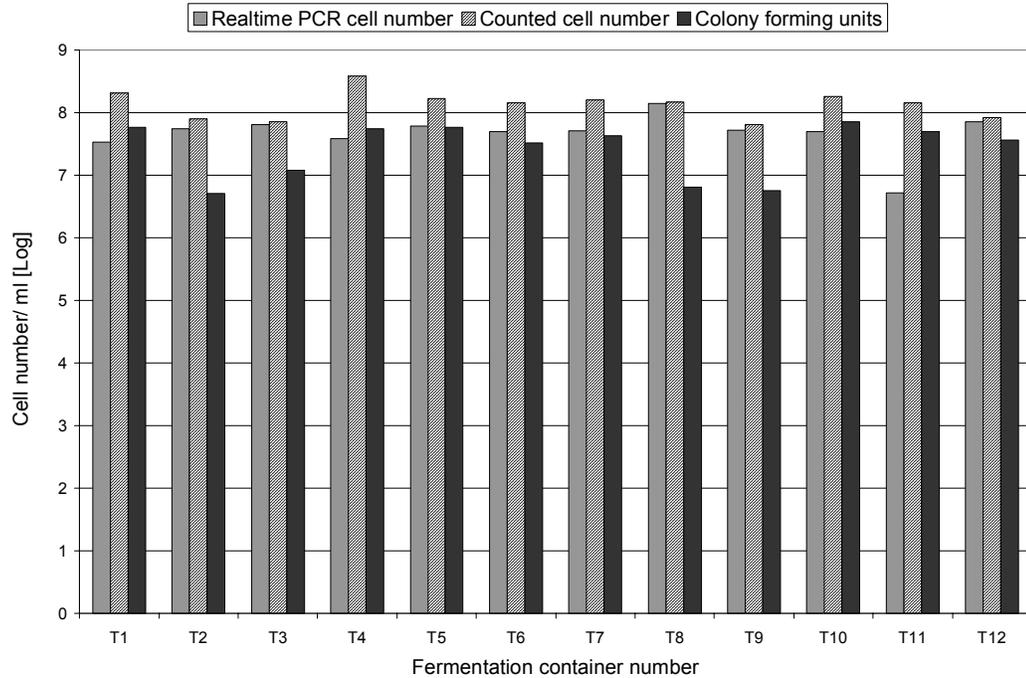


FIGURE 1.4 Müller-Thurgau, before malolactic fermentation. Comparison between the total cell number counted with a microscope, measured with realtime PCR and CFU from plate counts.

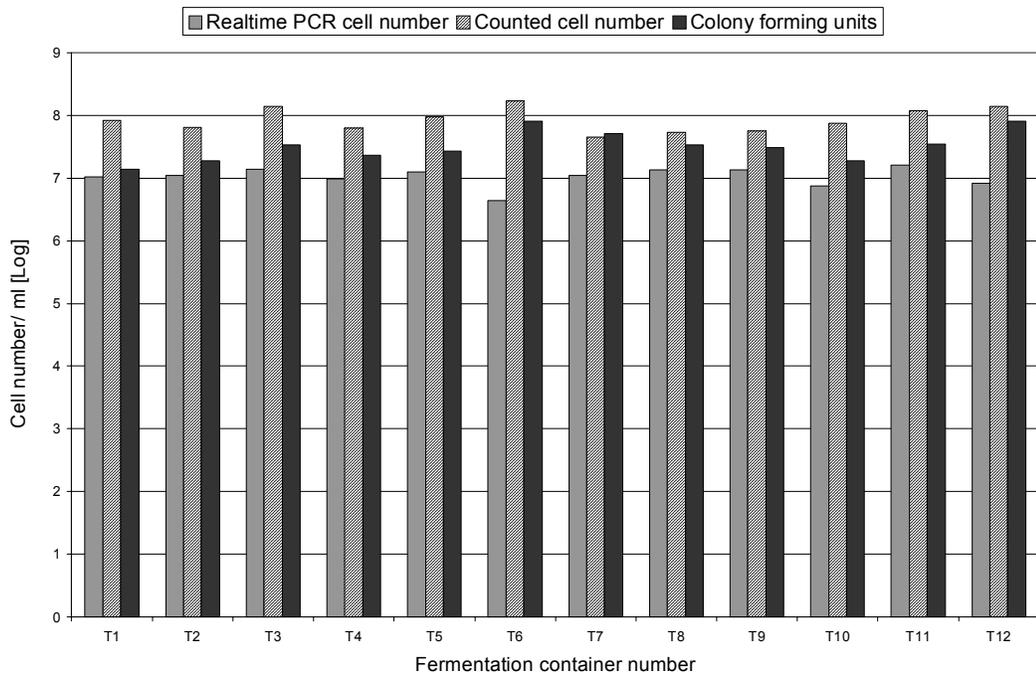


FIGURE 1.5 Pinot Noir, also called Clevner in Zürich, before malolactic fermentation. Comparison between the total cell number counted with a microscope and measured with realtime PCR and CFU from plate counts.

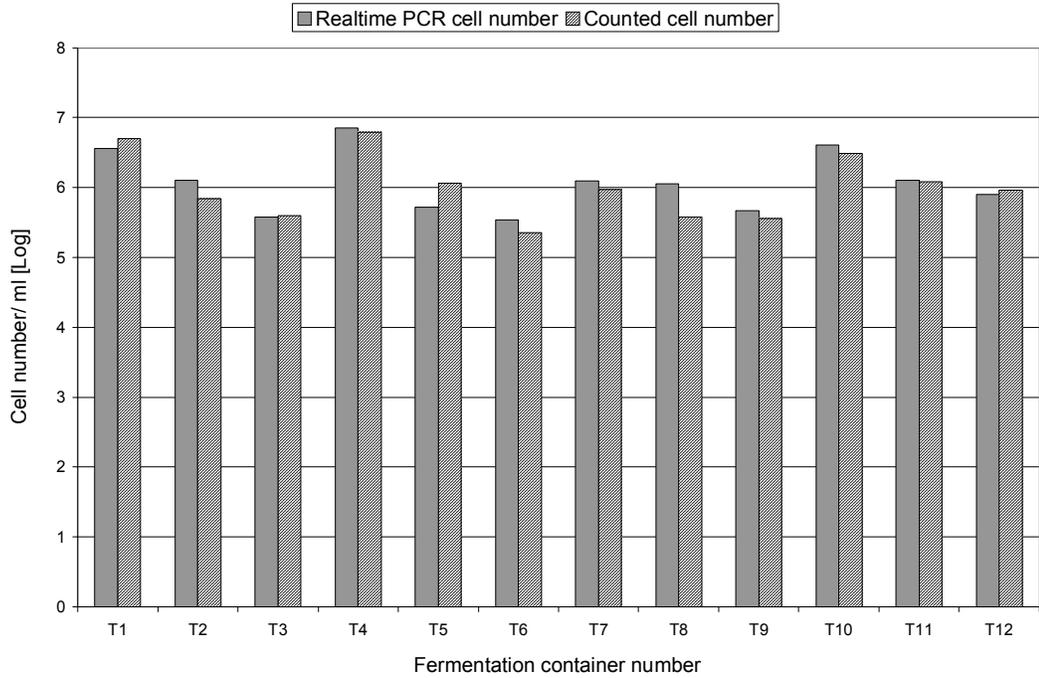


FIGURE 1.6 Müller-Thurgau, after malolactic fermentation. Comparison between the total cell number counted with a microscope and measured with realtime PCR

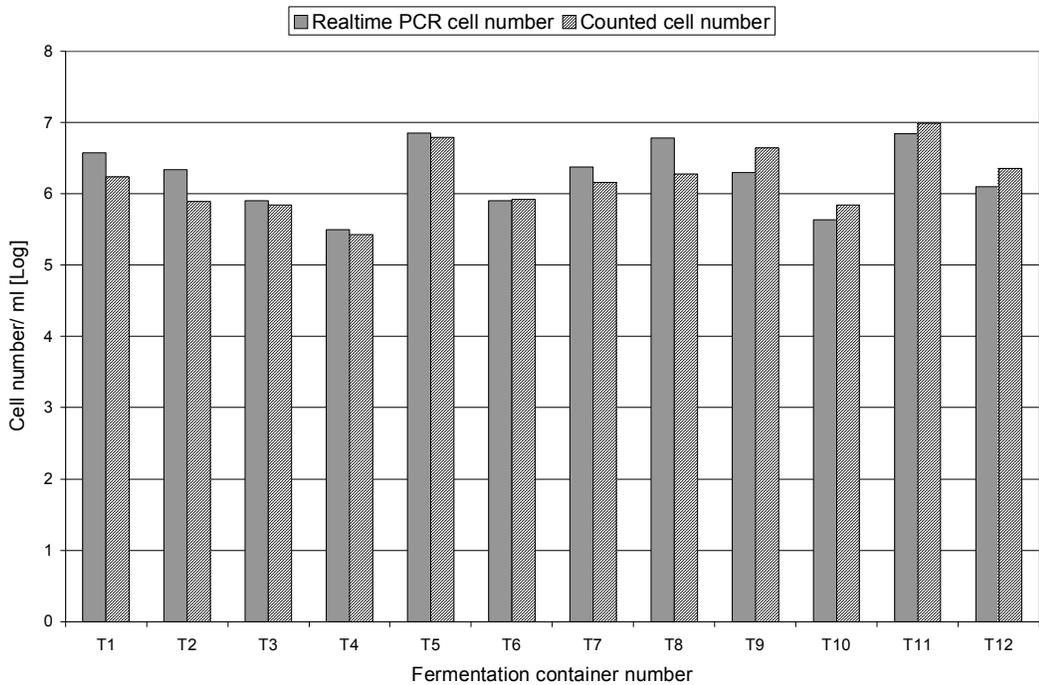


FIGURE 1.7 Pinot Noir, also called Clevner in Zürich, after the malolactic fermentation. Comparison between the total cell number counted with a microscope and measured with realtime PCR.

CHAPTER 2: VERTICAL DISTRIBUTION OF YEAST AND BACTERIA IN STAINLESS STEEL TANKS DURING WINE FERMENTATION

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Abstract

The wine fermentation process involves a succession of various microbial populations and at any one stage of fermentation a variety of microorganisms are present including those which can spoil the wine. Therefore, it is very important to monitor the microbial population all through the fermentation. The sampling process must ensure obtaining a representative sample. This study compares four different sampling locations in wine tanks, during both the alcoholic and the malolactic fermentation. Samples were taken in a sterile manner from the top, the middle, and the bottom of the tanks through the top hatch. Additionally, samples were taken from the sampling valve, after a short forerun. The samples were plated for viable *Saccharomyces cerevisiae* yeast and the wine lactic acid bacteria *Oenococcus oeni*. ANOVA was carried out to determine whether it makes a difference where in the tank a sample is taken, and whether samples taken from the sampling valve are representative of the yeast and bacteria population in the tank. This comparison showed that samples taken from the sampling valve are in fact representative of the tank microflora during alcoholic and malolactic fermentation.

Introduction

During the process of winemaking, microorganisms are involved at every step (Henick-Kling, 1995). The yeast *Saccharomyces* is essential for the alcoholic fermentation. In addition to transforming sugars into ethanol, the choice of the *Saccharomyces* strain also has a large influence on the aroma and mouthfeel of the

final product (Egli et al., 1998; Henick-Kling et al., 1998). After the alcoholic fermentation, red wines and some white wines undergo malolactic fermentation (MLF), where lactic acid bacteria transform malic acid into lactic acid, modify the wine aroma, and texture (Henick-Kling, 1995; Krieger et al., 1993; Lallemand, 1998; Laurent et al., 1994). The preferred lactic acid bacteria for MLF are *Oenococcus oeni*. Here, as in alcoholic fermentations, the strain that carries out the MLF also has a distinct flavor impact. Unfortunately, other yeast and bacteria species can grow on the surface of grapes, and so are carried into the winery with the grape juice. Some of them can add interesting characteristics to the wine, but their flavor contribution is very unpredictable because very little is known about the diversity and physiology of these micro-organisms. Most of the time, non-*Saccharomyces* yeasts, acetic acid bacteria, and lactic acid bacteria other than *O. oeni* damage the wine and can cause large losses to the wine producer. *Hanseniaspora uvarum* is the most common yeast in grape must, comprising 50 to 90% of the total yeast population (Gafner et al., 1996). The spoilage yeast *H. uvarum*, called *Kloeckera apiculata* in its asexual form, is capable of fully fermenting the sugar in grape must, but can produce up to 2 g/l of acetic acid during the process (Phister and Mills, 2003; Romano et al., 1992). Acetic acid undergoes esterification in the presence of alcohol, resulting in esters which can contribute agreeable flavors. Yet if the esterification includes the formation of ethyl acetate which attributes a nail polish remover odor to the wine, a wine can become un-salable. With the current knowledge about *Hanseniaspora* yeasts their flavor contribution can not be controlled. Therefore to avoid the risk of damage and even complete loss due to spoilage by *H. uvarum*, winemaking practice aims to suppress the growth of *H. uvarum* in grape must and in wine during fermentation. The yeast *Pichia anomala* can occur in grape musts during alcoholic and malolactic fermentations and later, during maturation. Although not a strong fermenter, *P. anomala* survives and

can form up to 500 mg/l ethylacetate. If *P. anomala* has some oxygen at its disposal, it can grow on the surface of the wine and produce large amounts of acetate, acetate esters, and oxidized flavors (Schnürer, 2003).

Three different genera of lactic acid bacteria occur on the grapes and in the must (Henick-Kling, 1995). These bacteria are able to perform a malolactic fermentation (MLF), transforming malic acid into lactic acid. To achieve desired aromas and texture, bacteria of *O. oeni* must outgrow the undesired *Pediococcus damnosus*, *P. parvulus*, *P. pentosaceus*, *Lactobacillus brevis* and *L. hilgardii*, which can produce unwanted compounds from the metabolism of sugars that remain in the wine. *L. brevis* strains can produce up to 5 g/l of acetic acid. *P. damnosus* and *L. brevis* can produce large amounts of lactic acid from sugar. Strains of *L. hilgardii* and *L. brevis* can produce the unpleasant 'mousy' flavor (Heresztyn, 1986), and *P. damnosus* can produce high concentrations of diacetyl and biogenic amines, as well as polysaccharides, the wine gets sticky or ropy (Lonvaud-Funel, 1999).

To avoid spoilage from the undesirable yeast and bacteria, grape must and wine must be monitored closely, by microbial examination, chemical measurements, and sensory checks. Particularly slow or sluggish fermentations have to be monitored carefully, because stuck fermentations can be caused by spoilage yeasts and some lactic acid bacteria and are very vulnerable to microbial spoilage and chemical oxidation (Bisson, 1999; Phowchinda et al., 1995). Wines should be checked regularly for progress of fermentation, formation of off-odors, and possible growth of spoilage microorganisms. Early detection of potential spoilage microorganisms allow the winemaker to intervene, remove or inactivate the unwanted microorganisms and avoid the formation of off-odors. Simple plating and microscopic examination can be carried out in most winery laboratories, yet rapid detection and identification of small numbers of spoilage microorganisms are typically carried out in specialized service laboratories.

Recently several molecular biological methods have been explored for the detection and quantification of wine microorganisms (Bleve et al., 2003; Bujdoso et al., 2001a, b; Egli and Henick-Kling, 2001; Esteve-Zarzoso et al., 1999; Guillamón et al., 1998; Mitrakul et al., 1999; Phister and Mills, 2003; Schütz and Gafner, 1993; Schütz and Gafner, 1994). With the methods available so far wine and must samples might receive a preliminary microscopic check, then the samples are cultured on various nutrient media plates and the culturable yeast and bacteria can be typed with molecular methods.

One part that has been neglected so far is the impact of the sampling method. It is unclear whether it makes a difference where in a tank a sample was taken. Many fermentation tanks are very large and reach several meters in height. It is not practical to stir the wine in the fermentation tanks before a sample is taken. The question that was asked in this project was whether there is a difference in the density of the yeast and bacteria population in the vertical location in a fermentation tank, during alcoholic fermentation through MLF. It is very important for a winemaker to know where in the fermentation tank a sample has to be taken for it to be representative. Before going through the time and expense of testing a sample for micro-organisms, a winemaker has to be confident that the sample was representative of the situation of the fermentation.

Materials and Methods

Fermentation assays and microbial strains. Three fermentations in two different

wineries were monitored from the beginning of the alcoholic fermentation to the end of the malolactic fermentation. All three musts were from Müller-Thurgau grapes. Two different barrel sizes were investigated. In one winery, 1,700 l must were fermented with the *S. cerevisiae* yeast Lalvin W15 (Lallemand, Montreal, Canada) in a 2,000 l stainless steel tank (trial MT1). After the alcoholic fermentation, 1,000 l of wine were transferred into another steel tank and inoculated for malolactic fermentation with *O. oeni* EQ 54 (Lallemand, Montreal, Canada). In another winery, two fermentations were followed: 9,000 l (MT2) and 8,000 l (MT3) of grape must, respectively, were fermented in 10,000 l stainless steel tanks. Both musts were inoculated with the *S. cerevisiae* yeast Lalvin W27 (Lallemand, Montreal, Canada). The malolactic fermentation was done in 6,000 l stainless steel tanks both for MT2 and MT3. MT3 did a spontaneous MLF. MT2 did not start malolactic fermentation spontaneously, and was inoculated with *O. oeni* by replacing 10% with another wine which was undergoing uninoculated malolactic fermentation.

Sampling. Samples were taken, as aseptically as possible, in the top part, the middle, and the lowest part of the tank. Additionally, a sample was taken from the sampling valve after a short forerun. The sampling valve always lay below the middle of the stainless steel tanks. A device was designed for aseptic sampling. A flexible tube was weighted with a stainless steel rod and cut at the appropriate length. The assembly was washed and autoclaved between each sampling. The samples were taken from the hatch at the top of the fermentation tanks with a sterile 50 ml syringe. During alcoholic fermentation, and through MLF, samples were taken regularly.

Measurements. Each sample was diluted according to an estimated cell number derived from microscopic count. The appropriate dilution was spread onto Phytone

Yeast Agar plates (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for the yeast counts and on Leuconostoc M5 agar (Scharlau, Barcelona, Spain) for the bacteria. They were incubated, at 25°C for the yeasts and 30°C for the bacteria, until colonies of the appropriate size had grown. The viable cell counts (CFU/ml) were determined for both yeast and bacteria. Usually six plates were counted for every sample, three each at two different dilutions. The must MT2 could not be tested for *O. oeni* because it had finished the malolactic fermentation too quickly. For each sampling series, the musts were analyzed by HPLC for glucose, fructose, ethanol, malic acid and lactic acid. The samples from the middle part of the fermentation tanks were used for the chemical analyses.

Analysis and statistics. The number of viable cells obtained from the separate locations are shown in relation to the date of sampling. Each growth curve was then compared to the other growth curves of the same fermentation. The trends of these location curves were analyzed (Ihaka and Gentleman, 1996). Differences of trends were assessed by the interaction of measurements with datasets (eg. MT1 yeasts, MT1 bacteria) and measurements with time, resulting in P values. For each measurement date, an analysis of variance was done with Microsoft Excel over the four tank locations. When the P-value was significant, another analysis of variance was added, using the three locations that are closest to the mean of the values.

Results

For each fermenter, the number of viable cells is shown in a bar diagram (FIGURES

2.1-2.5). The four bars at each date represent the different places where the samples were taken: at the top, in the middle, at the bottom, and from the sampling valve. The minimum and maximum values that were counted are indicated to show the range. In the diagrams for the yeasts, the measured concentrations of glucose, fructose, and ethanol are shown to mark the stage of the alcoholic fermentation. The bar diagrams for the number of bacteria are similar. To follow the progress of the malolactic fermentation we show the concentration of malic and lactic acid.

