CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA
PORTO

Survival, metabolism and production of volatile phenols by *Dekkera bruxellensis* in monovarietal wines

Thesis submitted to the Universidade Católica Portuguesa to attain the degree of PhD in Enology and Viticulture

Adriana Nunes de Lima

July 2019

CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA
PORTO

Survival, metabolism and production of volatile phenols by *Dekkera bruxellensis* in monovarietal wines

Thesis submitted to the Universidade Católica Portuguesa to attain the degree of PhD in Enology and Viticulture

Adriana Nunes de Lima

Supervisor: **Prof. Josè Antonio Couto**

Co-supervisor: **Prof. Francisco Campos**

July 2019

Dedico

Aos meus Pais, Januária e Genésio e meu irmão Adilson.

Abstract

The specific defect caused by the production of volatile phenolic compounds in wines is a major economic concern among wine producers at a global scale. Vinylphenols and, specially, ethylphenols may accumulate in wines imparting undesirable odours and flavours, which may strongly affect the wine quality. The main organisms responsible for the production of these compounds are the yeasts *Brettanomyces/Dekkera*. Several studies have been carried out on this subject over the last 20 years, but scientific work concerning the relationship between the grape varieties and the production of volatile phenols is extremely scarce. Thus, the overall aim of this work was to characterise the survival and metabolic behaviour of *Dekkera bruxellensis* in monovarietal wines and to evaluate whether certain grape varieties pose a higher risk to develop volatile phenols.

The work started by searching for contaminated wines and by isolating strains of *Brettanomyces/Dekkera* using selective and differential culture media. The percentage of contaminated wines was 37% and all strains isolated were identified as *Dekkera bruxellensis*. The results support the view that, among the *Brettanomyces/Dekkera* yeasts*, D. bruxellens*is is the main species responsible for the production of volatile phenols in wines. Phenotypic variation was found among the strains studied regarding the tolerance to ethanol. The most tolerant strains were able to grow at 14% v/v ethanol and the wine isolates were, in general, more tolerant than the reference strain *D. bruxellensis* PYCC 4801. Strains, from the most tolerant group, were selected to perform survival and metabolic studies in real wine conditions.

Prior to the metabolic studies, a chemical characterisation of monovarietal red wines from eight selected grape varieties (Cabernet Sauvignon, Syrah, Aragonez, Castelão, Touriga Franca, Touriga Nacional, Trincadeira and Vinhão) was performed, focusing on the volatile phenols and on the respective precursor compounds, both on the free and bond forms. It was found that the precursors exist mostly as esters of tartaric acid (caftaric, coutaric and fertaric acids). The predominant free hydroxycinnamic acid was p-coumaric acid, the highest concentrations being found in Syrah and Touriga Franca and the lowest in Touriga Nacional and Trincadeira. Touriga Nacional exhibited the highest difference between bound and free forms. Twenty two % of the wines analysed presented levels of volatile phenols above the perception threshold, the highest

values being found in Vinhão and Trincadeira. The results show relevant differences among grape varieties but the availability of precursors on the free form may not be the only factor explaining the potential of wines to develop volatile phenols.

The survival of *D. bruxellensis* in monovarietal wines (Touriga Nacional, Cabernet Sauvignon and Syrah) and the metabolism of hydroxycinnamic acids were evaluated. After the inoculation, yeast culturable populations were reduced to undetectable numbers in all wines, but viability measurement by flow cytometry showed that a significant part of the populations were in a viable-but-not-culturable state (VBNC). VBNC cells were metabolically active, causing the spoilage of the wine, and this physiological state favoured the accumulation of vinylphenols rather than ethylphenols independently of the grape variety. The survival capacity of *D. bruxellensis* was higher on Touriga Nacional wines than in Cabernet Sauvignon and Syrah. Wine susceptibility to *Dekkera* may be correlated with the phenolic composition, specifically the bound/free molar ratios of hydroxycinnamic acids. As stated above, Touriga Nacional exhibited the highest difference between bound and free forms and the level of free hydroxycinnamic acids is relatively low in comparison with other grape varieties. Therefore, the documented antimicrobial activity of free hydroxycinnamic acids may be expected to be lower in Touriga Nacional wines.

Besides the production of volatile phenols, *D. bruxellensis* showed esterase activity leading to the increase on the amount of ethyl esters, including ethyl acetate. The fatty acids isovaleric acid and caprylic acid also increased in the inoculated wines. The concentration of monoterpenes analysed increased in the Cabernet Sauvignon wines but not in Touriga Nacional wines. The grape variety effect observed can be related with different compositions of glycosidically bound terpenes subjected to the ß-glycosidase activity of *D. bruxellensis.*

Resumo

A produção de fenóis voláteis em vinhos tem sérias repercussões económicas nas empresas, pelo que continua a ser fonte de grande preocupação na indústria vínica a nível mundial. A presença de vinilfenóis e, em especial, os etilfenóis acima de determinados valores confere odores e sabores indesejáveis que podem afectar, drasticamente, a qualidade do vinho. Os principais organismos responsáveis pela produção desses compostos são as leveduras *Brettanomyces/Dekkera*. Diversos estudos foram realizados sobre este assunto nos últimos 20 anos, focando principalmente na origem/fontes de *Brettanomyces/Dekkera*, nas condições necessárias para o seu desenvolvimento no vinho, nos requisitos nutricionais, em métodos de detecção e identificação e em estratégias para controlar o problema. Mas, trabalho científico sobre a relação entre as castas de uvas e a produção de fenóis voláteis é escasso. Assim, o objetivo geral desta tese foi caracterizar a sobrevivência e o comportamento metabólico de *Dekkera bruxellensis* em vinhos monovarietais e avaliar se determinadas castas apresentam maior risco de desenvolvimento de fenóis voláteis.

O trabalho começou pela pesquisa de vinhos contaminados e pelo isolamento de estirpes de *Brettanomyces/Dekkera* usando meios de cultura seletivos e diferenciais. A percentagem de vinhos contaminados foi de 37% e todas as estirpes isoladas foram identificadas como *Dekkera bruxellensis*. Os resultados confirmam que, entre as leveduras *Brettanomyces/Dekkera*, *D. bruxellensis* é a principal espécie responsável pela produção de fenóis voláteis nos vinhos. Foi observada variação fenotípica entre as estirpes estudadas quanto à tolerância ao etanol. As mais tolerantes foram capazes de crescer na presença de 14% v/v etanol e, de uma forma geral, os isolados vínicos foram mais tolerantes do que a estirpe de referência *D. bruxellensis* PYCC 4801. Estirpes do grupo mais tolerante foram selecionadas para realizar estudos metabólicos e de sobrevivência em condições reais de vinho.

Antes dos estudos de metabolismo, foi feita a caracterização química de vinhos tintos monovarietais de oito castas selecionadas (Cabernet Sauvignon, Syrah, Aragonez, Castelão, Touriga Franca, Touriga Nacional, Trincadeira e Vinhão), focando nos fenóis voláteis e respectivos compostos precursores, tanto na forma livre como na forma ligada. Verificou-se que

os precursores existem principalmente como ésteres de ácido tartárico (ácido caftárico, coutárico e fertárico). O ácido hidroxicinâmico predominante foi o ácido p-cumárico, sendo as maiores concentrações encontradas em Syrah e Touriga Franca e as mais baixas em Touriga Nacional e Trincadeira. A casta Touriga Nacional exibiu a maior diferença entre formas livres e ligadas. Vinte e dois % dos vinhos analisados apresentaram níveis de fenóis voláteis acima do limiar de percepção, sendo os valores mais elevados encontrados em Vinhão e Trincadeira. Os resultados mostram diferenças relevantes entre as castas, mas a disponibilidade de precursores na forma livre não é o único fator que explica o potencial dos vinhos para desenvolver fenóis voláteis.