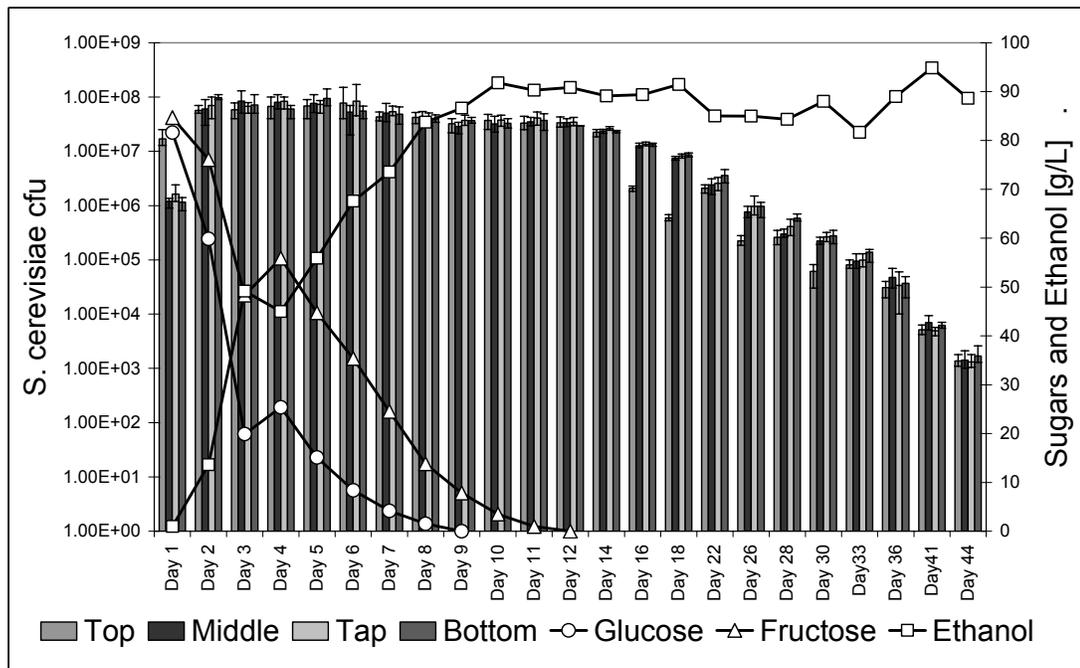


FIGURE 2.1 Dataset MT1 yeast: CFU for *S. cerevisiae*, at four locations in the tank (top, middle, tap, and bottom). Bars indicate the minimal and maximal values of CFU/ml. Amount of glucose, fructose and ethanol to indicate the progress of the alcoholic fermentation. Tap: sampling valve.

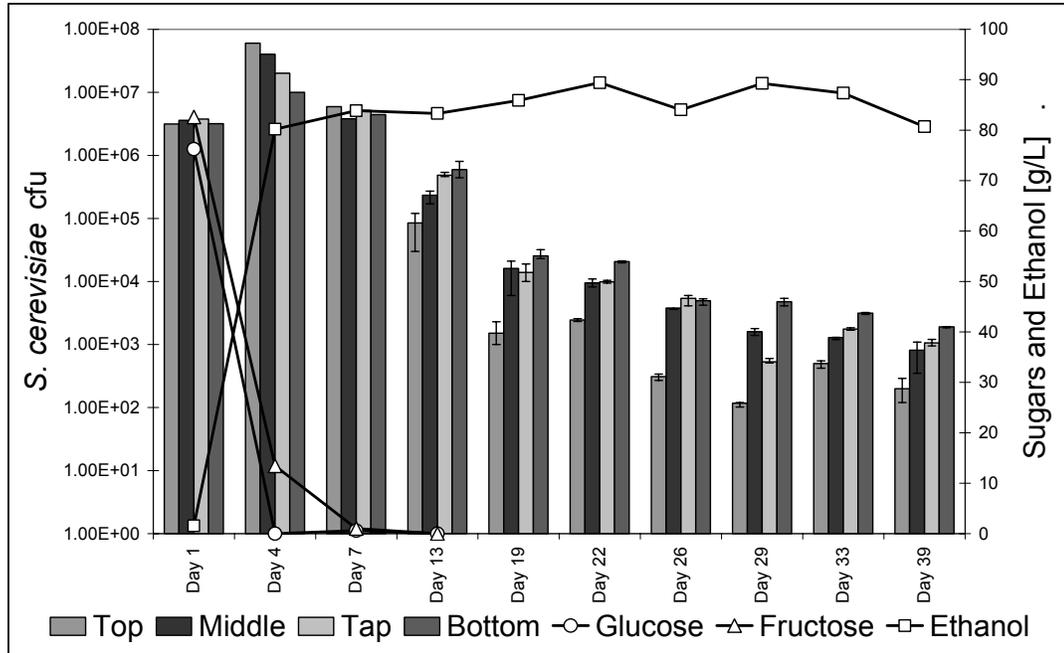


FIGURE 2.2 Dataset MT2 yeast: CFU for *S. cerevisiae*, at four locations in the tank (top, middle, tap and bottom). Bars indicate the minimal and maximal values of CFU/ml. Amount of glucose, fructose and ethanol to indicate the progress of the alcoholic fermentation. Tap: sampling valve.

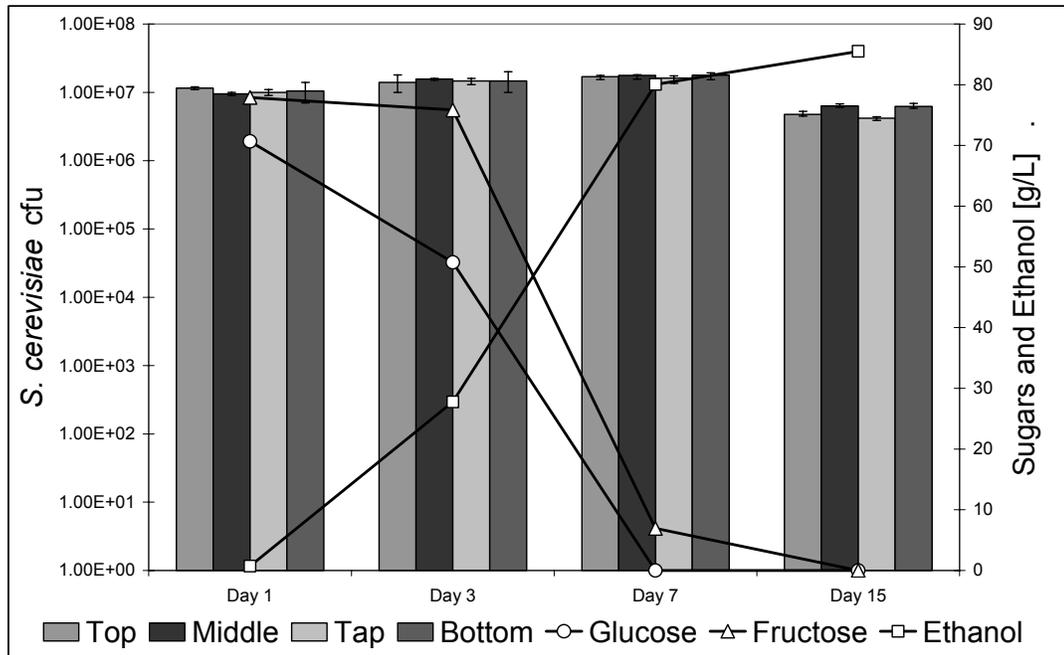


FIGURE 2.3 Dataset MT3 yeast: CFU for *S. cerevisiae*, at four locations in the tank (top, middle, tap and bottom). Bars indicate the minimal and maximal values of CFU/ml. Amount of glucose, fructose and ethanol to indicate the progress of the alcoholic fermentation. Tap: sampling valve.

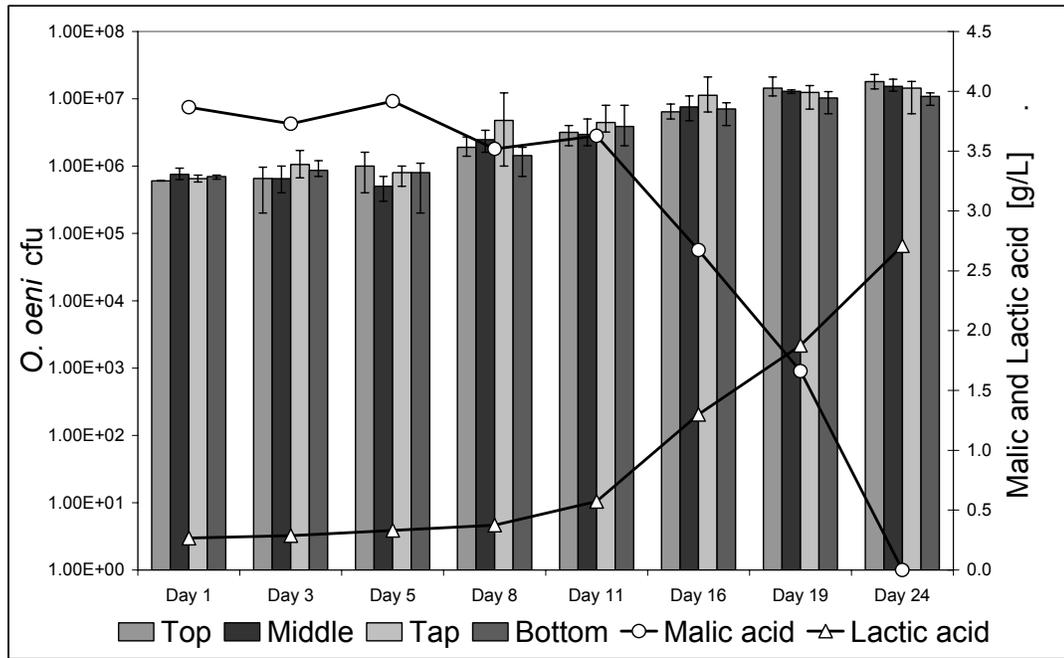


FIGURE 2.4 Dataset MT1 bacteria: CFU for *O. oeni*, at four locations in the tank (top, middle, tap and bottom). Bars indicate the minimal and maximal values of CFU/ml. Amount of malic acid and lactic acid to indicate the progress of the MLF. Tap: sampling valve

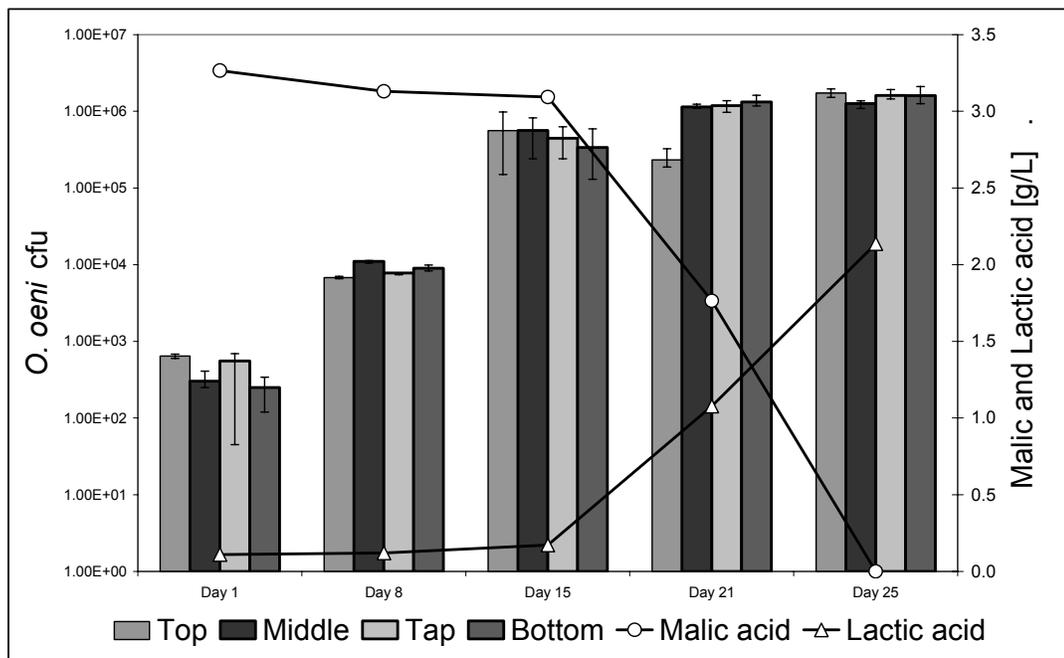


FIGURE 2.5 Dataset MT2 bacteria: CFU for *O. oeni*, at four locations in the tank (top, middle, tap and bottom). Bars indicate the minimal and maximal values of CFU/ml. Amount of malic acid and lactic acid to indicate the progress of the MLF. Tap: sampling valve.

The experiment comprised five datasets (FIGURES 2.1-2.5), observations at different times, and four measurements (top, middle, and bottom, and sampling valve (tap)). Samples were taken with replicate at each sampling time. Graphic displays of the mean measurements, shown with minimal and maximal values, show essentially similar trends for the datasets. Differences in trends were assessed using analysis of variance. Differences of the trends can be determined by the interactions of measurements with datasets and measurements with time. These interactions were not significant: $P > 0.9$ and $P = 0.5$ respectively over all five datasets.

For each of the five resulting curves (three yeasts and two bacteria), the measurements ‘top, middle, bottom, and sampling valve’ were compared and the P -values calculated. Additionally, P -values for the interaction between the viable cell number and time were calculated, to determine whether the curves show a trend that is different for each different location (TABLE 2.1).

TABLE 2.1 F- and P-values for separate fermentations, assess the trends for the growth curves at different locations within datasets

Dataset	Micro-organism	F-value	P -value
MT1	Yeast	0,19	0,9
MT1	Bacteria	2,05	0,11
MT2	Yeast	1,19	0,31
MT2	Bacteria	0,27	0,84
MT3	Yeast	0,36	0,77

For each date, the P -value was calculated to determine whether there was a difference between the locations of sampling. When the differences were significant, ANOVA was performed for the three values which were closest to the overall mean (TABLE 2.2). P -values that are significant, meaning $\alpha \leq 5\%$ are printed in bold.

Additionally, the ratio of the mean of the values Top, Middle, and Bottom to the value

of the sampling valve is shown (Tap).

TABLE 2.2 Statistical analysis for all fermentations, F- and P-values for each sampling date (ANOVA)

Datase t	Ferm. Day	<i>F-Value</i>	<i>P-Value</i> ¹				Mean/Tap ²
			Overall	No Top	No Middle	No Tap	
MT1 Yeast	Day 1	57.206	5.4E-10	0.024			3.96
	Day 2	1.909	0.207				1.02
	Day 3	0.804	0.515				1.06
	Day 4	0.615	0.624				0.83
	Day 5	1.539	0.237				1.08
	Day 6	1.492	0.247				0.74
	Day 7	0.988	0.418				0.88
	Day 8	0.242	0.866				1.05
	Day 9	3.175	0.047	0.018	0.296		0.88
	Day 10	0.722	0.551				0.90
	Day 11	0.995	0.417				0.85
	Day 12	0.761	0.531				0.94
	Day 14	1.343	0.327				0.88
	Day 16	88.573	1.8E-06	0.691			0.70
	Day 18	120.91	5.3E-07	0.152			0.68
	Day 22	1.129	0.388				1.05
	Day 26	2.945	0.067				0.67
	Day 28	5.192	0.010	0.209			0.92
	Day 30	3.011	0.054				0.71
	Day33	4.459	0.015	0.478			1.06
Day 36	0.487	0.695				1.13	
Day41	0.555	0.651				1.23	
Day 44	0.447	0.722				1.13	
MT2 Yeast	Day 1	0.527	0.687				0.88
	Day 4	n. d.	n. d.				1.83
	Day 7	1.296	0.391				0.92
	Day 13	12.493	8.0E-05	0.014			0.63
	Day 19	11.332	0.003	0.088			1.03
	Day 22	404.852	1.6E-11	1.3E-05			1.09
	Day 26	20.287	2.8E-06	0.105			0.56
	Day 29	227.448	4.1E-12	7.0E-08			4.09
	Day 33	68.540	1.1E-10	6.7E-07			0.93
	Day 39	55.413	7.2E-10	6.8E-06		2.0E-05	0.91
MT3 Yeast	Day 1	1.243	0.337				1.05
	Day 3	1.049	0.393				1.01
	Day 7	0.470	0.707				1.08
	Day 15	18.606	0.001			0.012	1.38

TABLE 2.2, continued

MT1 Bacteria	Day 1	1.172	0.386		1.06
	Day 3	3.389	0.038	0.012	<i>0.68</i>
	Day 5	0.689	0.584		0.96
	Day 8	0.608	0.629		0.41
	Day 11	1.187	0.340		0.75
	Day 16	2.229	0.116		0.62
	Day 19	1.260	0.332		1.00
	Day 24	2.360	0.123		1.03
MT2 Bacteria	Day 1	11.543	0.003	0.031	<i>0.72</i>
	Day 8	7.176	0.005	0.522	<i>1.15</i>
	Day 15	1.370	0.299		1.09
	Day 21	25.108	2.0E-04	0.144	<i>0.76</i>
	Day 25	0.376	0.771		0.95

¹⁾ *P*-values for all four sampling locations (overall), and for the three values closest to the mean of all values (without top, without middle, without tap). Significant values ($P \leq 0.05$) are shown in **bold**.

²⁾ Ratio of mean cfu of the measurements (top, middle, bottom) and tap. Ratios of data points with significant *P*-values are in *italic*.

Discussion

The results of the experiments clearly show that, during active fermentation, both bacteria and yeast cell density are very similar in the different parts of the fermentation tanks. Occasionally, there was some mold growth on plates which were drawn from the tap, but this did not interfere with the detection or quantification of either yeast or bacteria. The results for the bacteria are very straightforward. Only four measurements show significant differences. Although these differences are statistically significant, they do not impact the validity of the statement that samples can be taken from the tap to obtain a representative sample of the bacteria. The ratio ‘mean of three measurements top, middle and bottom’ divided by ‘tap’ lies between 68% and 115%. If the cells were perfectly uniform, this ratio would be 100%. *S. cerevisiae* showed differences, particularly between the top measurements and the others. In the fermentation MT1, the measurement from the top part of the tank is markedly higher

than that of the others on the first day. The reason for that is that the yeast culture was inoculated from the top hatch, and took a day to distribute evenly throughout the tank. The alcoholic fermentation took 11 days to finish, and 13 days later, the wine was transferred into the smaller stainless steel tank for the MLF. During that time, the yeasts began to settle. They started to settle again after inoculation with the bacteria, because the MLF did not start immediately. In both cases, the measurements from the top part of the stainless steel tanks dropped compared to the other locations. MT2 and MT3 were fermented in much larger stainless steel tanks, reaching 6 meters in height. For MT2, the measurements from the top part of the tank started dropping as soon as the alcoholic fermentation was finished. Even leaving the top part of the tank out of the equation, the measurements middle, tap and bottom were still significantly different from each other. But again, the ratio 'mean of three measurements top, middle and bottom' divided by 'tap' stays within reasonable limits, at 56% to 409%. MT3 started MLF quickly, and so the yeast did not have time to settle before its end. So it is better to avoid taking samples from the hatch when a representative image of the microorganisms is the aim of sampling. Samples taken with a short forerun from the tap, using a sterile container but without special treatment of the sampling valve, are adequate to for wines during alcoholic and malolactic fermentation.

Conclusions

Samples taken from the sampling valve of stainless steel fermentation tanks, after a short forerun, are representative for the population of micro-organisms during active alcoholic and malolactic fermentation. Even if there are occasionally molds that grow

on samples taken from the sampling valve, this does not interfere with the quantifying process, because they cannot be mistaken for either yeasts or bacteria. This allows taking only one sample from a fermentation tank rather than sampling top, middle and bottom to assess the microbial population. Sampling from the top of a tank is only representative during active alcoholic and malolactic fermentation when the CO₂ evolution ensures good mixing.

CHAPTER 3: VALIDATION OF A REALTIME PCR SYSTEM FOR FAST DETECTION OF THREE WINE YEAST SPECIES

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Abstract

Realtime PCR systems for the detection and quantification of three wine yeast species, *Hanseniaspora uvarum*, *Pichia anomala*, and *Brettanomyces bruxellensis*, were developed. These three spoilage yeasts can occur in wine before, during, and after the alcoholic fermentation. Current detection techniques are time consuming because they rely on growth of the yeast on nutrient media: a fast and accurate system would be very valuable for assuring wine quality. A sequence from the transcribed part of the actin gene was used as DNA template for the realtime PCR. The validation of the three detection systems was carried out with special consideration for the extraction method used and the wine matrix. All three yeast species were validated on the GeneAmp5700 instrument from Applied Biosystems. Additionally, the detection of *B. bruxellensis* was adapted for use with the SmartCycler[®] I from Cepheid and the iCycler iQ real-time PCR detection system from Bio-Rad. The specificity, linearity, sensitivity, and trueness were determined for each system. All three systems proved to be very robust, particularly regarding the specificity and linearity. An assay takes about 6 hours from start to finish, including the DNA extraction. Cell concentrations from 10^3 to 10^8 cells/ml can be quantified reliably. At 10^2 cells/ml the result of the test is positive, but not quantitative.

Introduction

Microorganisms are essential for the production of wine. Among the various yeast and bacteria which enter the winemaking process from the grapes and from the winery

environment are several potent spoilage yeasts. In order to produce high quality wines which express fruit and regional flavor characteristics the winemaker must guide the development of yeast and bacteria to prevent production of off-flavors by unwanted microorganisms. Since spoilage yeasts such as *Hanseniaspora uvarum*, *Pichia anomala*, and *Brettanomyces bruxellensis* may be present in the incoming grapes, good winemaking practices aim to ensure that the desired wine yeast *Saccharomyces* is dominant during alcoholic fermentation. To ensure that off-flavors are not produced, the microbial population in the grape must before and during fermentation must be monitored and controlled.

H. uvarum is the most common yeast in grape must, comprising up to 50 to 90% of the yeast flora. Also called *Kloeckera apiculata* in its asexual form, it is capable of fully fermenting the sugar in grape must to ethanol, but can produce up to 2 g/l of acetic acid during the process. Acetic acid undergoes esterification in the presence of alcohol, producing a number of flavor active compounds, some with pleasant and some with undesirable flavor impact. One such compound is ethyl acetate, of which *H. uvarum* can produce up to 870 mg/l (Mateo et al., 1991). It gives a wine a nail polish remover type of odor and may make it unsalable. Acetoin can be produced up to 187 mg/l by this yeast (Romano et al., 1993), which is present in high amounts at the beginning of the alcoholic fermentation (Mortimer, 1995). Cold settling or bentonite treatments have little influence on the concentration of *H. uvarum* at the beginning of the fermentation (Mora and Mulet, 1991). *H. uvarum* tends to decrease sharply during the alcoholic fermentation (Lema et al., 1996).

The yeast *P. anomala* can occur in grape musts, during alcoholic and malolactic fermentations and later, during maturation. Although not a strong fermenter, *P. anomala* can survive alcoholic fermentation and can form up to 1200 mg/l of ethyl acetate (Romano et al., 1997). If *P. anomala* has some oxygen at its disposal, it can

grow on the surface of the wine and produce other unpleasant aromas.

Before fermentation and during aging of wines in barrels and in tanks, the yeast *B. bruxellensis* (also called *Dekkera bruxellensis* in its imperfect form) can produce strong ‘bretty’ off-odors (Bravo-Plasencia and Bravo-Abad, 1996; Chatonnet et al., 1997; Llanos Company, 1997). Bretty wines are described as having horse stable, horse sweat, leather, band aid®, dried cow manure, mousy, medicinal flavors, smoky and spicy aromas and at times a distinct metallic bitter taste (Egli et al., 1998; Henick-Kling et al., 2000; Licker, 1998; Licker et al., 1999). Additionally, *B. bruxellensis* can produce large amounts of acetic acid (Ciani and Ferraro, 1997; Freer, 2002). *B. bruxellensis* also can produce biogenic amines (Caruso et al., 2002). Studies on the occurrence of *B. bruxellensis* in different grape musts and wines were carried out (Larue et al., 1991), and the influence of different treatments of wine barrels were investigated. Currently, three marker substances produced by *B. bruxellensis* are used to monitor wines for growth and activity of this yeast: 4-ethylphenol, 4-ethylguaiacol (Chatonnet et al., 1992; Pollnitz et al., 2000), and 4-ethylcatechol (Hesford et al., 2005). Small concentrations of bretty aroma compounds, especially the smoky and spicy aromas, may increase the wine aroma complexity (Fugelsang, 1998). Some winemakers are convinced that the presence of any *Brettanomyces* yeast is bad for a winery, others like the character that some *B. bruxellensis* strains may give an under-ripe, simple fruity wine. Because “Brett” is present worldwide and can grow in many different wines (Fugelsang, 1997), it is important to develop better detection methods and ways to control its activity in wine.

Current detection and quantification techniques

Most commonly, traditional plating techniques using semi-selective nutrient media are used. The two major problems with all plating techniques are the delay in getting

results due to the long time required for the yeast to grow, and the non-detection of viable but not culturable cells: some yeasts will not grow on the nutrient media employed (Millet and Lonvaud-Funel, 2000). Several advanced molecular biological methods were evaluated to detect *B. bruxellensis* in wines (Egli and Henick-Kling, 2001; Mitrakul et al., 1999). In surveys of Cabernet Franc and Pinot Noir in the Finger Lakes, NY region, the 4-ethylphenol and 4-ethylguaiacol concentrations were related to growth of *B. bruxellensis* isolates on nutrient plates and the presence of bretty odors in the wines. Isolated yeasts were characterized using molecular methods (Conterno and Henick-Kling, 2003; Egli et al., 2000). These studies showed that both the detection of *Brettanomyces* by analysis of the chemical indicator substances and the detection by traditional plating method are inaccurate. About 10% of wines which had bretty off-odors were detected neither with the 4-ethylphenol and 4-ethylguaiacol analysis nor with the plating technique. Recently, realtime PCR was used to detect *B. bruxellensis* in wine (Delaherche et al., 2004) using the *rad4* gene and the 26S rRNA gene (Phister and Mills, 2003).

Actin as a target for realtime PCR

The actin gene has been studied intensively (Daniel and Meyer, 2003; Daniel et al., 2001; Gallwitz and Seidel, 1980; Gallwitz and Sures, 1980; Kang et al., 2001; Nellen et al., 1981; Okeke et al., 2001; Poch and Winsor, 1997; Thompson-Jäger and Domdey, 1990). Actin is a highly conserved housekeeping gene, which makes it possible to design robust, specific PCR primers. There are only rare point mutations within the coding region of the gene. Due to its housekeeping functions, the mRNA of the *ACT1* gene is present in every phase of the cell cycle (Riou et al., 1997). The mRNA of this gene yields solid systems to quantify the living organisms because the expression does not vary very much with external influences or the cell phase. mRNA

from the actin gene was used for quantitative PCR to detect yeasts and molds in foods other than wine (Bleve et al., 2003). These new detection methods have the potential to surpass the traditional methods of microorganism detection, like microscopy or enrichment with subsequent microscopy and antibody based detection systems, in rapidity and sensitivity. With realtime PCR, samples can be analyzed within 6 hours, including DNA extraction and RT-PCR analysis.

Important considerations for a realtime PCR validation for microorganisms

Specificity

For any detection method of microorganisms, it must be determined whether non-targeted species might be detected. Therefore, microorganisms that are likely to occur within the target environment need to be tested for possible interference. Several yeast and bacteria species found in grape must and in wine were used for this purpose.

Linearity

The quantification of microorganisms with realtime PCR is based on the linearity of the standard curve. Both the range and the linearity of the standard curve need to be determined using dilution series of DNA of known cell numbers. The quality of the final quantification system is determined by the efficiency of the PCR.

When using realtime PCR as a quantification method, the cell number is calculated by quantifying the number of copies of the target sequence. Therefore, the extraction method is of paramount importance for a reliable quantification of the cells. It is very important to know within which range the extraction is quantitative. Just as importantly, the influence of the matrix (growth media, physiological salt solution,

wine) on the PCR, as well as the sample size, need to be investigated.

Threshold

The threshold of a detection system is the lowest concentration at which a reliable signal is produced. The threshold can be outside of the quantitative range of the realtime PCR.

Sensitivity

The sensitivity describes which difference in quantification can be measured with a given system. In this case, the sensitivity should be 1 log unit of cells.

Trueness

The results of a test using a new detection system need to be compared to another detection method that is already validated. Using spikes of yeast and bacteria suspensions containing known cell numbers, a graph is drawn (log of known cell number against log of measured cell numbers). The resulting curve of a regression analysis is required to have a slope that is very close to one, the y-axis intercept should be very close to zero.

Materials and methods

Microbial strains and growth conditions

The microbial strains used in these experiments are listed in TABLE 3.1. Each

detection system was tested for the microorganisms listed. The strains marked as standard or positive are those which are expected to amplify during PCR, while those

TABLE 3.1 Tested organisms for the detection system of *H. uvarum*, *B. bruxellensis* and *P. anomala*

Yeasts	Strain	Detection System		
		<i>H. uvarum</i>	<i>B. brux.</i>	<i>P. anomala</i>
<i>Hanseniaspora uvarum</i>	DSM 2768	standard	negative	negative
	FAW98/10-4	positive	negative	negative
	FAW75 403H	positive	negative	not tested
	FAWRbst9/00/4	positive	negative	not tested
	FAW74	positive	negative	not tested
	Rst 9/10	positive	negative	not tested
	DSM 70739	negative	standard	negative
<i>Brettanomyces bruxellensis</i>	Jodat95	negative	positive	negative
	Buess a	negative	positive	not tested
	Vallellina	negative	positive	not tested
	Malanser	negative	positive	not tested
	Cor94-41	negative	positive	not tested
<i>Saccharomyces cerevisiae</i>	Lalvin W15	negative	negative	negative
	Lalvin W27	negative	negative	not tested
	Ceppo 20	negative	negative	not tested
	DSM 4266	negative	negative	negative
	Lalvin EC 1118	negative	negative	not tested
<i>Saccharomyces bayanus</i>	FAW 43	negative	negative	negative
<i>Saccharomyces uvarum</i>	S6U	negative	negative	negative
<i>Metschnikowia pulcherrima</i>	Rst6	negative	negative	negative
	DSM 70321	negative	negative	negative
<i>Pichia anomala</i>	FAW10	negative	negative	positive
	DSM 6866	not tested	not tested	standard
<i>Candida stellata</i>	FAW3	negative	negative	negative
	Rst 98/10/7	negative	negative	negative
Bacteria				
<i>Oenococcus oeni</i>	DSM 20257	negative	negative	negative
<i>Pediococcus damnosus</i>	DSM 20331	negative	negative	negative
<i>Lactobacillus brevis</i>	DSM 2647	negative	negative	negative

Negative: no signal at 45 cycles

Standard: the strain is used for the standard curves

Positive: signal at the appropriate cycle number for the respective detection system

labeled negative are the negative controls which should not yield a signal in the realtime PCR assay. The yeasts were grown in liquid YPD media (2% peptone, 1% yeast extract and 2% glucose) at 25°C, and the bacteria in liquid MRS (*Lactobacilli* MRS broth, Difco, Detroit MI, USA) at 30°C.

Optimization of the DNA extraction

Using *B. bruxellensis* suspensions at defined cell densities (5×10^9 , 10^9 to 10^2 cells/ml in growth media, diluted with physiological NaCl solution), four different DNA extraction kits employing three different methods of DNA collection (two with precipitation and centrifugation, and one each with fiberglass and silica gel columns) were evaluated. In the end, the Qiagen DNeasy Tissue Kit was used for further extractions, with a slightly modified protocol: the lysis buffer is adjusted to pH 7 (not adjusted in original protocol), the lysis with lyticase at 37°C instead of 30°C. All incubation steps are carried out using the Thermomixer from Eppendorf: the first step at 700 RPM, the others at 1,000 RPM. The DNA is eluted in 100µl elution buffer.

Realtime PCR design and amplification conditions

The oligonucleotide primers and probes were designed using sequences obtained previously. The dual-labeled TaqMan® TAMRA™ probes were modified with FAM (5'), and TAMRA (3') as quencher for the *B. bruxellensis* and *P. anomala* detection systems. For the *H. uvarum* system, a TaqMan® MGB Probe labeled with FAM was used. The realtime PCR was performed with the GeneAmp5700 instrument from

Applied Biosystems, using the TaqMan[®] Universal PCR Master Mix, no AmpErase[®] UNG from the same company. Each PCR was done in a 20 µl reaction mix, including 5 µl of DNA, 900 nM of each primer and 100nM of the probe. The PCR conditions were as follows: activation of the Taq polymerase for 10 min at 95°C, then dissociation of the DNA at 95°C for 10 sec and annealing plus elongation at 60°C (58°C for *H. uvarum*) for 1 min for 45 cycles.

For testing with the SmartCycler[®] I from Cepheid, OmniMix[®] HS Reagent was used for the realtime PCR. The PCR run conditions were changed to suit the different PCR chemistry and instrument. Initial denaturation at 95°C for 2 min, denaturation at 94°C for 2 sec and annealing plus elongation at 60°C for 30 sec for 45 cycles.

Additionally, the *B. bruxellensis* realtime PCR system was adapted to the iCycler iQ real-time PCR detection system from Bio-Rad. The same PCR chemistry, concentrations and PCR conditions were used as for the GeneAmp5700, except that 16µl reactions were used instead of 20µl.

Validation of the detection systems

The samples for the realtime PCR and the sensitivity of the DNA extraction were taken from cell suspensions in YPD growth medium and diluted in tenfold steps with physiological NaCl solution. The yeast cells in the samples were counted under the microscope with an improved Neubauer counting chamber. The standard curves, which show the linearity, efficiency, and range of the quantitative PCR, were generated by diluting extracted DNA from a known cell number, in these cases 10^7 cells/ml, down to 10 or 10^2 cells/ml in ten-fold steps. Standard curves were calculated for suspensions from 10^7 down to 10^3 cells/ml. For the calculation of the linearity,

range, threshold, sensitivity, and trueness of the whole detection system, cell suspensions of 10^7 cells/ml were used for a dilution series down to 10^1 or 10^2 cells/ml. Each cell suspension was then extracted with the DNeasy Tissue Kit as described above, and the resulting DNA used for realtime PCR. Both, for the standard curve and the validation of the whole system, each cell density (from 10^7 to 10^3 cells per ml) was amplified in three replicates.

Results

DNA extractions with different methods and media

Four different extraction kits were tested with *B. bruxellensis*. For each kit, 5×10^9 , 10^9 and in tenfold step dilutions down to 10^1 cells/ml were extracted from a physiological NaCl solution and amplified in duplicate (one kit with fiberglass columns, one with precipitation and centrifugation and one with a silica gel column, from Qiagen) and are shown in FIGURE 3.1. The linearity and range were calculated. The kits based on precipitation of the DNA and centrifugation did not allow quantitative DNA extraction. The kit using a fiberglass column yielded varying correlations for the linearity. Between 10^8 and 10^3 cells/ml, the linearity typically had a value of 0.812. The DNA extraction kit based on a silica gel column (Qiagen DNeasy Tissue Kit) yielded by far the best values for linearity and reproducibility. The correlation between 10^7 and 10^3 cells/ml had a value of 0.996. Similar correlation values were obtained with the other media and other yeast species tested.

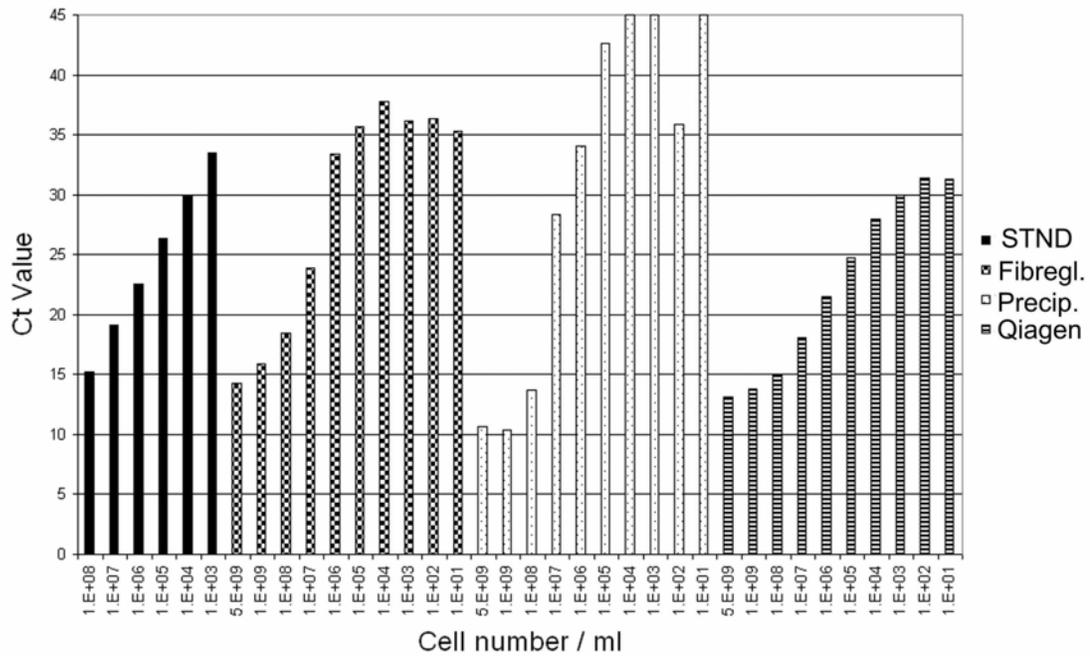


FIGURE 3.1 Ct-values from different DNA extractions of *B. bruxellensis*. For each extraction series, 5×10^9 , 10^9 in tenfold dilution steps down to 10^1 cells/ml were extracted, and the realtime PCR run on the GeneAmp5700 instrument from Applied Biosystems. The standard was diluted from the DNA of 10^8 cells/ml extracted with the Qiagen Kit. STND: Standard; Fibregl.: Extraction with Fibreglass column; Precip.: Extraction based on precipitation and centrifugation; Qiagen: Extraction with DNeasy Tissue Kit from Qiagen (silica gel column).

With the silica gel column based kit (Qiagen), further trials were performed to discover whether wine sediments can interfere with the DNA extraction or amplification, and whether in a sample with few cells (10^2 cells/ml) the sample size (50ml versus 1ml) lowers the threshold of detection. To test this, 0, 10^2 and 10^6 cells/ml of *B. bruxellensis* were inoculated into three media: physiological NaCl solution, wine without sediments and wine with sediments and left overnight. The wine with sediments was an unfiltered wine from Spain which contained proteins, phenolics and dead microorganisms. From each spiked sample, 1 ml and 50 ml were extracted and quantified with the realtime system.

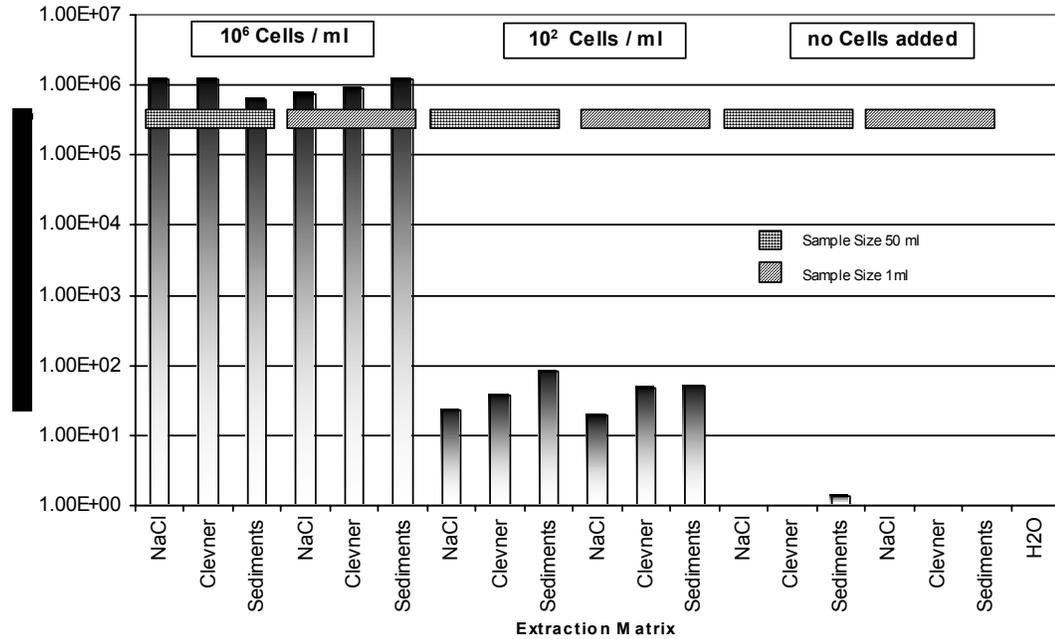


FIGURE 3.2 Trials with different media and extractions of 50 ml and 1 ml samples for *B. bruxellensis*. NaCl: physiological salt solution, sediments: spanish wine with a lot of sediments, Clevner: negative control, filtered wine. The realtime PCR was run on the GeneAmp5700 instrument from Applied Biosystems.

As can be seen in FIGURE 3.2, the measured cell numbers correspond well to the inoculated cell numbers. The standard deviation over all samples was 13 % for 10⁶ cells/ml and 10 % for 10² cells/ml. The extraction of 50 ml samples did not yield better results, but are more labor-intensive to process than 1 ml. Based on these results, only samples of 1 ml were extracted for the subsequent tests.

Realtime PCR validation of three wine yeasts

All the strains of the targeted yeast for the realtime PCR detection system are

considered 'positive' organisms for the test of specificity. All other species are the 'negative' (non-target) organisms. For each strain mentioned, 10^6 cells/ml were extracted and three realtime PCR performed. The 'positive' organisms were required to have the same Ct values ± 1.0 . The Ct values for the 'negative' organisms had to be higher than 35. All the extracted yeast DNAs underwent Internal Transcribed Spacer (ITS) PCR (Esteve-Zarzoso et al., 1999; Guillamón et al., 1998) and PCR-RFLP analyses and were compared to known patterns to ensure correct identification. The mean Ct values for the 6 different *H. uvarum* strains at 10^6 cells/ml was 21.55. The maximum difference between the mean and any single Ct value was 1.04, and the relative standard deviation for the Ct values was 2.9%. The average of the Ct values for the 6 *B. bruxellensis* strains at 10^6 cells/ml was 22.98. The maximum difference between the average and any single value was 0.89. The relative standard deviation was 2.36% for the Ct values. The average of the Ct values for the two different *P. anomala* strains at 10^6 cells/ml was 23. The maximal difference between the mean and a single value is of 0.19. The relative standard deviation is 0.63% for the Ct values. All non-targeted organisms tested for each species were negative after 45 cycles (TABLE 3.1).