A sobrevivência de *D. bruxellensis* em vinhos monovarietais (Touriga Nacional, Cabernet Sauvignon e Syrah) e o metabolismo dos ácidos hidroxicinâmicos foram avaliados. Após a inoculação, as populações de leveduras cultiváveis foram reduzidas para números indetectáveis em todos os vinhos, mas a determinação da viabilidade por citometria de fluxo mostrou que uma parte significativa das populações estava em no estado viável mas não cultivável (VNC). Células VNC mostraram ser metabolicamente ativas causando a deterioração do vinho, e este estado fisiológico favoreceu a acumulação de vinilfenóis, independentemente da casta. A capacidade de sobrevivência de *D. bruxellensis* foi maior nos vinhos Touriga Nacional do que em Cabernet Sauvignon e Syrah. A susceptibilidade do vinho a *Dekkera* pode estar correlacionada com a composição fenólica dos vinhos, especificamente a proporção de ácidos hidroxicinâmicos livres/ligados. Tal como acima referido, a casta Touriga Nacional exibiu a maior diferença entre as formas livres e ligadas e concentração de ácidos hidroxicinâmicos livres é relativamente baixo em comparação com as outras castas. Assim, podemos esperar que a reconhecida atividade antimicrobiana dos ácidos hidroxicinâmicos livres seja menor nos vinhos Touriga Nacional.

Além da produção de fenóis voláteis, *D. bruxellensis* mostrou atividade esterásica levando ao aumento da quantidade de ésteres etílicos, incluindo o acetato de etilo. Os ácidos gordos isovalérico e caprílico também aumentaram nos vinhos inoculados. A concentração de monoterpenos analisados aumentou nos vinhos Cabernet Sauvignon mas não nos vinhos Touriga Nacional. A influência da casta pode estar relacionada com a composição de terpenos ligados a açúcares, sujeitos à atividade ß-glicosidásica de *D. bruxellensis*.

Agradecimentos

A DEUS, autor e consumador da minha Fé, sem o qual nada na minha vida seria possível. Pelas bençãos derramadas a cada dia.

A Escola Superior de Biotecnologia da Universidade Católica Portuguesa, pela oportunidade de realizar este trabalho.

Aos meus Orientadores José António Couto e Francisco Campos, pela competência científica, pelo incansável e incondicional apoio, pela disponibilidade que sempre tiveram comigo, pela dedicação, ensinamentos transmitidos, paciência e orientação deste trabalho. Obrigada por tudo.

Ao Programa Ciências sem Fronteiras - do Governo Brasileiro e ao Conselho Nacional de Desenvolvimento Cientifico e Tecnológico - CNPq [248796/2013-5], pelo financiamento deste trabalho.

Ao Projeto "ferramentas biológicas para agregar e defender valor nas principais cadeias agroalimentares (bio – N2 – valor)", NORTE-01-0145-FEDER-000030.

Ao CBQF (Centro de Biologia e Química Fina) no âmbito da FCT (Agência Nacional de financiamento para a ciência e tecnologia) do projecto UID/multi/50016/2013.

A minha Família, em especial aos meus Pais (meu Porto Seguro) por todo amor, carinho, por suportarem à ausência, pelas orações diárias, pelo apoio e incentivo em busca dos meus sonhos, por todo esforço e por acreditarem na minha capacidade de vencer. Por estarem ao meu lado em todos os momentos. Amo Vocês. **OBRIGADA POR TUDO**.

Ao meu Irmão preferido e minha Cunhada Letícia, pelo carinho, amor e amizade. Pela força nos momentos que mais precisei e por presentear a minha vida com a maior felicidade, minha sobrinha Maria Cecília.

Ao meu Tio Tonho, por todo amor, carinho, por estar em minha vida, pela vontade de vencer e por sua risada tao inocente. Mesmo diante de todos os problemas, nunca deixou de sorrir.

Ao meu Tio Elias (*In memorian*), um exemplo de bondade, que com seu olhar terno demonstrava tanto orgulho do meu sucesso profissional e sempre incentivou-me a lutar pelos meus sonhos e vencer os obstáculos da vida.