To test the linearity and threshold of the PCR, DNA was extracted from suspensions of 10^7 cells/ml, and diluted twice in double distilled water in tenfold steps down to 10^1 cells/ml. The same cell suspension to evaluate the linearity of the PCR was used for the test for linearity of the whole system. The suspension was diluted twice in physiological NaCl solution in tenfold steps down to 10^1 cells/ml. From each cell suspension a 1 ml sample was used to extract the DNA and perform three realtime PCR. The quantification was required to be linear in the range of 10^7 to 10^4 cells/ml, but the realtime PCR results were linear between 10^7 and 10^3 cells/ml (TABLE 3.2). At 10^2 cells/ml, all three standards and two of three PCR amplified. Thus results were

nonlinear in this range. Only some amplifications occurred at 10^1 cells/ml; the negative controls never amplified. Thus, the threshold of quantification is 10^3 cells/ml, and detection 10^2 cells/ml.

TABLE 3.2 Slope and correlation of the standard curves

Standard curve:	<i>H. uvarum</i>		<i>B. bruxellensis</i>		<i>P. anomala</i>	
	Slope	Corr.	Slope	Corr.	Slope	Corr
Diluted DNA 1	-3.51	-0.998	-3.46	-0.999	-3.88	-0.999
Diluted DNA 2	-3.48	-0.999	-3.45	-0.998	-3.62	-0.997
Extraction 1	-3.56	-0.997	-3.46	-0.999	-3.86	-0.997
Extraction 2	-3.75	-0.998	-3.51	-0.999	-3.87	-0.998

Standard curves between 10^7 and 10^3 cells/ml for the linearity of the realtime PCR and the whole system. The realtime PCR was run on the GeneAmp5700 instrument from Applied Biosystems.

One log of CFU/ml can be clearly differentiated within the results of the extractions for all three yeast species within the linear range (10^7 to 10^3 cells/ml), satisfying the requirements for sensitivity.

TABLE 3.3 Results of the trueness for both extractions, shown for each detection system.

Trueness	<i>H. uvarum</i>			<i>B. bruxellensis</i>			<i>P. anomala</i>		
	Slope	Corr	Y-sect	Slope	Corr	Y-sect	Slope	Corr	Y-sect
Extraction 1	0.996	0.993	-0.057	1.002	0.999	-0.25	1.064	0.990	-1.62
Extraction 2	0.946	0.999	0.380	0.984	0.999	-0.175	1.000	0.997	2.51

The realtime PCR was run on the GeneAmp5700 instrument from Applied Biosystems.

For the determination of the trueness the results of the test for the linearity were used. The obtained logarithmic values of the cell numbers were plotted against the expected values. In the resulting plot the regression analysis is required to have a slope that is very close to 1. The y-axis intercept should be very close to zero (TABLE 3.3,

FIGURE 3.3). Both extractions for all three yeasts yielded slopes and y-axis intercepts which were well within range, and the r^2 value ranged from 0.990 to 0.999.

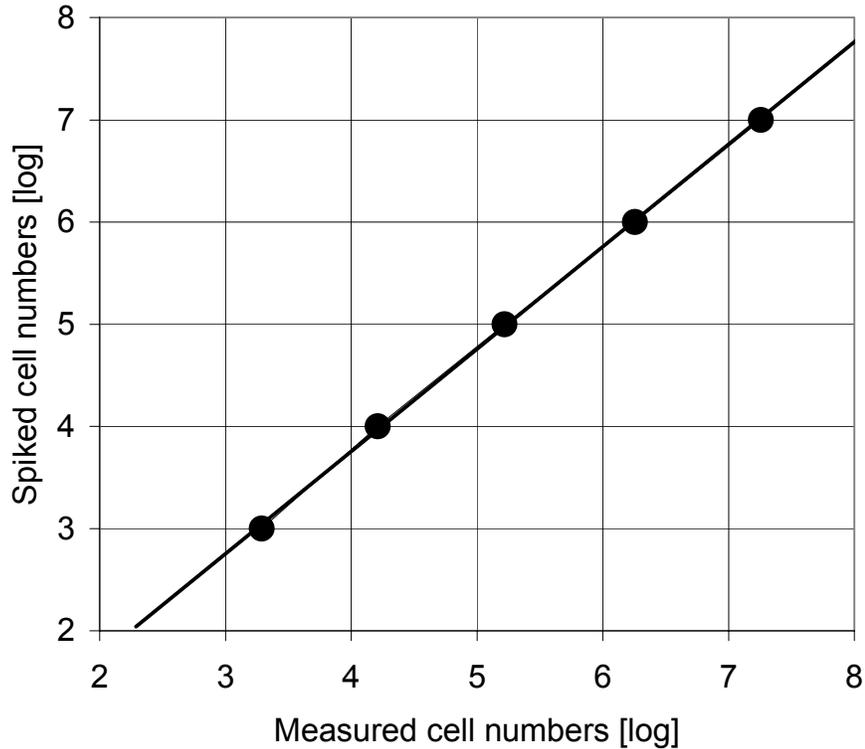


FIGURE 3.3 Trueness of the detection system for *B. bruxellensis*, extraction 1. The slope of the curve of regression analysis is 1.002, and the correlation 0.993. The realtime PCR was run on the GeneAmp5700 instrument from Applied Biosystems.

Realtime PCR with the SmartCycler[®] I from Cepheid

In order to adapt the method for *B. bruxellensis* to the SmartCycler, we validated the DNA extractions only. We considered it redundant to repeat the validation for the diluted DNA and for the method specificity since these are not affected by the system. As the SmartCycler has only 16 places for simultaneous Realtime PCR, it is not

possible to run standards with the samples on each run. Therefore, it is very important, in order to perform a correct validation, to use standards from several runs. The cell suspension was diluted three times from 10^7 to 10^2 cells/ml in ten-fold dilution steps. The cell suspensions were then extracted and three reactions performed from each dilution series (two for one of them). With one exception, the replicates were amplified in separate runs.

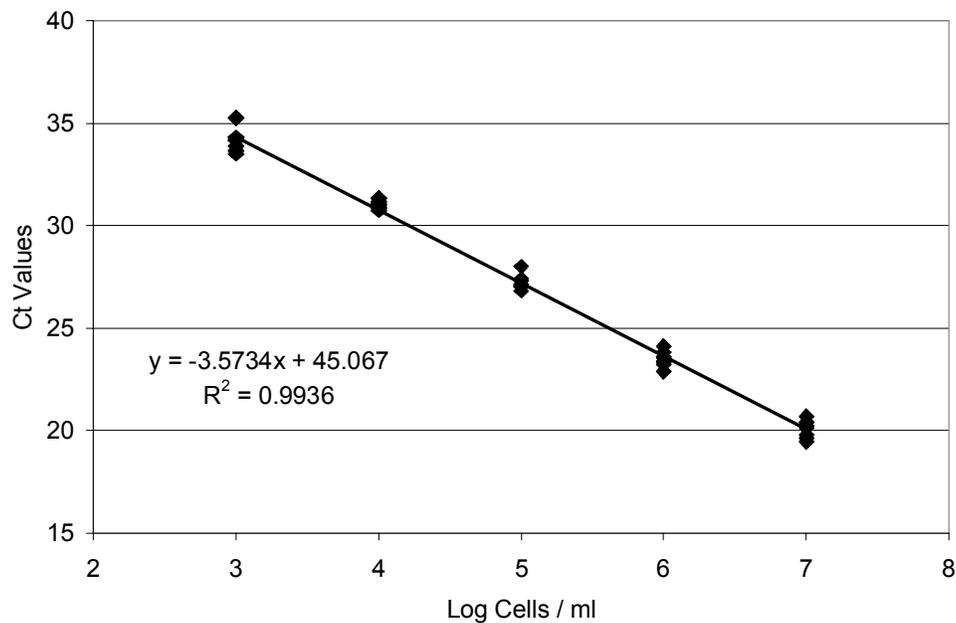


FIGURE 3.4 Standard curve for *B. bruxellensis* on the SmartCycler[®] I from Cepheid. The slope is -3.57 and the correlation 0.994.

Taking into account all the obtained Ct values from 10^7 to 10^3 , the standard curve has the following equation (FIGURE 3.4):

$$Y = -3.57X - 45.07$$

$$R^2 = 0.994$$

Where:

Y = Ct value

X = Log (cells/ml)

DNA is extracted from samples with unknown cell numbers and three realtime PCR are performed. To calculate the cell concentration using the standard curve, the following equation is applied:

$$\text{Log (cells/ml)} = -[(\text{Ct value} - 45.07) / 3.57]$$

Realtime PCR with the the iCycler iQ real-time PCR detection system from Bio-Rad

The iCycler has a 96-well format, so that a standard curve can be added for each realtime PCR run. The specificity was not tested again, but the linearity of the PCR was ascertained.

TABLE 3.4 Slope and correlation of the standard curves between 10^7 and 10^3 cells/ml for the linearity of the realtime PCR and the whole system. The realtime PCR was run on the iCycler iQ real-time PCR detection system from Bio-Rad.

Standard curve:	Slope	Correlation
Diluted DNA 1	-3.46	-0.998
Extraction 1	-3.59	-0.994
Extraction 2	-3.56	-0.994
Extraction 3	-3.56	-0.997

The realtime PCR results between 10^7 and 10^3 cells/ml are linear (TABLE 3.4). 10^2

cells/ml amplified, but the results were not always quantitative compared to the linear range. At 10^1 cells/ml, amplification occurred in at least one out of three replicates.

The same extractions and amplifications as for the linearity were used to test for the sensitivity (FIGURE 3.5). The sensitivity of the realtime is required to be 1 log unit. The measured values for the different cell numbers can be clearly differentiated down to 1 log unit.

TABLE 3.5 Results of the trueness for all three extractions, plus all points together

Trueness	Slope	Correlation	Y-axis section
Extraction 1	1.04	0.994	-0.142
Extraction 2	1.03	0.994	-0.159
Extraction 3	1.05	0.996	-0.250
Extractions all	1.04	0.994	-0.184

The realtime PCR was run on the iCycler iQ real-time PCR detection system from Bio-Rad.

The threshold, which has to lie between 10^3 and 10^4 cells/ml, is within this requirement at 10^2 cells/ml. The negative controls never amplified. The trueness was determined in the same way as for the GeneAmp5700. TABLE 3.5 shows the results of the trueness.

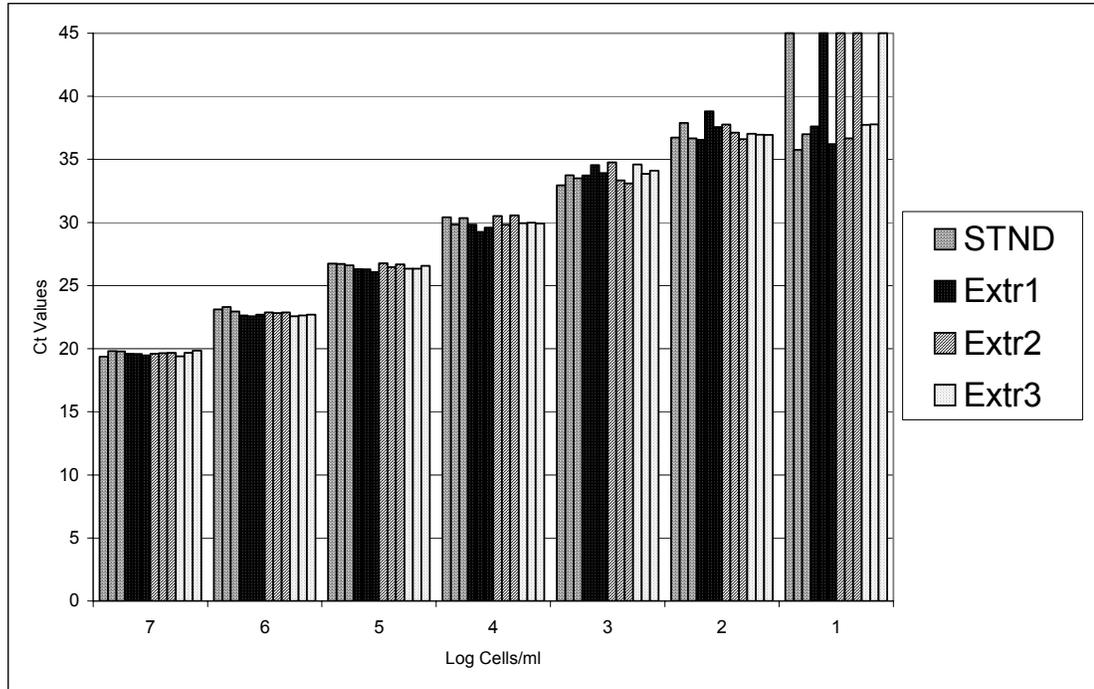


FIGURE 3.5 Ct values of all realtime PCR repeats on the iCycler iQ real-time PCR detection system from Bio-Rad. STND: standards (diluted DNA), Extr1-3: three DNA extraction series, ranging from 10^7 to 10^1 cells/ml.

Discussion

The realtime PCR systems we designed proved to be very robust. Unspecific amplification never occurred, with either bacteria or non-targeted yeast species. Different strains from the targeted species amplified within one PCR cycle, which is good, considering the level of polyploidies within the targeted species is unknown. The extraction kits that we tried yielded very unequal results. Kits based on the precipitation with subsequent centrifugation did not yield quantitative amounts of DNA yields, as shown in the example in FIGURE 3.1. The linearity of the cell suspension dilution series extracted with the kit based on a fiberglass column, at -

0.812 between 10^8 and 10^3 cells/ml and of -0.68 between 10^7 and 10^3 cells/ml, did not fulfill the expectations either. For this type of experiment, the linearity should lie above -0.98. Only the Qiagen DNeasy Tissue Extraction Kit, which uses a silica gel column to collect the DNA, was acceptable with a correlation of -0.994 between 10^8 and 10^3 cells/ml and of -0.996 between 10^7 and 10^3 cells/ml. The second important point about the DNA extractions was the matrix. Wine contains a lot of polyphenols, which notoriously disturb PCR. In particular unfiltered wines are difficult to extract since they can contain a high amount of sediments. The direct comparisons between spiked examples of cell suspensions in physiological NaCl, a filtered wine and a wine with a high amount of sediments clearly showed that neither the matrix nor the sample size influence the result of the realtime PCR. Because the handling of 1 ml samples is much simpler than 50 ml, only 1 ml samples were used after this experiment.

The range of the linearity of a realtime PCR has to be determined as well as the slope and correlation. The slope of a standard curve is acceptable between -3.12 and -3.9. The slopes for all three species, both for the standard curves and the extraction series, were between -3.45 and -3.88 from 10^7 to 10^3 cells/ml. The corresponding correlations varied between -0.997 and -0.999. Therefore the range, the PCR efficiency and the correlation for each detection system was within the requirements. With these slopes and correlations, the sensitivity of one Log unit that was expected for the detection systems were satisfied. The comparison of the Log of the measured cell numbers to the Log of expected cell numbers, counted with a microscope, results in a measure of the trueness. The slopes of these comparisons lie in all cases between 0.946 and 1.064, with a correlation of at least 0.99. This means that the Log of measurements result show between 94.6 and 106.4% of the Log of the spiked cell number, with a good correlation. The Y-axis intercept varies between -1.62 and 2.51, meaning that $10^{2.51}$ is

the maximum number of cells that do not get measured, although 10^2 cells always gave a signal before 45 cycles.

The system for *B. bruxellensis* worked accurately on three different realtime PCR instruments: The GeneAmp5700 from Applied Biosystems, the SmartCycler[®] I from Cepheid and the iCycler iQ real-time PCR detection system from Bio-Rad. All standard curves using diluted cell suspensions were well within the requirements for their parameters.

Delaherche *et al.* (Delaherche et al., 2004) designed a realtime PCR system for *B. bruxellensis* in wine using the *rad4* gene. Their detection limit was much higher at 10^4 CFU/ml. They used CFU to quantify their standards, which means that they detected both dead and living organisms, but counted only the culturable yeast cells. Therefore their detection threshold is probably even higher. Phister *et al.* (Phister and Mills, 2003) used the 26S rRNA gene, and they found that they can detect one cell per ml. They also compare DNA copies to CFU. But it is known that not all *B. bruxellensis* cells present in wines or cultures effectively grow on plates (Millet and Lonvaud-Funel, 2000). Additionally, even if there are several copies of the rRNA gene in each cell, it would be difficult to reliably detect one cell per ml by extracting 700 μ l of sample. Bleve *et al.* (Bleve et al., 2003) used actin mRNA as template for realtime PCR. Their matrix was yogurt and pasteurized food products. They obtained a much lower threshold at 10 CFU/ml, but their system is designed to detect a wide range of contaminating yeast and mould species. These systems were carried out using SybrGreen assays.

After the basic validation described in this paper, we made trials with our realtime PCR systems using wine samples from different origins, particularly for *B. bruxellensis*. The next portion of this project will be concerned with the detection of actin mRNA, using our specific primer-probe systems, to determine the viable cell

numbers. Our goal to surpass the currently available detection systems was achieved. The samples can be processed within 6 hours, and the results are very reliable. This is particularly important for a yeast like *B. bruxellensis*, which can be disastrous even in small amounts and is often difficult to culture (Millet and Lonvaud-Funel, 2000). Although using DNA as a template shows dead as well as viable organisms, these results are an early indication that there is a potential problem in the winemaking.

CHAPTER 4: CONNECTION BETWEEN *BRETTANOMYCES BRUXELLENSIS*
CELL NUMBERS, MARKER PHENOLS AND SENSORY IMPRESSION OF
WINE

Running Title: *Brettanomyces* and marker phenols

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Abstract

The spoilage yeast *Brettanomyces bruxellensis* produces potent off-odors in wine. These range from horse stable, mousy, medicinal and leather to smoky and spicy. These odors are caused, in part, by three volatile phenols: 4-ethylphenol, 4-ethylguajacol, and 4-ethylcatechol. These phenols are widely used as marker substances to determine *B. bruxellensis* contamination in wines. The aim of this study was to determine whether there is a quantitative connection between *B. bruxellensis* contaminations, the marker phenols, and the sensory impression. Additionally, the influence of sugar and the length of growth in wine on *B. bruxellensis* and the presence of the marker phenols was investigated. The effect of cleaning procedures on the cell count was determined by testing rinsing water from cleaned oak barrels for *B. bruxellensis*. The method used to quantify this yeast was a realtime PCR system which specifically targets *B. bruxellensis*. Two or three of the marker phenols were analyzed by GC-MS, and the wines were also tasted to get a complete picture of the contaminated wines. Although there was clearly a connection between *B. bruxellensis* contamination and the marker phenols, there was no quantitative correlation. This yeast is unpredictable, it can not be inferred from a cell quantification what the marker phenol concentrations will be, and neither can the marker phenol infer the cell number. The cleaning of oak barrels needs to be investigated further, because conventionally cleaned barrels still contain residual populations of *B. bruxellensis*. The length of time that *B. bruxellensis* is present in wine is not necessarily correlated with the concentration of marker phenols, and neither does increase in biomass. Apparently there are other physiological factors which determine how much of the marker phenols are produced.

Introduction

The characteristics of wines are determined by a wide range of influences. Among them are the grape variety, site, comprising weather (temperature during vegetative growth of grapes) and soil characteristics, as well as the fermentation conditions and maturation of the wines. The alcoholic fermentation of the grapes should be carried out by strains of the yeast genus *Saccharomyces*. There are other yeast species in grape must which are capable of transforming glucose and fructose into ethanol, however, these yeast usually produce increased amounts of acetic acid, ethyl acetate or other unwanted substances. These non-*Saccharomyces* yeasts are present on the grapes and comprise a large part of the native yeast flora of the grape must. Therefore, it is common either to inoculate with a commercial *Saccharomyces* yeast starter culture, or use a pied-de-cuve (enhancing the native *Saccharomyces* flora in a portion of the grape juice) and then inoculating the bulk of the grapes (Jackson, 1994). After the wine is fermented, the risk of contamination of the young wine is reduced to few yeast species because most yeasts are sensitive to the combination of alcohol and low pH that characterizes wine. There are two yeast genera which can still damage the finished wine: *Pichia* and *Brettanomyces*. *B. bruxellensis*, whose anamorph is called *Dekkera bruxellensis*, can produce a variety of mostly unpleasant odors in wines. They have been described as horse stable, horse sweat, leather, band aid®, dried cow manure, mousy, medicinal flavors, smoky and spicy aromas and at times a distinct metallic bitter taste (Egli et al., 1998; Henick-Kling et al., 2000; Licker, 1998; Licker et al., 1999). Wines with this particular off-flavor are often referred to as ‘bretty’. Additionally, *B. bruxellensis* can produce large amounts of acetic acid (Ciani and Ferraro, 1997; Freer, 2002) and biogenic amines (Caruso et al., 2002). It is controversial among winemakers whether the influence of *B. bruxellensis* is always

negative (Fugelsang, 1998), but it is known that the effects are unpredictable and uncontrollable, and when left uncontrolled they can cover up all grape varietal and regional flavor characteristics of a wine.

Volatile phenols have a very strong influence on the aroma and flavor of wines. Two such phenols can be produced in large quantities by *B. bruxellensis*: 4-ethylphenol (4-EP) and 4-ethylguajacol (4-EG) (Chatonnet et al., 1992). P-coumaric acid can be transformed into 4-vinylphenol and ferulic acid into 4-vinylguajacol by a range of yeasts and bacteria which are able to grow in wine, using the cinnamate decarboxylase. These two compounds then are converted into 4-EP and 4-EG, respectively, by a vinylphenol reductase (Chatonnet et al., 1992). Only *Brettanomyces* yeasts produce these substances in large amounts in wine. These two phenols are widely used as marker substances, to determine the presence of *B. bruxellensis* and the potential of spoilage. Recently, a third marker phenol was discovered: 4-ethylcatechol (4-EC). Caffeic acid is probably transformed first into 4-vinylcatechol by a decarboxylase and then into 4-EC by a reductase similarly as with 4-EP and 4-EG (Hesford et al., 2005). The sensory threshold in wine of these phenols is: 300 µg/l (4-EP), 50 µg/l (4-EG) and 50 µg/l (4-EC). The sensory impact of these phenols on wine are varied: 4-EP has a cow barn and baid-aid[®] flavor, while 4-EG smells spicy and 4-EC smoky, like barbeque. In combination, the result is the typical horsy and leather impression, which can become overwhelming in large amounts.

The present study attempts to determine whether there is a quantitative connection between *B. bruxellensis* contamination and the three mentioned marker phenols. To that end, analyses are made using various wines, comparing *B. bruxellensis* cell numbers, marker substances and subjective flavor descriptions.

Materials and Methods

Wines and sensory evaluation. For all experiments, the samples were taken after the alcoholic fermentation. Sampling took place either from bottled wines or from oak barrels, as described for each test. The tastings were done informally, and therefore only give subjective impressions. The results of tastings by expert tasters are included because it provides valuable information that the chemical analysis might not reveal. Each set of wine used for individual experiments was tasted by the same panel, except for the experiment ‘bottled wines from varied sources’, where only the impression of ‘brettiness’ from different experts was relevant.

For the trial ‘Sensory data, *B. bruxellensis* and filtration’, 15 wine barrels from one winery in the Thurgau district (Switzerland) were investigated, as well as two of these wines after filtration and wash water from these two barrels after cleaning. The experiment ‘Incubation time and phenol quantities’ investigated the changes in *B. bruxellensis* cell numbers and marker phenols after two months residence time of *Brettanomyces* in the wine. To this end, eight oak barrels from a winery in Tuscany (Italy) were analyzed twice, with a two months interval. Additionally, wines from different vintages were tested to find out whether the influence of *B. bruxellensis* changes over time. The experiment ‘Complete set of barrels from a winery’ describes how *B. bruxellensis* can be distributed in a winery. To accomplish this goal, all 52 barrels of wine in one winery from the region of Zürich (Switzerland), all with wine from the same vintage, were analyzed. In ‘bottled wines from various sources’, a collection of 58 wines were analyzed. They were collected on the sensory impression of *B. bruxellensis* contamination. The analyses were done to give an overview of the quantification of *B. bruxellensis* and marker phenols in unrelated wine samples.

Quantification of *B. bruxellensis*. Total cell numbers of *B. bruxellensis* were determined by realtime PCR. One ml of the wine samples was extracted with the DNeasy Tissue Kit from Qiagen (Valencia, California, USA) with a slightly modified protocol: the lysis buffer was adjusted to pH 7, the lysis with lyticase at 37°C. All incubation steps were carried out using the Thermomixer from Eppendorf: the first step at 700 RPM, the others at 1000 RPM. The DNA was eluted in 100µl elution buffer. Quantitative PCR were performed either with the GeneAmp 5700 or the AB 7500 realtime PCR instrument from Applied Biosystems. The TaqMan[®] Universal PCR Master Mix, no AmpErase[®] UNG from Applied Biosystems was used for the amplifications. Each PCR was done in a 20 µl reaction mix, including 5 µl of DNA, 900 nM of each primer and 100 nM of the probe. For the AB7500 instrument, a total of 12 µL of the same reaction mix was used. The *B. bruxellensis* strain DSM70739 was used as standard. DNA of the actin gene was used as template. The realtime PCR system and its validation were described in detail in another publication (Porret et. al., submitted), and the primer sequences in an European patent (Brodmann et al., 2004).

Measurement of phenols. The three phenols 4-ethylphenol, 4-ethylguajacol and 4-ethylcatechol were quantified with GC-MS. The calibrations were carried out with 4-EP, supplied by Sigma-Aldrich, Buchs, Switzerland, 4-EG by Morecambe, Lancaster, UK, and 4-EC was obtained from Alfa Aesar (Johnson Matthey), Karlsruhe, Germany. 4-ethylresorcinol, used as internal standard, was purchased from Acros Organics, New Jersey, USA. Analytical grade acetic acid anhydride, pyridine, pentane and diethyl ether were obtained either from Fluka, Buchs, Switzerland or from VWR International GmbH (formerly Merck Europe), Darmstadt, Germany. Each extraction was done as follows: 10 ml of the wine sample were used, with 50µL of 4-ethylresorcinol (100 mg/l, in 1% diethyl ether and 99% pentane) added as internal standard. After addition

of 5 ml of pentane/diethyl ether 1:1 solution, the phenols were extracted by inverting the flask 20 times. The upper, organic phase was then transferred to a 5 ml ReactiVial™ and dried down to approximately 1 ml under nitrogen in an ice bath. 1.5 ml of acetic anhydride and 30 µL of pyridine were added as catalyst, and the contents mixed well. The vial was then placed in a water bath at 70 °C for 60 minutes. After cooling, the contents were transferred to an autosampler vial and analyzed within 24 hours.

The calibration points were 50, 250, 500, 750 and 1000 µg/l of the three phenols in a wine without detectable phenols. The calibration was carried out using the internal standard method (with 4-ethylresorcinol). For each calibration point, five injections were performed and the mean value taken for the calibration. The gas chromatography was performed with a Saturn 2000 GC-MS system with ion-trap detector, supplied by Varian AG, Scientific Instruments, CH-6303, Zug, Switzerland. The following conditions were employed: Injector temperature was set at 250 °C and the split ratio adjusted to 1:120. The carrier gas used was helium 5.0 at a nominal flow rate of 1 ml/minute. The DB 5, 60 m x 0.32 mm ID gas chromatography column with a film thickness of 1 µm (Column #123-5063) was supplied by J&W Scientific, Folsom, CA 95630-4714, USA. Column temperature was programmed from 65°C at 6°C/min to 140°C, held at 140°C for 10 min, then at 4°C/min to 240°C and held at this temperature for 10 min.

MS conditions: electron ionisation with automatic gain control mode, with detection of the single substances in SIS (Selected Ion Scan) mode to reduce effects from matrix ions and increase sensitivity. Seven segments were programmed as follows. Segment 1 (0 to 28.0 minutes): No ionization or ion preparation technique, since first section of

TABLE 4.1 Mass ions used for analysis of acetylated 4-ethylphenols.

Substance	Mass ions (m/z): (determination)	M ⁺ , molecular ion (confirmation)
4-ethylphenol acetate	107, 122	164
4-ethylguaiacol acetate	137, 152	194
4-ethylcatechol diacetate	123, 138	222
4-ethylresorcinol diacetate	123, 138	222

chromatogram contains no compound of interest. Segment 2 (28.0 to 31.5 minutes): SIS (selected ion segment) ion preparation with a mass range of $m/z = 74$ to 125 and concomitant 100% ejection of mass $m/z = 101$ from a matrix compound. Segment 3 (31.5 to 38.0 minutes): No ionization. Segment 4 (38.0 to 39.5 minutes): SIS ion preparation with a mass range of $m/z = 134$ to 155. Segment 5 (39.5 to 42.5 minutes): No ionization. Segment 6 (42.5 to 50.0 minutes): SIS ion preparation with a mass range of $m/z = 120$ to 143. Segment 7 (50.0 to 57.5 minutes): No ionization. The mass ions used for quantitation as well as the molecular ions are shown in TABLE 4.1.

Results

Sensory data, *B. bruxellensis* and filtration. From a winery in the Thurgau district (Switzerland), 15 wines were evaluated sensorially, and chemically for 4-EP, 4-EG and 4-EC. All wines were from Pinot Noir grapes except barrel B13, which contained a Cabernet Sauvignon and Merlot blend. *B. bruxellensis* was quantified with realtime PCR.

Five of the wines contained *B. bruxellensis*, including the three described as bretty. And two out of those five also had a measurable amount of the marker phenols.

After filtration of B3 and B13 with a 0.45 μm filter, no cells of *B. bruxellensis* were detected in the wine, while the two marker phenols 4-EP and 4-EG remained unchanged (data on 4-EC were not available). After the wines were removed from the barrels for bottling, the barrels B3 and B13 were carefully washed with warm water and treated with a SO_2 solution, and then rinsed with water. The final rinse water still contained *B. bruxellensis* cells TABLE 4.2.

TABLE 4.2 Quantification of *B. bruxellensis* by realtime PCR and of 4-EP, 4-EG and 4-EC by GC/MS. Wines from one winery, including tasting notes.

Barrel number	<i>B. bruxellensis</i>	Concentration [$\mu\text{g/l}$]			Degustation
		4-EP	4-EG	4-EC	
B1	0.00E+00	n.d.	n.d.	n.d.	clean
B2	0.00E+00	n.d.	n.d.	n.d.	clean
B3	3.33E+03	831	147	177	bandaid, may be bretty
B4	0.00E+00	n.d.	n.d.	n.d.	clean
B5	0.00E+00	n.d.	n.d.	n.d.	clean
B6	1.60E+01	n.d.	n.d.	n.d.	dull, may be bretty
B7	0.00E+00	n.d.	n.d.	n.d.	clean
B8	0.00E+00	n.d.	n.d.	n.d.	clean
B9	0.00E+00	n.d.	n.d.	n.d.	smoky, not bretty
B10	0.00E+00	n.d.	n.d.	n.d.	clean
B11	0.00E+00	n.d.	n.d.	n.d.	medicinal, not bretty
B12	7.27E+01	n.d.	n.d.	n.d.	little dull, not bretty
B13	3.84E+04	169	49	27	bretty
B14	1.87E+01	n.d.	n.d.	n.d.	clean
B15	0.00E+00	n.d.	n.d.	n.d.	clean
B3 filtered wine	0.00E+00	797	135	n.a.	
B13 filtered wine	0.00E+00	187	52	n.a.	
B3 rinsing water	1.77E+02				
B13 rinsing water	5.34E+04				

n.d.: phenols not detectable. < 50 $\mu\text{g/l}$ for 4-EP and 4-EG, <25 $\mu\text{g/l}$ for 4-EC

n.a.: not available

Rinsing water: water used to rinse barrels after cleaning

Incubation time and phenol quantities. Samples from Sangiovese wines were taken from 8 oak barrels in a winery in Tuscany (Italy). The wines were tasted, and were found to have a strong bretty flavor. Total cell numbers from *B. bruxellensis* were determined with realtime PCR, and the two marker phenols 4-EP and 4-EG measured using GC/MS. All samples contained large numbers of *B. bruxellensis*, ranging between 5×10^4 and 5×10^5 cells/ml (Fig. 1). These wines also contained a high concentration of the chemical indicators, around 450 $\mu\text{g/l}$ for 4-EP and 150 $\mu\text{g/l}$ for 4-EG. Approximately two months later, samples from the same barrels were measured in the same way. The cell numbers strongly increased over that time period, by about 100-fold, now ranging from about 10^6 to 10^7 cells/ml. The comparison showed that the content in the phenolic compounds in all barrels except one remained unchanged

FIGURE 4.1.

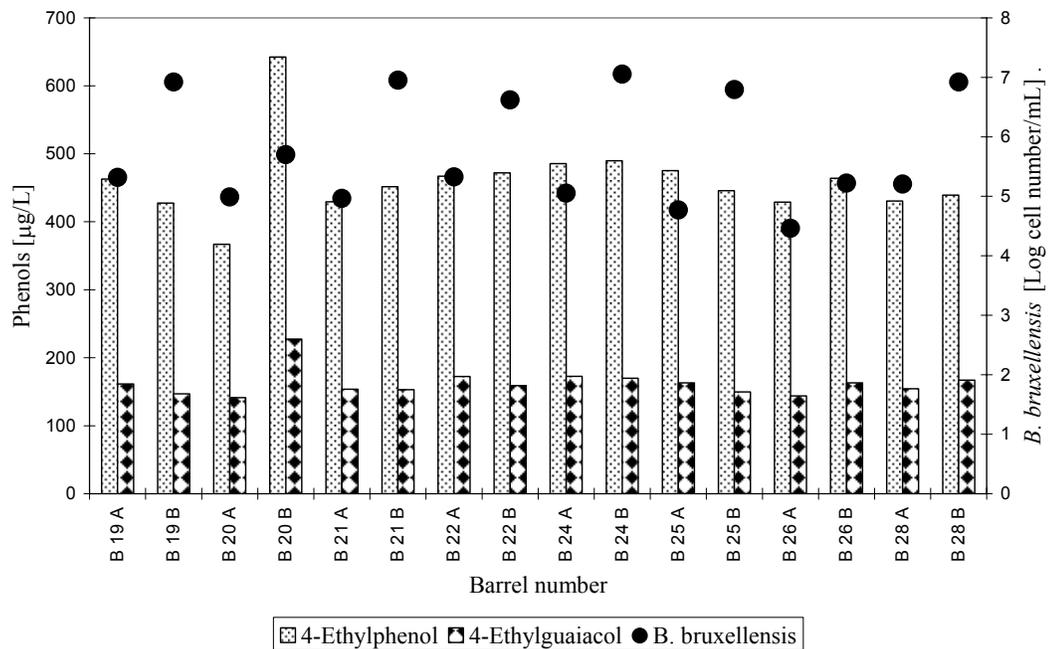


FIGURE 4.1 Samples from 8 oak barrels, each taken with and interval of about 2 months. A: First sampling; B: sampling after two months.

After the wines were bottled, the barrels were carefully cleaned and treated with a SO₂ solution. Tests for *B. bruxellensis* were carried out for 33 different rinse waters. Of those, 12 samples did not contain any cells. The cell numbers in the other samples ranged from about 10 to 10⁴ cells/ml (data not shown).

From the same winery, bottled wines from 1998, 1999, and 2000 were investigated. *B. bruxellensis*, 4-EP, 4-EG and 4-EC were quantified. Tastings revealed a distinctly bretty aroma. There was a steady increase both in phenols and in cell numbers over the vintages, with the largest amount of both in the oldest bottle. These wines had not been filtered before bottling.

TABLE 4.3 Wines from the Tuscany. Measurements of *B. bruxellensis*, 4-EP, 4-EG and 4-EC concentrations.

Vintage	<i>B. bruxellensis</i> [cells/ml]	4-EP [$\mu\text{g/l}$]	4-EG [$\mu\text{g/l}$]	4-EC [$\mu\text{g/l}$]
1998	3.97E+05	902.3	170.8	213.6
1999	3.34E+03	418.3	67.3	109.3
2000	3.57E+02	225.2	44.9	49.4

Complete set of barrels from a winery. All 52 oak barrels from a winery from the region of Zürich (Switzerland) were measured for the content of *B. bruxellensis*, 4-EP, 4-EG and 4-EC. The barrels had different ages (from new to five years) and different provenances (oak source: American, European and Russian oak). The ages and provenances of the barrels were distributed in a random pattern. The wines were Pinot Noir all from the same year (2001). The fermentations were performed in two batches, and then the wines distributed into the oak barrels. One batch was fermented with the commercial *S. cerevisiae* strain Lalvin W15 (Lallemand, Germany) and had no

residual sugar and the other with Lalvin W27 and retained 2 g/l of fructose after the alcoholic fermentation.

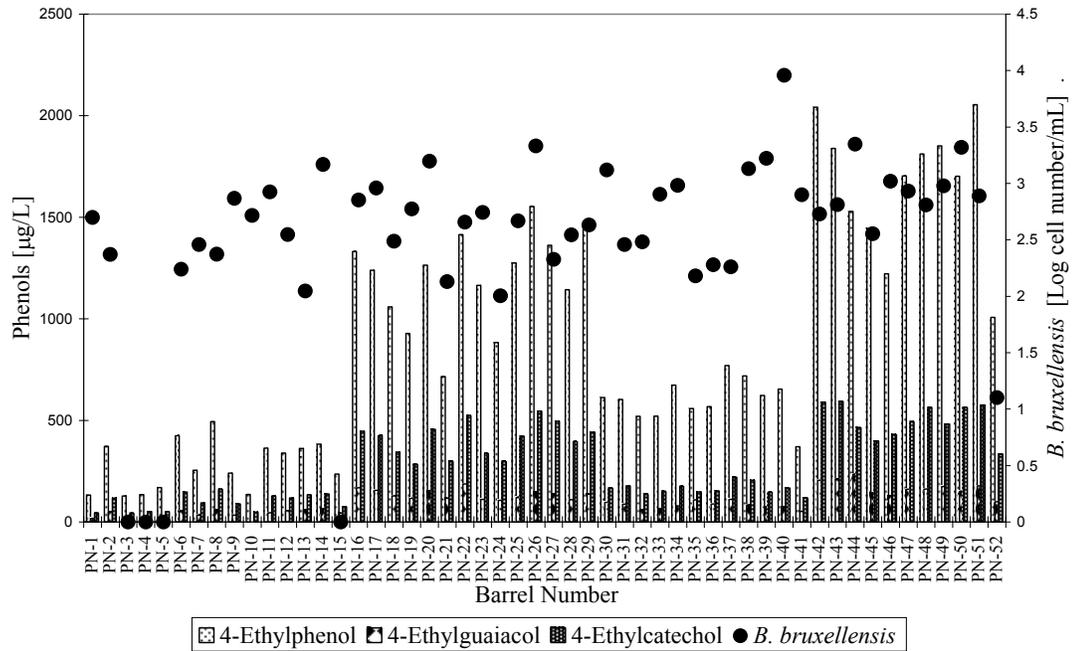


FIGURE 4.2 All oak barrels from a winery, containing maturing wines made of Pinot Noir grapes.

The samples that were positive for *B. bruxellensis* had a total cell number ranging from about 100 to 5000 cells/ml, with one exception at about 10^4 and another around 10 cells/ml FIGURE 4.2. Four samples did not have detectable *B. bruxellensis*. The phenol contents of the wines separated them into four distinct groups. PN 1-15 were the lowest, with an average of 278 µg/l of 4-EP, 41 µg/l of 4-EG and 95 µg/l of 4-EC. PN 16-29 were high, with an average of 1200 µg/l of 4-EP, 135 µg/l of 4-EG and 408 µg/l of 4-EC. PN 30-41 were low, with an average of 600 µg/l of 4-EP, 81 µg/l of 4-EG and 163 µg/l of 4-EC. The highest phenol concentration were found in the samples PN 42-52 with an average of 1655 µg/l of 4-EP, 168 µg/l of 4-EG and 499 µg/l of 4-EC.

Bottled wines from various sources. All 58 samples described in this experiment came from wine bottles. At different tastings, they had been suspected to be bretty. Realtime PCR was performed on all samples to confirm the presence of *B. bruxellensis* cells. 4-EP and 4-EG were also measured. The bottles were collected and tested over a period of two years. The origins of these wines are Switzerland, Spain, France, Italy and Austria. With four exceptions, each sample contained at least traces of 4-EP. These four wines did not contain any *B. bruxellensis* cells. Thirteen wines contained no detectable *B. bruxellensis*, but showed various quantities of 4-EP and 4-EG. All wines which contained *B. bruxellensis* a minimum of 10µg/l of 4-EP FIGURE 4.3.

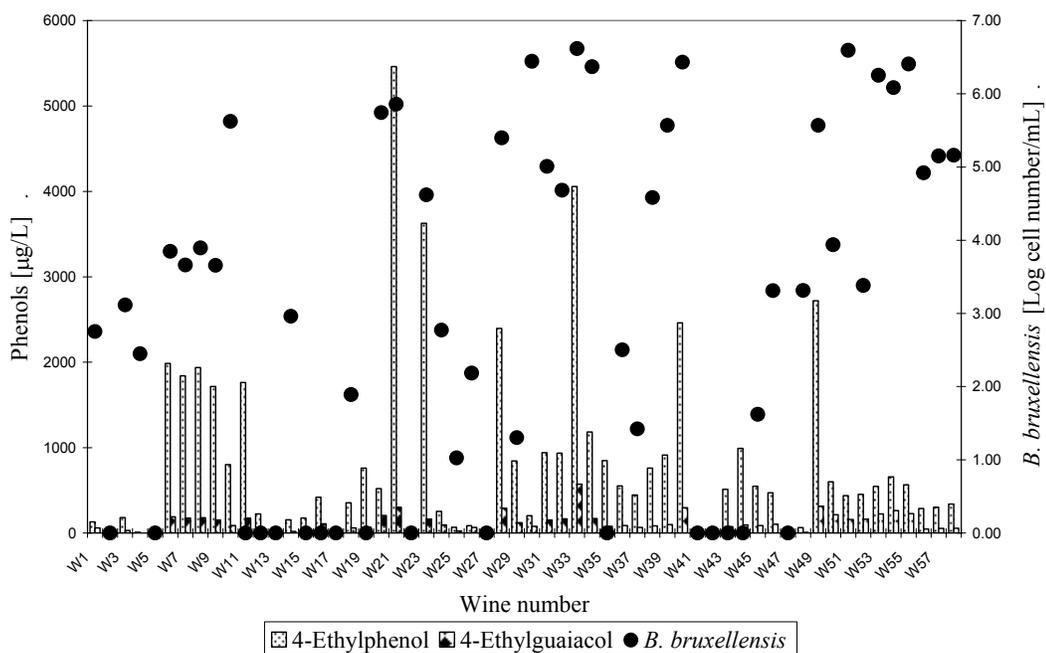


FIGURE 4.3 Wines from bottles form various sources. *B. bruxellensis* was measured with realtime PCR, and the two marker phenols with GC-MS.

Discussion

The experiments shown in this study were selected to cover a variety of different situations in which *B. bruxellensis* may occur in wines. For each situation, the content of marker phenols were measured to compare against the occurrence of *B. bruxellensis*. Ultimately, the aim was to better understand which factors influence the production of the phenols and the presence of *B. bruxellensis* in wines, and how the cell number and phenol concentration are linked. The question was also whether the cell number can be inferred from the phenol concentration. Conversely, the cell number of *B. bruxellensis* might be used to estimate the phenol concentration. It would also be very valuable to be able to judge the impact of a *B. bruxellensis* contamination, including knowledge of other factors, to predict how much the wine will be spoiled in the future and perhaps take steps to keep spoilage to the minimum.

In the example ‘Sensory data, *B. bruxellensis* and filtration’, two wines were strongly bretty by sensory assessment. These two correspond to those samples with large numbers of *B. bruxellensis* cells and marker phenols. B12 and B14 likely had been contaminated from B13, which contained a fair amount of *B. bruxellensis* cells. These contaminations happen when barrels stand next to each other in a winery, and not enough care is taken at tastings, leading to inadvertent contamination of the neighboring barrels. B6 was very lightly contaminated with *B. bruxellensis*, and although the volatile phenols were not measurable, the sensory impression was of ‘possible brettiness’. From this experience we can infer that, the sensory impression does not rely only on the marker phenols. Filtration of the wines removes the cells, but of course does not change the phenol concentration. After a vigorous cleaning of the empty barrels, there were still *B. bruxellensis* cells present on the surface of the wood. The second experiment, ‘Incubation time and phenol quantities’, was designed to find

out how time affects the phenol production of *B. bruxellensis*. The yeast multiplied over a period of two months, but with one exception the concentration of volatile phenols remained constant. In barrel 20, *B. bruxellensis* cell numbers increased slightly, but the content of marker phenols increased strongly. Only barrel 26 had a similarly low *B. bruxellensis* growth, but without the high increase in phenols. The explanation of this phenomenon might be that the production of phenols is low during the growth of *B. bruxellensis*, and increases during the stationary phase, at least under certain circumstances. *B. bruxellensis* seemed to grow well in bottled, unfiltered wines and produced increasing amounts of volatile phenols over the years. The older the wine was, the more *B. bruxellensis* and marker phenols were present. Yet, because we do not have any measurements on the wines from before bottling, this is not proof of what happened in the bottles. Again, the cleaning of the barrels was not consistently effective in this winery, as most rinse waters still contained *B. bruxellensis* after cleaning.

To investigate how the occurrence of *B. bruxellensis* is distributed in a winery and how it affects the wines, a winery was selected in which we checked all barrels for tests on *B. bruxellensis* and its marker phenols ('Complete set of barrels from a winery'). *B. bruxellensis* was present, with four exceptions, in every barrel, with the cell numbers spread over a range of 100 to 5000 cells/ml. Because of the generally low cell densities it may be assumed that the barrels without detectable cells in fact contained very low numbers *B. bruxellensis*, too low to be detected. The barrels themselves were of various origins and ages, but they were not stacked in groups. Yet the sugar content and yeast strain used matched the two groups of phenol concentration. There was no residual sugar in the barrels 1-15 and 30-41 but 2 g/l of sugar had remained in the barrels 16-29 and 42-52. Therefore it seems that sugar promotes the production of the marker phenols by *B. bruxellensis*, even while the cell

numbers remained largely unchanged. It is also possible that the sugar actually promotes the production of the vinylphenols by other microorganisms like *Lactobacillus*, *Pediococcus*, *Saccharomyces* or others (Chatonnet et al., 1992), and so activate the production of ethylphenols indirectly, by increasing their precursors. It is also possible that the *S. cerevisiae* strain in some other way influences the production of the volatile phenols by *B. bruxellensis*.

‘Bottles of wine from various sources’ were collected on the suspicion from different people who thought that these wines had a bretty off-flavor. The ‘histories’ of these wines were not known, and different wines were tasted by different people. These wines give a good overview of what can be found on the market. There was clearly no direct correlation between cell numbers and marker phenol concentrations. Only four of the 58 bottles were not bretty at all, containing neither *B. bruxellensis* cells nor marker phenols. All wines which had a detectable level of *B. bruxellensis* cells also had a detectable level of marker phenols. This shows that there is a tangible connection between *B. bruxellensis* and these marker phenols, even though it is not a quantitative connection. The twelve wines containing phenols but no detectable *B. bruxellensis* cells were almost certainly wines which had been filtered before bottling. Supposedly clean barrels can be an important source of contamination, as shown in the two experiments which contained realtime PCR analyses of rinse water from cleaned barrels. *B. bruxellensis* can survive in tiny cracks in the wood for a long time. Very stringent cleaning methods carry the risk of flushing out aroma components of the wood, which are essential for oak barrel aging of wines. It is clear that better cleaning techniques will have to be investigated thoroughly, possibly barrels will have to be shaved and retoasted.

Overall, the experiments show that although *B. bruxellensis* does in fact produce the known marker phenols, there is no correlation between cell number and concentration

of marker phenols. It must be assumed that in a winery all wine become infected with *B. bruxellensis*. Under some, still not well understood conditions *B. bruxellensis* can grow rapidly. Since not all contaminated wines also contain the marker phenols, and since there is no correlation between cell number and phenol concentration, it is best to monitor wines for the presence of *B. bruxellensis* cells, not for concentrations of volatile phenols. Sufficient SO₂ should always be maintained and wines should be filtered before bottling. In cases of high *B. bruxellensis* contamination, the wines should be filtered while still held in the barrels. Time and residual sugar may influence the amount of marker phenols produced by this yeast. The effect of *B. bruxellensis* on wine is unpredictable and so cannot be deduced accurately.

CHAPTER 5: WINERY WINERY HYGIENE AND OCCURRENCE OF
BRETTANOMYCES

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identification.

Keywords: winery sanitation, *Brettanomyces*, yeast contamination, realtime PCR

Abstract

Brettanomyces bruxellensis is a major wine spoilage yeast which can produce volatile phenols and give the wine a horsy, smoky flavor. The influence of winery sanitation practices on the presence of *B. bruxellensis* in wineries was investigated. Eight winemakers completed a questionnaire about cleaning practices. In four of these, the occurrence of *B. bruxellensis* on different surfaces and in maturing wines was measured. Realtime PCR results showed that *B. bruxellensis* is present on most winery surfaces and wines. Even if *B. bruxellensis* can not be completely eliminated from the cellar environment, winery sanitation remains an essential aspect of *Brettanomyces* control.

Introduction

Wine is susceptible to spoilage by microorganisms. Because wine has a low pH, a low sugar and a high alcohol content, only few microorganisms can survive and grow (Kalathenos et al., 1995). During alcoholic fermentation in wine, the desired wine yeast *Saccharomyces* should predominate, and for the malolactic fermentation the bacteria *Oenococcus oeni* should predominate (Krieger et al., 1993). The main spoilage yeasts in wine are *Hanseniaspora uvarum* (*Kloeckera apiculata*), *Pichia* spp., and *Brettanomyces bruxellensis* (*Dekkera bruxellensis*). *H. uvarum* is found frequently in grape musts and can disturb the fermentation (Velazquez et al., 1991). When present in high numbers, it can produce up to 2 g/l of acetic acid. *H. uvarum* tends to decrease sharply during the alcoholic fermentation because of its lower alcohol tolerance (Lema et al., 1996). *P. anomala* is not a very strong fermenter, but it can produce ethyl acetate through esterification of acetic acid (Rojas et al., 2003). During

the aging of red wines in barrels, the yeast *B. bruxellensis* can lead to the ‘bretty’ off flavor (Chatonnet et al., 1992; Licker et al., 1999). Bretty wine aroma has been compared to a horse stable, horse sweat, Band-Aid[®], medicinal, spicy and others. It is controversial whether *B. bruxellensis* in wine is always bad. Most winemakers want to avoid *B. bruxellensis* because it is impossible to control.

Materials and Methods

The aim of this project was to determine the effectiveness of winery sanitation practices and their influence on *Brettanomyces* occurrence. Eight wineries were selected and the winemakers interviewed by questionnaire about their sanitation and winemaking practice. The questions were focused on the size of the winery, the origin of the barrels, the wines aged in barrels, and the length of wine maturation. The flowchart of the processing of red grapes which are aged in barrels was determined from the press to the bottling. An important part of the questionnaire asked about cleaning habits, and types of chemicals used for sanitation.

In each winery, samples were analyzed using a luminometer (Luminator, by Charm Sciences Inc.). This instrument measures the content of ATP in the sample and estimates the level of contamination of any living cells on the tested surface.

Four wineries with different general hygiene practices were selected for more detailed analyses. For each of the four selected wineries, 4 strategic spots were investigated in triplicate: drain, full barrel surface near the bung hole, clean stainless steel tank (inside) and floor. The samples were taken with sterile cotton swabs and streaked onto agar plates: Lysine agar (from Sigma, media for non-*Saccharomyces* yeasts), YM + cycloheximide (YM from Difco[™], Yeast media. Cycloheximide inhibits most yeasts

but not *Brettanomyces*) and MLB + antifungal agent (MLB is a lactic acid bacteria media). In parallel, one sample was resuspended in PBS (physiological buffer) and analyzed directly with Real Time PCR. Three wine samples from barrels from each winery were filtered and incubated on YM + cycloheximide (YM+C). In parallel, samples from the same barrels were centrifuged and tested for *B. bruxellensis* with Real Time PCR. Real Time PCR is a molecular method which quantifies cells by measuring the DNA content of the organisms that are present in the tested sample. The colony and cell morphology of the yeasts which grew on the plates (YM+C and Lysine) were studied by microscopy, and yeasts that were not clearly different from *B. bruxellensis* were further identified by sequencing of a portion of the 26S rDNA gene (Kurtzmann and Robnett, 1998).

Results

The differences among the wineries in the processing of red wines were relatively small. None of the wineries bought new wine to top up the barrels: most used either wine from a previous year or kept a separate tank for topping. The barrels were usually bought new, made of either American or European oak (also Hungarian in one case). No winery used heat for the grape color and flavor extraction. All inoculated both yeast and bacteria starter cultures for the alcoholic and malolactic fermentations, respectively. The cleaning procedures were very similar. Most wineries used large amounts of water (hot and cold, as well as hot water under pressure) for cleaning. The sanitizers used were preparations of chlorine, citric acid, iodine, peroxide and NaOH in various concentrations and frequencies (TABLE 5.1). Some of the wineries followed very strict cleaning practices, others a less strict, or less frequent.

TABLE 5.1 Sanitation procedures in eight wineries for four strategic surfaces

Winery	Sanitation Procedure			
	Drain	Floor, sink	Barrel	Steel tank
Chem. W 1	Brush + detergent	Broom, detergent	Hot water, sulphur for storage, spray ball	TSP/NaOH, spray ball
Freq. W 1	When dirty	When dirty	Before and after use	After use
Chem. W 2	Destain (Sodium hydroxid)	Destain and Hypochlorate	Hot water with barrel washer	NaOH + Citric
Freq. W 2	After work	After work	Before use	Before and after use
Chem. W 3		water	Gamma jet, hot water/ burning sulphur	Na-Peroxycarbonate, NaOH, water
Freq. W 3	Never	After use, more deeply every 6 months	Racking/ storage	Before and after use
Chem. W 4	Hot water	Hot water	Hot water outside: sodium percarbonate	Sodium percarbonate or caustic or iodophor
Freq. W 4				
Chem. W 5	Cleaned by flow of water.	Hot and cold water	Hot (60°C) water, cold water/ sulphur burn	Brush and rinse/ brush and caustic
Freq. W 5	Continuously	Continuously	Before/ for dry keeping	Before and after use
Chem. W 6	Water	Water with a little chlorine	Hot water, sulphur for storage	NaOH + Cit
Freq. W 6	After work	When dirty, deeper cleaning before harvest	Every raking + bef. storage, watering every month	When emptying the tank
Chem. W 7	iodine		Hot water (160°), burnt sulphur for dry keeping	Sprayball (Iodofore)/ rinse with hot water
Freq. W 7	After work	After work		Before and after use
Chem. W 8	Hot water and peroxy carb	Hot water (pressure), Peroxy carb	Iodine, hot water, peroxy carb	NaOH + Citric acid, sometimes Peroxy carb
Freq. W 8	Once a month or When dirty	Every stacking time	Every racking time (twice a year)	When empty

Chem.: Chemical used

Freq.: Frequency

W: Winery number

All the selected wineries (FIGURE 5.1) carried a high load of microorganisms in the drain. The floor revealed a different picture: only wineries 1, 2, 3 and 6 had high microbial loads all through the winery. The bottling lines were generally very clean, only traces of microorganisms were found in winery 3 and 6. The empty, clean stainless steel tanks generally had a low contamination on the inside. Winery 2 had more contamination and wineries 1, 3 and 4 were clean. Contamination on the outer

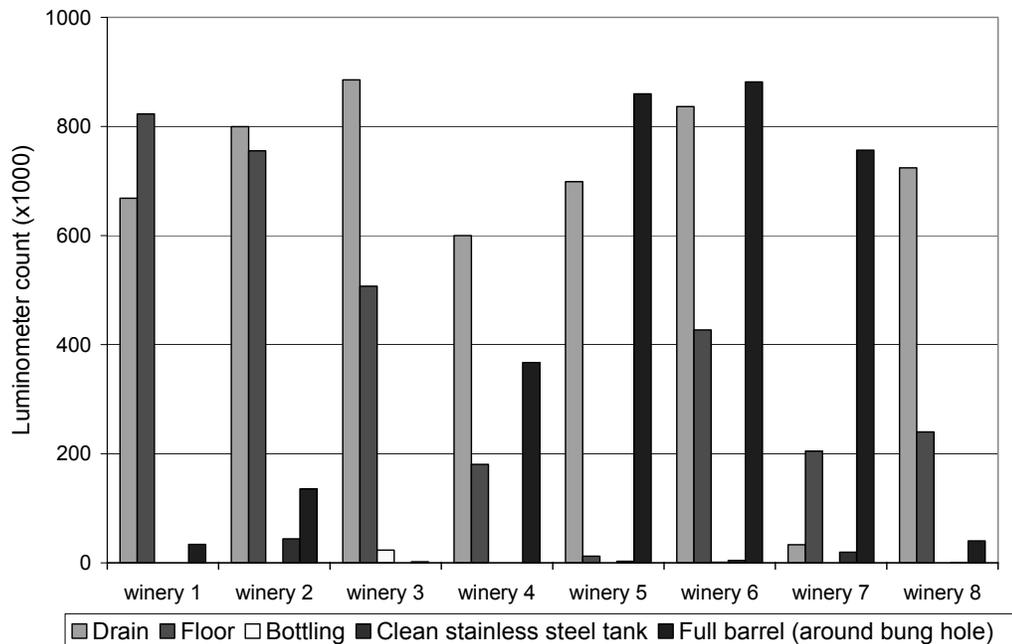


FIGURE 5.1 Contamination of different winery surfaces, measured with a luminometer. Values not in the chart: winery 7, drain had a very low value, possibly due to detergents which interfere with the analysis; winery 3, barrel was not recorded; winery 8, bottling line was not tested because bottling is carried out in another winery.

surface of the barrels was highly variable. Wineries 1, 2, and 8 carried low numbers of microorganisms, whereas wineries 4, 5, 6, and 7 had highly contaminated surfaces.

This is possibly due to the high porosity of the wood and to the sampling practice (wine spillage on the outside of the barrel during filling or racking). From this data, it

seems that the barrel surface can become an important source of contamination. For the next step of the project, wineries 4, 5, 6 and 8 were selected.

The number of different yeast colonies (morphological groups) that grew on Lysine agar and YM plus cycloheximide agar are described in FIGURE 5.2. The colony numbers

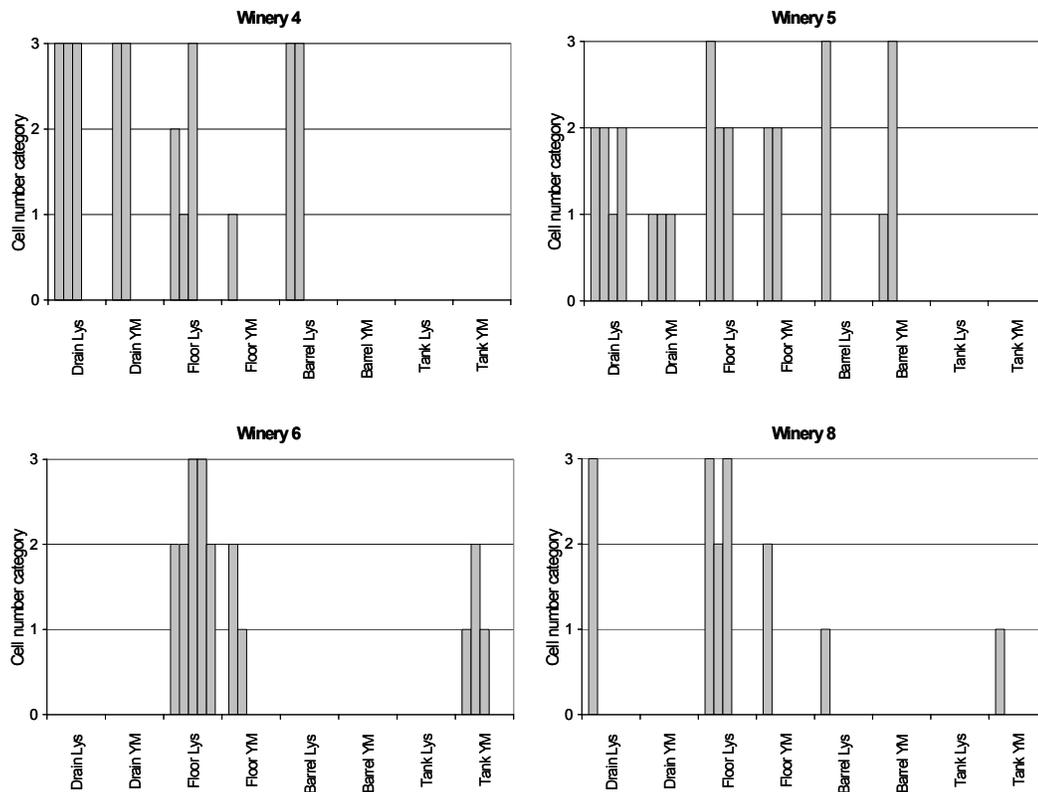


FIGURE 5.2 Number of different yeast colonies sampled from strategic surfaces in 4 wineries (Lys: Lysine agar; YM: YM agar plus cycloheximide)

for each different yeast were separated into three categories: 1-10 colonies, 11-50 and more than 51 colonies. The yeast contamination of the different surfaces was highest in the wineries 4 and 5. Winery 6 had only a high level of yeasts on the floor and in

the stainless steel tank, but was otherwise very clean. Wineries 4, 6 and 8 had yeasts in the stainless steel tank. The problem areas in winery 4 were the floor and drain, and the floor and barrel in winery 5.

Yeast grew from the wine samples A and B from winery 4, A, B and C from winery 5 and A, B and C from winery 6, as detected from the plate. Seven of these different morphological types of yeast that grew were identified as *B. bruxellensis* (TABLE 5.2). The same wine samples were analyzed by Real Time PCR. *B. bruxellensis* was found in all samples except for the wines A from winery 5 and C from winery 6 (FIGURE 5.3). The severity of contamination ranged from about 25 to 10,000 cells/ml.

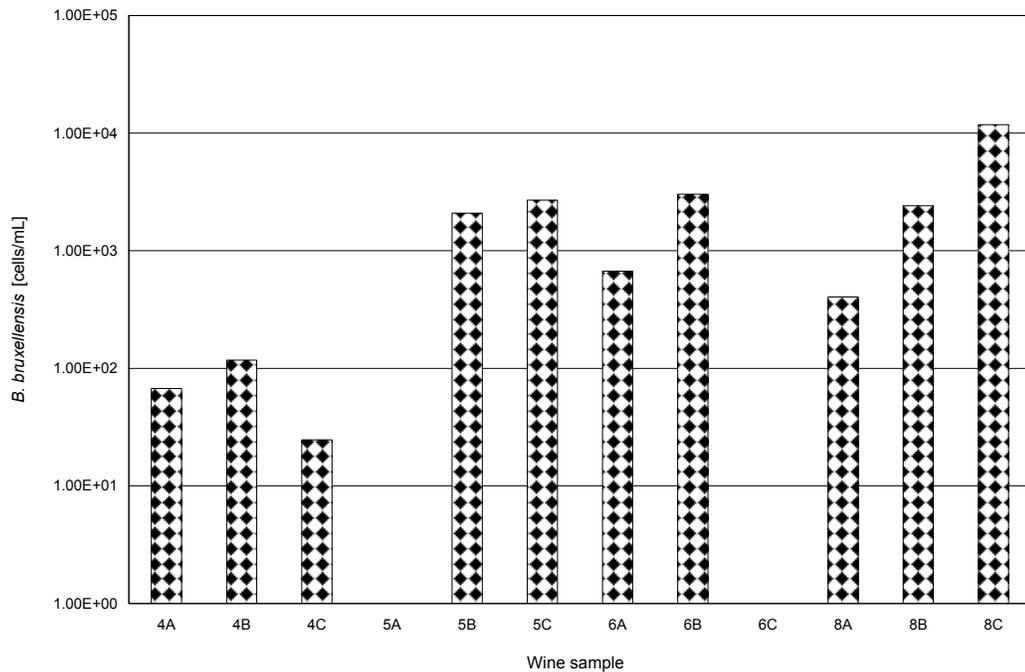


FIGURE 5.3 *B. bruxellensis* contamination in wines measured by Real Time PCR. Three samples (A, B and C) from different barrels taken from the four wineries (4, 5, 6 and 8)

There was no correlation between *B. bruxellensis* contamination of the wines and contamination measured with the luminometer. Bacteria were present in all locations in all wineries except for the stainless steel tank in winery 4. None were lactic acid bacteria, and they were not further studied. The yeast species identified by sequencing are shown in TABLE 5.2. None of the isolates from samples taken from floors, drains, stainless steel tanks of the outside of barrels were identified as *B. bruxellensis*. The other identified yeast species pose no threat to winemaking because they can not grow in wine.

TABLE 5.2 Yeast isolated from the winery environment and wines

Winery	Yeast species	Sampling spot	plating media
4	<i>Yarrowia lipolytica</i>	Drain	Lysine
4	<i>Yarrowia lipolytica</i>	Drain	YM-Cy
4	<i>Lodderomyces elongisporus</i>	Steel tank	YM-Cy
5	<i>Candida boidinii</i>	Drain	Lysine
5	<i>Cryptococcus surugaensis</i>	Floor	Lysine
6	<i>Zygoascus hellenicus</i>	Drain	YM-Cy
5	<i>Cryptococcus laurentii</i>	Barrel	YM-Cy
6	<i>Cryptococcus laurentii</i>	Floor	YM-Cy
8	<i>Cryptococcus laurentii</i>	Floor	Lysine
8	<i>Cryptococcus laurentii</i>	Floor	YM-Cy
4	<i>Dekkera bruxellensis</i>	Wine B	YM-Cy
5	<i>Dekkera bruxellensis</i>	Wine B	YM-Cy
5	<i>Dekkera bruxellensis</i>	Wine C	YM-Cy
5	<i>Dekkera bruxellensis</i>	Wine D	YM-Cy
6	<i>Dekkera bruxellensis</i>	Wine A	YM-Cy
6	<i>Dekkera bruxellensis</i>	Wine B	YM-Cy
6	<i>Dekkera bruxellensis</i>	Wine C	YM-Cy

YM-Cy: YMagar containing cycloheximide; Lysine: and Lysine Agar

The yeast species identified by sequencing are shown in TABLE 5.2. None of the isolates from samples taken on surfaces were identified as *B. bruxellensis*. The other

identified yeast species pose no threat to winemaking because they can not grow in wine.

The presence of *B. bruxellensis*, measured by Real Time PCR in the samples taken from winery surfaces is shown in FIGURE 5.4. These results show that winery 4 is at a higher risk of damaging its wines with *B. bruxellensis* compared to the other wineries, followed by winery 5. Winery 6 had the lowest overall contamination with *B. bruxellensis*. Winery 8 did not have *B. bruxellensis* in the drain sample, nor in two of the samples taken from the floor, but the contamination on the barrel surface in this winery appears dangerously high, as did winery 5. The comparison of the population

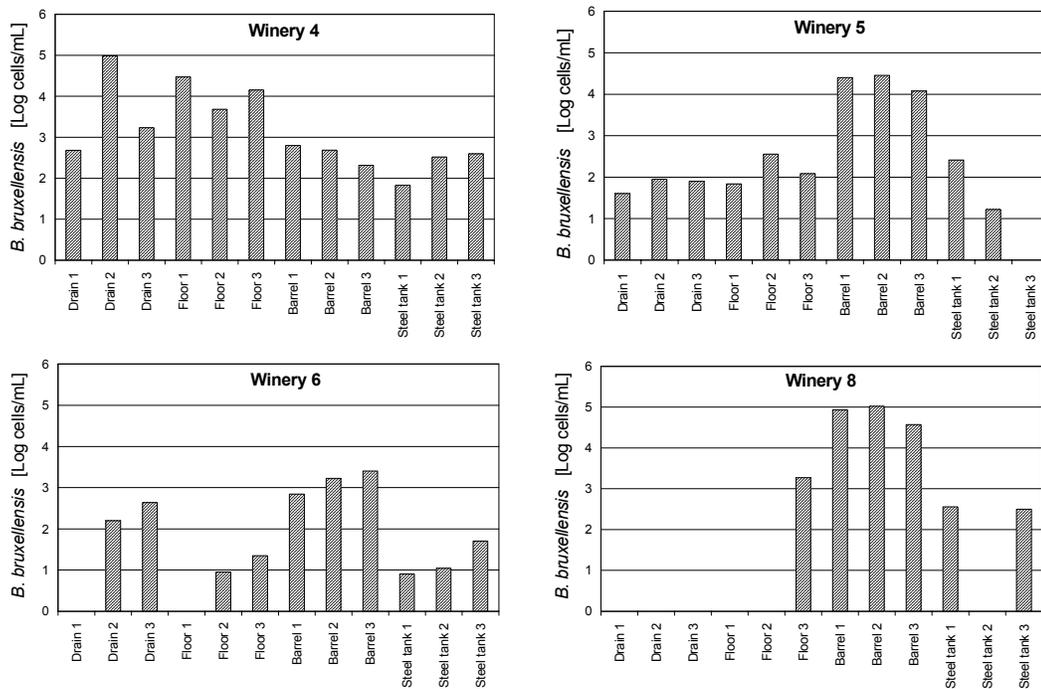


FIGURE 5.4 Contamination load of *B. bruxellensis* on strategic surfaces of the four wineries, measured with Real Time PCR. Samples taken with sterile cotton swabs.

density and the distribution of three of these four wineries shows that the population

can be successfully reduced with a good general cellar hygiene. It is possible to keep floors, drains, and stainless steel tanks nearly free of *B. bruxellensis*. Barrels must be monitored carefully because individual barrels can support very high populations of *B. bruxellensis*. This first survey does not tell us how much the microbial population and specifically *B. bruxellensis* varies in a variety over the course of a productive year.

This comparison shows that *B. bruxellensis* is present in all wineries. Regular sensory and microbial monitoring is necessary to detect wines in which the *Brettanomyces* population starts to increase. The Real Time PCR method which we used has the potential to detect very low cell densities, thus giving the winemaker time to intervene by SO₂ addition and/or filtration to limit *B. bruxellensis* growth. Even if *B. bruxellensis* can not be completely eliminated from the cellar environment, winery sanitation remains an essential aspect of *Brettanomyces* control. Before bottling, all wines should be carefully checked for *B. bruxellensis*. Filtration of red wines will further help to prevent spoilage control in the bottle, and to assure the wine quality.

CHAPTER 6: VARIATION WITHIN THE PROMOTER OF *VvmybA1*
ASSOCIATES WITH FLESH PIGMENTATION OF INTENSELY COLORED
GRAPE BERRIES

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Abstract

Grapevine (*Vitis vinifera* L.), one of the oldest domesticated crops, is mainly grown to produce different kinds of wine. Grape pigmentation shows a wide variety of colors. For most grapevine cultivars, the pigments which define the color are located in the skin of the berry. One of the primary determinants of color is the gene *VvmybA1*, with the black allele being dominant. Teinturier grapevines contain anthocyanins both in the skin and the flesh of the grape berries, whereas in other red or black grapes only the berry skin is pigmented. This character is distinct. A cross between a heterozygous teinturier (white × teinturier) and a white fleshed variety was studied to determine the segregation of this gene. Leaf discs were cut from the seedlings, incubated in a sucrose solution and exposed to light, and visually compared to the teinturier, red berried and white berried controls. Teinturier leaf discs turn red with this treatment. Of 62 seedlings, 29 showed the teinturier character. All 29 also contained a 408 bp insertion in the upstream region of the *VvmybA1* gene. The leaf disk of one seedling did not turn red but contained the 408 bp indel. These results suggest a 1:1 segregation, suggesting that the same gene which controls grape color also controls the berry flesh color. However, the population of seedlings was too small to confirm this theory. The role of the 408 bp duplication in the promoter of the teinturier varieties is unknown.

Introduction

Grapevine (*Vitis vinifera* L.) is one of our oldest domesticated crops and economically the most important cultivated fruit crop in the world. Cultivated grapes show substantial diversity in fruit color, including: varying shades of black, red, pink, grey,

white, and types with pigmented berry flesh. Diversity in fruit color has led to significant definition of market classes of wine, juice, and table grape cultivars and has cultural significance that extends thousands of years into human history. The majority of *V. vinifera* cultivars only possess anthocyanin pigmentation in the skin of the berry. However some cultivars possess berries with intensely pigmented flesh as well as skin which is often also associated with greater pigmentation of vegetative tissues. These varieties are often termed teinturiers and have commercial importance as a source of intensely pigmented juice for blending of wines (Becker and Ries, 1986). For example, the red-fleshed cultivar 'Rubired' is currently the 6th most important grape grown in the state of California for wine production (CA agricultural statistics service). The genetic control and inheritance of fruit color in grapevine is poorly understood despite evidence that the primary determination of anthocyanin production appears to be controlled by a single dominant locus in *V. vinifera* (Doligez et al., 2002; Riaz et al., 2004) with white fruit being a recessive character. Recently, it has been shown that the presence of *Gret1*, a Ty3-gypsy-type retro-transposon in the promoter region of a *myb*-like regulatory gene is present in white-fruited cultivars of *V. vinifera* and that allelic variation in this gene associates with several qualitative classes of grape fruit color (Kobayashi et al., 2004; Owens et al., 2006 in review). It has been suggested that the red-flesh berry phenotype is similarly controlled by a single dominant locus (Boyden, 2005; Galet, 2000). Considering the association of variation in *VvmybA1* with grape berry skin color, it was hypothesized that DNA sequence variation in *VvmybA1* would also be associated with genotypes showing intensely pigmented berry flesh.

In this study we show that allelic variation in *VvmybA1* associates with the teinturier phenotype both in a panel of accessions possessing red-flesh as well as in a population of full-sibs segregating for the red-flesh phenotype.

Materials and Methods

Plant Material and Phenotypic Analysis: Sixty-two seedlings of a cross of Viognier × Scarlet were generated and the phenotype was determined by excising 1 cm square leaf pieces and submerging the leaf segments in a 100g/l sucrose solution for 3 weeks in the presence of ambient light in a greenhouse following previously reported protocols (Becker and Ries, 1986; Carbonneau, 1980; Pirie and Mullins, 1976). Pinot Blanc, Picolit, Tempranillo, Mataro, Petit Bouschet, and Alicante Bouschet were used as negative and positive controls for the leaf disc assay.

Either leaf tissue or DNA was obtained for additional red-fleshed accessions were obtained from the US. National Clonal Germplasm Repository, Davis, CA and the French National Grape Collection, Vassal, France TABLE 6.1.

Amplification and Sequencing: Genomic DNA was isolated following the protocol of Lodhi et al (Lodhi et al., 1995). The *VvmybA1* gene and promoter region was amplified and directly sequenced utilizing the following primers: VvmybA1 morepromof 5'-TCAAAATTTTCAAAGGTTTCATGTG-3' and VvmybA1 morepromor 5' ATGGTGCATTACTGGCTTCC-3'. Nucleotide positions referred to in the text correspond to alignment positions from the reference sequence AB111101 submitted by Kobayashi et al. (2004). Sequence data included in this article have been deposited with the EMBL/GenBank data libraries.

Results

Sequence polymorphisms within promoter of *VvmybA1*

No significant associations were detected between polymorphisms within the coding region of *VvmybA1* and the red-fleshed phenotype. However, a 408 bp indel was detected (FIGURE 6.1) within the promoter of all teinturier varieties tested that are derived from Teintrurier du Cher (TABLE 6.1).

TABLE 6.1 Accessions assayed for presence of sequence polymorphisms.

Accession	Red-fleshed	Presence of 408 bp indel
Alicante Bouschet	+	+
Alicante Ganzin	+	+
Royalty	+	+
Teinturier du Cher	+	+
Scarlet	+	+
Rubired	+	+
Petit Bouschet	+	+
Pinot Teinturier	+	-
Gamay Teinturier	+	-
Gamay Freaux	+	-
Zinfandel	-	-
Tempranillo	-	-
Cabernet Franc	-	-
Pinot noir	-	-
Mataro	-	-
Dolcetto	-	-

Interestingly, a few red-fleshed varieties that are hypothesized to be independent mutations of the red-fleshed phenotype (Pinot teinturier and the Gamay teinturiers) did not show the presence of this 408 bp indel (TABLE 6.1). The 408 bp indel is a direct duplication of the 408 bp immediately upstream of this indel. The 408 bp sequence upstream of the indel is present in all *V. vinifera* cultivars tested to date.

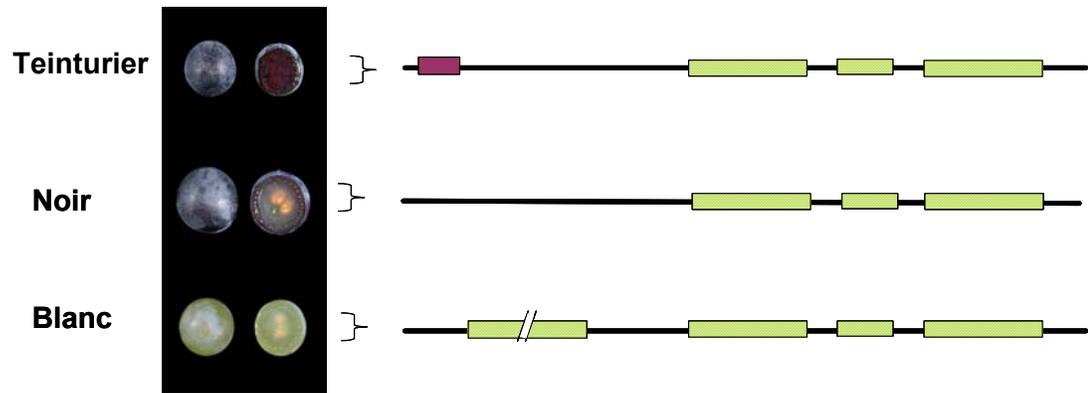


FIGURE 6.1 Red fleshed phenotype and associated 408bp indel within promoter of *VvmybA1*.

Co-segregation of leaf disc phenotype with Teinturier allele

The transmission of the teinturier phenotype and cosegregation with the 408 bp indel within *VvmybA1* was tested by observing the inheritance of these two characters within the progeny of a cross of Viognier × Scarlet. Viognier is a white skinned, clear fleshed cultivar, while Scarlet is a black skinned, red-fleshed cultivar derived from a cross between a white skinned and a teinturier variety (FIGURE 6.2).

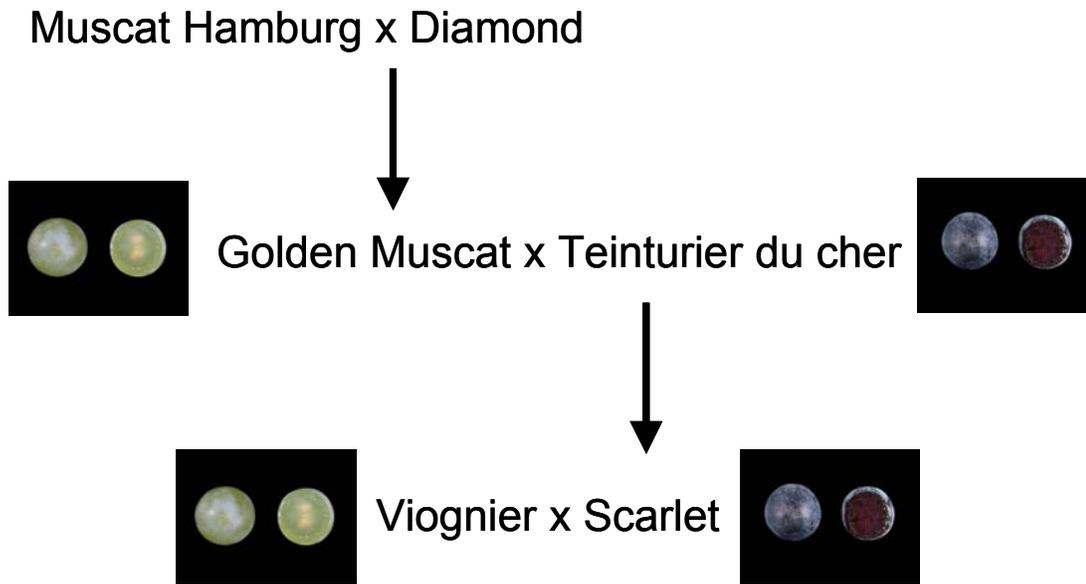


FIGURE 6.2 Pedigree of Viognier x Scarlet cross.

Sixty-two seedlings from the cross of Viognier × Scarlet were tested for the presence of intense red coloration of leaf discs following immersion of the leaf disc in a sucrose solution and exposure to light for 3 weeks and for the presence or absence of the 408 bp indel previously identified in teinturier varieties derived from Teinturier du Cher. Leaf discs from 29 of the progeny showed intense purple pigmentation, and 33 progeny showed no accumulation of anthocyanin (TABLE 6.2). All 29 progeny with

TABLE 6.2 Co-segregation of leaf disc phenotype with teinturier allele.

	No. of progeny with 408 bp indel	No. progeny without 408 bp indel
Red leaf discs	29	0
Un-pigmented leaf discs	1	32

red leaf discs also showed the presence of the 408 bp promoter indel. All of the progeny with unpigmented leaf discs, except 1, did not possess the 408 bp promoter indel.

TABLE 6.3 Sequence polymorphism at site 600^a within promoter of *VvmybA1*.

Phenotype	Polymorphism
Red fleshed – black skin	TTCTACGTAATGTCCCATCA.. [408 bp].. TCCTACCAATGTCCATATGA
Clear fleshed – black skin	TTCTACGTAATGTCCCATCA.. [0 bp].. TCCTACCAATGTCCATATGA

Discussion

It has been previously shown that DNA sequence variation within the promoter of *VvmybA1* is associated with several qualitative classes of fruit color in grapevine, primarily in differentiating white, red, and pink skinned varieties from black skinned varieties (Kobayashi et al., 2004; Owens et al., 2006 in review). The results presented show that DNA sequence variation further upstream of the previously identified mutations (TABLE 6.3) is associated with the red-fleshed phenotype present in several commercially important grape cultivars.

Interestingly, this polymorphism was only detected within red-fleshed cultivars derived from Teinturier du Cher, most notably several cultivars generated by the French father and son grape breeders Louis and Henri Bouschet de Bernard in the early to mid-19th century (Galet, 1998). Additional red-fleshed mutations that are speculated to be independent origins of the ref-flesh phenotype: Pinot Teinturier, and the Gamay Teinturiers did not possess the same mutation. This provides additional

support to the hypothesis previously put forth that the Pinot and Gamay teinturiers are indeed mutations arisen from Pinot Noir and Gamay Noir and not the result of hybridization with other teinturier varieties derived from Teinturier du Cher (Galet, 1998). This hypothesis could be more rigorously tested by examining the relationship between Pinot noir, Gamay Noir, and their putative teinturier mutations through the use of microsatellite marker fingerprinting (Bowers et al., 1999; Bowers et al., 1993). Alternatively, the mutation observed within the promoter of the teinturier varieties derived from Teinturier du Cher could be a spurious association. This explanation seems unlikely considering the number of cultivars derived from Teinturier du Cher that all possess the red-fleshed character as well as the promoter mutation. Similarly, the promoter mutation co-segregates with a red-leaf assay conducted on the Viognier × Scarlet progeny and existing in approximately a 1:1 ratio. Scarlet, a red-fleshed variety, was derived from a parent that was red-fleshed, as well as a white fruited variety with no presence of the red-fleshed character in its pedigree (1997). In the present cross, if the teinturier character is controlled by the same locus controlling skin color, the progeny would segregate in a 1:1 teinturier:white ratio, as observed here. If the teinturier trait was independently assorting from the skin color locus, then it is hypothesized that both a dominant black skin allele at the skin color locus and a dominant flesh coloration allele at the teinturier locus would be required to observe the teinturier character and would exist in a 1:3 ratio of teinturier:non-teinturier. The assay used to detect was an indirect leaf-disc assay. This assay has been reported to be 100% effective in determining the presence of red-fleshed berries in progeny of multiple crosses (Becker and Ries, 1986; Carbonneau, 1980) and as observed within the controls utilized in the present study. In the present study, there is a clear differentiation between teinturier varieties in which leaf discs turn an intense purple and non-teinturiers, regardless of skin color, which remain green.

The population size tested in the present study was small, and is not large enough to easily identify rare recombinants and it remains possible that the teinturier locus is tightly linked to *VvmybA1* and the mutation noted in this study is a spurious correlation. One seedling from the Viognier × Scarlet cross did show the presence of the 408 bp indel with the promoter of *VvmybA1* but did not show an intense purple leaf disc in the sucrose assay. It is unlikely that this is a rare recombinant, as this plant also exhibited intense purple leaf coloration during leaf senescence in autumn. Purple leaf coloration is often observed in teinturier varieties and typically becomes more pronounced during leaf senescence in autumn (Galet, 2000). Notably all the progeny that show the purple leaf disc assay also exhibit purple leaf coloration in the autumn. It seems likely that this one seedling is therefore a true teinturier variety.

The role of the 408 bp duplication in the promoter of the teinturier varieties is unknown. The basis of tissue specificity that typically results in the expression of anthocyanins only in the skin of the berry could be a complex one. It is tempting to hypothesize that a portion of this tissue specificity is determined by cis-acting regulatory elements within the promoter of *VvmybA1*. The mutation in the promoter may have eliminated the tissue specificity and allowing for expression of *VvmybA1* in tissues in which it is not normally expressed. As mentioned earlier, teinturier varieties often display accumulation of anthocyanins in berry flesh as well as vegetative tissues. The most direct way to test these hypotheses would be to transform non-teinturier grapevines with alleles of *VvmybA1* containing the 408 bp insertion.

CHAPTER 7: FURTHER STUDIES; POTENTIAL EXTENSIONS TO OTHER BEVERAGES

Non-alcoholic beverages like fruit juices are prone to microbial contamination, among other things because they lack the protection of ethanol and low pH. *Clostridium acetobutylicum* is an anaerobic bacterium which can grow in fruit juices. Before World War II, it was widely used for the biotechnological industrial production of acetone and butanol. However, the discovery of more cost-effective petrochemical procedures to obtain solvents rendered the use of *C. acetobutylicum* obsolete (Bahl, 2004). Still this spore-forming bacterium has been studied intensively, and is often used as a model organism. Publications about its biochemical pathways are numerous. *C. acetobutylicum* is neither heat-resistant nor pathogenic.

Molds produce ascospores or similar structures with heat resistance, enabling them to survive the thermal processes given to some fruit products. *Byssochlamys fulva* is an acidophilic heat-resistant mold that can be highly problematic in fruit juices and various non-carbonated soft drinks (Back, 2001). *B. fulva* produces an enzyme which clots milk similar to animal rennet (Chu et al., 1973; Sun and Chu, 1979). In fruit juices, some *B. fulva* strains can form the toxic and carcinogenic mycotoxin patulin (Rice et al., 1977). In 2001, the US Food and Drug Administration (FDA) set the acceptable limit of patulin in fruit juices at 50 ppb (McLellan and Padilla-Zakour, 2004). *B. fulva* is present in soils, contaminating fruits upon contact. All current methods for the quantitative detection of *B. fulva* rely on its cultivation for several days, which can result in spoiled beverages by the time the microbial tests are ready. A faster test is necessary to detect and prevent spoilage by *B. fulva*.

Another microbial concern in fruit juices, and other beverages, is the bacterium *Alicyclobacillus acidoterrestris*. *A. acidoterrestris* was found in apple juice, white grape juice, tomato juice and various blends (Splitstoesser et al., 1998). Spoilage of

fruit juice by *A. acidoterrestris* is characterized by a distinct medicinal or antiseptic off-odor attributed to guaiacol, a metabolic by-product of the bacterium (Orr et al., 2000). It also produces 2,6-dibromophenol (2,6-DBP) and 2,6-dichlorophenol (2,6-DCP) (Jensen and Whitfield, 2003). It is Gram positive, with a growth temperature optimum of 35-55°C and a pH optimum of 2.2-5.8 (Chang and Kang, 2004). Although it grows at very low pH, it is sensitive to certain organic acids (Hsiao and Siebert, 1999). Current methods to determine *A. acidoterrestris* are very accurate with a low threshold (1 cell/ml), but slow (Pettipher et al., 1997). Ultra High-Temperature heat-treatment (UHT) can not eliminate *A. acidoterrestris*. As an alternative, juices can be treated with antibacterial substances from agricultural products. Semi-quantitative reverse transcription PCR (Funes-Huacca et al., 2004) and quantitative PCR (Luo et al., 2004) have been developed for *Alicyclobacillus* spp.

CHAPTER 8: SUMMARY

The quality of beverages, particularly wine, depends largely on their microflora. Yeast and bacterial fermentations shape the product flavor profile, and can cause spoilage, unwanted compounds and product loss. Current detection methods for microorganisms are usually either inaccurate, slow, or both. In this project, realtime PCR systems were designed to test wines for the presence of certain microorganisms.

Before analyzing samples, it is essential to know whether the samples are representative of the entity that is tested. Some wines are fermented in large steel tanks. We asked the question of whether the yeast or bacteria cell density is similar at different levels in the fermenting tanks. Four different sampling spots within the vertical axis of steel tanks, during both the alcoholic and the malolactic fermentation, were chosen to analyse and compare. Samples were taken in a sterile manner from the top, the middle and the bottom of the tanks through the top hatch. Additionally, samples taken from the sampling valve, with a short forerun. The samples were plated and colonies counted for *Saccharomyces cerevisiae* and *Oenococcus oeni*. It is safer to avoid taking samples from the hatch when a representative image of the microorganisms is the aim of sampling, but values obtained from samples taken with a short forerun from the tap, using a sterile container but without special treatment of the tap assembly, is adequate to sample wines during alcoholic fermentation and MLF (see chapter 2).

Realtime PCR systems to detect the three spoilage yeasts *B. bruxellensis*, *H. uvarum* and *P. anomala* were developed. The template for these amplifications was the actin gene DNA. The three systems were specific for the targeted organisms. The extractions were tested to ensure that they are quantitative and useful in direct wine sampling. The range, the PCR efficiency, and the correlation for each detection system

was within the requirements. The detection level for these yeasts was at 100 cells/ml, and the linear range from 1000 to 10^7 cells/ml (see chapter 3).

The realtime PCR system for *B. bruxellensis* was used to determine whether there is a quantitative connection between *B. bruxellensis* contamination, the marker phenols and the sensory evaluation. The tastings were done informally, and so give only subjective impressions of the participants, yet the results are still valuable as a description of the wines. The three phenols 4-ethylphenol, 4-ethylguaiacol and 4-ethylcatechol were quantified with GC-MS at the chemistry laboratory from the Agroscope Federal Research Institute, Wädenswil, Switzerland. The experiments in this study were designed to cover a variety of different situations in which *B. bruxellensis* may occur in wines. The experiments show that although *B. bruxellensis* does in fact produce the known marker phenols, there is no correlation between the cell number and the amounts of marker phenols (see chapter 4).

The influence of winery sanitation practices on the presence of *B. bruxellensis* in wineries was investigated. Eight winemakers from eight wineries completed a questionnaire about cleaning practices. Four of these were chosen to investigate the occurrence of *B. bruxellensis* on different surfaces and in maturing wines. Parallel samples were evaluated by plating on semi-selective media and with realtime PCR analysis. Although only some plated samples detected the presence of *B. bruxellensis*, realtime PCR results showed that *B. bruxellensis* is present on most winery surfaces and wines. Even if *B. bruxellensis* can not be completely eliminated from the cellar environment, winery sanitation remains an essential aspect of *Brettanomyces* control. Filtration of red wines will further help to prevent spoilage control in the bottle (see chapter 5).

Teinturier grapevines contain anthocyanins both in the skin and the flesh of the grape berries, whereas in other red or black grapes only the berry skin is pigmented. This

character is distinct. A cross between a heterozygous teinturier (white X teinturier) and a white berried variety was studied to determine the segregation of this gene. Leaf discs were cut from the seedlings, incubated in a sucrose solution and exposed to light, and visually compared to the teinturier, red berried and white berried controls. Teinturier leaf discs turn red with this treatment. Of 62 seedlings, 29 showed the teinturier character. All 29 also contained a 408 bp insertion in the upstream region of the *VvmybA1* gene. These results suggest a 1:1 segregation, meaning that the same gene which controls grape color also controls the berry flesh color. However, the population of seedlings was too small to confirm this theory (chapter 6).

CHAPTER 9: SUMMARY IN GERMAN

Die Qualität von Getränken, insbesondere Wein, ist stark abhängig von deren Mikroflora. Hefe- und Bakterienfermentationen beeinflussen den Geschmack und Geruch des Produktes und können zu unerwünschten Substanzen, Verderbnis oder gar zum Verlust des Produktes führen. Gegenwärtige Detektionsmethoden sind zumeist entweder ungenau, langsam oder beides. In diesem Projekt wurden realtime PCR Systeme entwickelt, um die Anwesenheit von bestimmten Mikroorganismen quantitativ nachzuweisen.

Bevor eine Probe analysiert wird, ist es wichtig zu wissen, ob die Probe für die getestete Einheit repräsentativ ist. Da manche Weine in grossen Edelstahl Gärtanks fermentiert werden, sollte die Frage geklärt werden, ob die Hefe- und Bakterienzell-dichte in verschiedenen Tankhöhen die selbe ist. Vier verschiedene Lagen in der vertikalen Achse der Tánke wurden während der alkoholischen Gärung und der Milchsäuregärung untersucht und verglichen. Die Proben wurden steril aus der oberen Tanköffnung - von den Tankhöhen 'oben', 'mitte' und 'unten' - entnommen. Zusätzlich wurden Proben mit einem kurzen Vorlauf aus dem Tankhahn entnommen. Die Proben wurden auf verschiedenen Medien ausplattiert und die Kolonien von *Saccharomyces cerevisiae* und *Oenococcus oeni* ausgezählt. Es hat sich gezeigt, dass es 'besser ist, die Proben nicht aus der oberen Partie des Gärtanks zu entnehmen. Dadurch konnte zu Beginn und Ende der Gärung kein repräsentatives Bild der Mikroorganismen erhalten werden. Proben die nach einem kurzen Vorlauf ohne besondere Vorkehrungen aber in ein steriles Gefäss aus dem Tankhahn entnommen wurden, sind für die Untersuchung während der alkoholischen Gärung und der Milchsäuregärung gut geeignet (Kapitel 2).

Realtime PCR Systeme wurden entwickelt, um die drei unerwünschten Hefen *Brettanomyces bruxellensis*, *Hanseniaspora uvarum* und *Pichia anomala* zu detektieren. Als Basis dafür wurde das Gen für Actin (DNA) verwendet. Alle drei Systeme waren spezifisch für ihre jeweilige Art. Die DNA Extraktion wurde getestet, um sicher zu gehen, dass die Resultate quantitativ sind und Weine direkt extrahiert werden können. Der lineare Bereich, die PCR Effizienz und die Korrelation entsprechen für jedes Detektionssystem den Anforderungen. Der Schwellenwert lag für diese Hefen bei 100 Zellen/ml, und der lineare Bereich umfasste 10^3 bis 10^7 Zellen/ml (Kapitel 3).

Es wurde untersucht, ob ein quantitativer Zusammenhang zwischen *B. bruxellensis* Kontaminationen, deren spezifischen Phenolen und einer sensorischen Evaluierung besteht. *B. bruxellensis* Zellzahlen wurden mittels realtime PCR gemessen und die drei Phenole 4-Ethylphenol, 4-Ethylguajacol und 4-Ethylcatechol durch Gaschromatographie-Massenspektroskopie (GC-MS) analysiert. Die Degustationen wurden nicht formell durchgeführt und geben deshalb nur die subjektiven Eindrücke der Degustatoren wieder. Die Resultate sind jedoch trotzdem für die Beschreibung der Weine nützlich. Die Experimente, die in dieser Arbeit gezeigt werden, wurden entwickelt, um ein möglichst breites Spektrum von Situationen, in denen *B. bruxellensis* in Weinen vorkommt abzudecken. Es zeigte sich, dass *B. bruxellensis* zwar die bekannten spezifischen Phenole produziert, dass jedoch keine Korrelation zwischen deren Konzentrationen und *B. bruxellensis* Zellzahlen besteht (Kapitel 4). Im Weiteren wurde der Einfluss von Weinkeller-Hygiene auf das Vorkommen von *B. bruxellensis* untersucht. Acht Kellermeister aus den jeweiligen Weinkellereien füllten einen Fragebogen über ihre Kellerreinigungspraktiken aus. Von diesen wurden vier ausgewählt, um verschiedene Keller-Oberflächen und Weine aus Barriques auf *B.*

bruxellensis zu untersuchen. Die Proben wurden parallel durch Ausplattieren auf semi-selektiven Medien und mit realtime PCR untersucht. Obwohl nur wenige *B. bruxellensis* auf den Platten wuchsen, zeigte es sich, dass sie mit realtime PCR fast überall nachgewiesen werden konnten. Auch wenn diese Hefe nicht vollständig aus dem Keller eliminiert werden kann, ist die Kellerreinigung ein unerlässliches Mittel zur Eindämmung von *Brettanomyces* Kontamination. Das Filtrieren von Rotweinen vor dem Abfüllen ist ein wichtiger Schritt, um nachteilige Veränderung der Weine in der Flasche zu verhindern (Kapitel 5). Die vorgelegte Arbeit vermittelt die Grundlagen zur Erstellung eines effizienten und kostengünstigen Detektionssystems zur Anwendung in der Getränkeherstellung, wodurch in Zukunft relevante ökonomische Schäden markant reduziert oder gar vermieden werden können.

Färbertrauben (Teinturier) haben Anthocyanin in den Häuten und im Fruchtfleisch der Trauben, während bei anderen roten Trauben nur die Haut pigmentiert ist. Eine Traubensorte verfügt entweder eindeutig über das Teinturier Merkmal oder hat dieses nicht. Es existieren keine Mischformen. Eine Kreuzung zwischen einem heterozygoten Teinturier (weiss x Teinturier) und einer weissen Sorte (Viogner) wurde untersucht, um die Segregation dieses Gens zu ermitteln. Blattmaterial der Jungpflanzen wurde in einer Zuckerlösung und mit natürlichem Licht inkubiert und visuell mit Teinturier, roten und weissen Kontrollsorten verglichen. Teinturier Blätter verfärbten sich mit dieser Behandlung tiefrot. Von den 62 untersuchten Jungpflanzen zeigten 29 dieses Teinturier-Merkmal. Diese Jungpflanzen hatten eine zusätzliche eine DNA Sequenz von 408 Basenpaaren im Gen *VvmybA1*. Dieses Resultat legt nahe, dass das Teinturier-Merkmal 1:1 segregiert, was darauf hinweist, dass dasselbe Gen sowohl für die Traubenfarbe, wie auch die Fruchtfleischfarbe verantwortlich ist. Das Ergebnis ist, aufgrund der geringen Jungpflanzenpopulation statistisch nicht signifikant (Kapitel 6).

CHAPTER 10: SUMMARY IN FRENCH

La qualité de certaines boissons telles que le vin dépend en grande partie de leur microflore. Les fermentations conduites par les levures et les bactéries agissent sur le goût du vin ; elles peuvent produire des substances indésirables, altérer le produit ou provoquer sa perte. La plupart des méthodes de détection courantes sont imprécises ou lentes, ou présentent les deux défauts. Le projet dont il est question ici consistait à élaborer des systèmes de realtime PCR (réaction en chaîne de la polymérase en temps réel), afin de tester la microflore du vin.

Avant d'analyser, il est essentiel de savoir si les échantillons sont représentatifs de la situation microbienne dans toute la cuve à tester. Beaucoup de vins sont fermentés dans de grandes cuves en acier inoxydable. La question était de savoir si la densité des levures et des bactéries est similaire dans toute la hauteur des cuves. Quatre points échelonnés dans l'axe vertical de la cuve ont été testés durant les fermentations alcoolique et malolactique, et comparés entre eux. Des échantillons ont été prélevés stérilement en haut, au milieu et en bas de la cuve depuis l'ouverture du haut. En plus, des échantillons ont été pris par le robinet, en laissant couler le vin un court instant avant prélèvement. Les échantillons ont été incubés sur des plaques d'agar nutritif, et l'on a compté les colonies de *Saccharomyces cerevisiae* et de *Oenococcus oeni*. Il est plus prudent de ne pas prélever d'échantillons dans le haut de la cuve si l'analyse a pour but une image représentative de la microflore durant la fermentation. En revanche, le prélèvement d'échantillons au robinet – en laissant couler le vin un moment et en utilisant un récipient stérile, mais sans autres précautions – est approprié durant les fermentations alcoolique et malolactique (chapitre 2).

Des systèmes de realtime PCR ont été développés pour détecter les trois levures

indésirables *Brettanomyces bruxellensis*, *Hanseniaspora uvarum* et *Pichia anomala*.

Le diagnostic s'est fait en utilisant le gène codant pour l'actine. Chacune des trois levures a donné lieu à un système spécifique. Les extractions ont été testées afin de s'assurer que l'ADN peut être extrait directement du vin, et que les résultats sont quantitativement fiables. La plage de linéarité, l'efficacité de la RCP et la corrélation correspondaient aux exigences, avec un seuil de détection situé vers 100 cellules/ml, et une RCP linéaire de 10^3 à 10^7 cellules/ml. (chapitre 3).

Le système realtime PCR a servi à comparer le nombre de cellules de *B. bruxellensis*, la concentration des phénols indicateurs et l'évaluation organoleptique. N'étant pas formelles, les dégustations ne reflètent que l'impression subjective des participants ; mais la description des vins est en soi un résultat utile. Les trois phénols 4-éthylphénol, 4-éthylguajacol et 4-éthylcatéchol ont été analysés par GC-MS. Les expériences choisies servaient à examiner différentes situations dans lesquelles *B. bruxellensis* peut être présente dans des vins. Alors que *B. bruxellensis* produit bien les phénols en question, les résultats indiquent qu'il n'y a pas de corrélation entre le nombre de cellules et les concentrations en phénols (chapitre 4).

Ce projet a également étudié l'influence de l'hygiène générale sur la présence de *B. bruxellensis* dans des caves de vinification. Huit vinificateurs ont rempli un questionnaire sur leurs méthodes de nettoyage. Quatre ont été choisies pour analyser la quantité de *B. bruxellensis* présente sur différentes surfaces importantes des caves, et dans des vins en barriques. Les échantillons ont été analysés en parallèle par RCP en temps réel et par incubation sur milieux semi-sélectifs. Peu de *B. bruxellensis* croissaient sur les plaques, mais avec la RCP on constatait leur présence sur la plupart des surfaces et dans les vins. Même si *B. bruxellensis* ne peut pas être complètement éliminée des caves, l'hygiène générale est importante pour contrôler cette levure. La filtration des vins rouges contribue à éviter la perte de vin en bouteilles (chapitre 5).

Les cépages teinturiers contiennent des anthocyanines dans la peau et la pulpe du raisin, alors que les variétés rouges ne sont pigmentées que dans la peau. Un cépage est teinturier ou ne l'est pas ; il n'y a pas de demi-teinturiers. On a recouru à un croisement entre un cépage teinturier hétérozygote (blanc x teinturier) et un cépage blanc (viognier) pour étudier la ségrégation de ce gène. Les feuilles des plantules ont été découpées en bandes et incubées dans une solution de sucrose à la lumière naturelle, puis comparées à des contrôles teinturiers, blancs et rouges. Des bandes de feuilles ainsi traitées virent au rouge sombre. Des 62 plantules, 29 avaient le caractère teinturier, et présentaient également une insertion de 408 bases dans le gène *VvmybA1*. Ce résultat suggère une ségrégation de 1:1, ce qui signifierait que le même gène détermine la couleur de la peau et celle de la pulpe du raisin. La population de plantules était trop peu importante pour confirmer cette théorie (chapitre 6). Ce travail présente le principe d'un système de détection efficace, permettant de réduire ou même de prévenir entièrement des dommages économiques substantiels dans la production de boissons.

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