Aos tios (as) e aos primos (as), por entenderem que nas reuniões familiares eu não estava presente fisicamente, e que sempre faziam conferencias pelo skype para que eu participasse de tudo e me sentisse um pouco mais próxima. Em especial a Carol por sua risada mais gostosa, característica dela e por alegrar-me com isso.

Um agradecimento ***ESPECIAL*** aos **Brazucas**, Glenise, Giovania, Isabella, Andréia, Cristiane, Erick, Fernanda, Karol, Priscila, Suelen, Antonio, Evla, pela agradável convivência, conversas diárias e força nos momentos mais difíceis, pelo carinho e amizade, pelas viagens juntos, pela alegria de viver os melhores momentos com **AMIGOS**.

A João Nunes, por toda dedicação, apoio, compreensão, incentivo, conselhos, por partilhar sonhos e acreditar em minha capacidade de vencer mesmo quando eu queria desistir e por estar presente em todos os momentos da minha vida. Por todos os maravilhosos dias em sua companhia. Meu sincero OBRIGADA.

Aos amigos Flaviana e Manuel, por todo apoio, carinho, amizade, cumplicidade, força e incentivo nos momentos de querer desistir, pelos puxões de orelhas nos momentos em que foi necessário, pela confiança e amor, por fazerem sentir-me em casa e em família mesmo estando tão longe. Á Sasha, por alegrar meus dias, nos momentos de convívio.

A Manuel Ferreira por todos os maravilhosos momentos de convívio, pela amizade verdadeira, carinho, brincadeiras, conselhos, compreensão, palavras de incentivo e apoio nos momentos difíceis. Pelas viagens inesquecíveis, pelo seu humor contagiante e seu sorriso lindo.

A Carla Oliveira e a Cristina Santos, pelo apoio prático, paciência, por todos os ensinamentos partilhados, pela amizade e carinho.

A Ana Martins, por todo carinho e amizade, apoio, por escutar meus desabafos em momentos de tristeza com saudades de casa, pelas boas conversas e conselhos em todos os momentos nesses anos de convivência.

A Eduardo Freitas, pela atenção e disponibilidade, nas inúmeras vezes que contactei os serviços de informática.

A Ricardo Amadeu, pela alegria em cada "Bom dia", seu bom humor e otimismo contagiantes, pela amizade carinhosa.

A Rui Magalhães, pelo apoio, ajuda técnica, amizade.

A Profª Teresa Brandão pelo profissionalismo, carinho, suporte nas Análises Estatísticas.

A todos os Professores, pelos ensinamentos, dedicação, atenção e carinho.

A todos os amigos do Laboratório pelo maravilhoso ambiente de trabalho, apoio, ajuda e incentivos nos momentos de menor motivação. Sendo um agradecimento especial dirigido a Ingrid Collombel, pela amizade verdadeira e companheirismo, experiências partilhadas, pelos momentos divertidos, pela alegre convivência, pela energia positiva e por todo carinho.

A Poliana Silva, Rita Monforte pela ajuda e apoio, pelas conversas e boas risadas, pela amizade e carinho.

Aos amigos do Gabinete 1.6, pela ótima convivencia, alegria, boas conversas e descontração.

Aos amigos “estrangeiros” em especial, Pompeyo, Edgar, Nuário, Adrian e Nazzareno pela troca de experiências, ótima convivencia, alegria e amizade.

Aos amigos distantes, Adrícia, Bruno, Chris, Cybelle, Cíntian, Erika, Flávia, Kaline, Katia, Lívio, que mesmo ausentes fisicamente, sempre estiveram presentes em minha vida, o meu muito obrigada pela amizade, carinho e apoio em todos os momentos.

A todos os funcionários da Universidade Católica.

“Experience is not what happens to you; it's what you do with what happens to you”

Aldous Huxley.

Table of Contents

List of Figure

List of Tables

Abbreviations

CFU Colonys Formings Units

FID Flame Ionization Detector

GC Gas Chromatography

Log Logarithm to the base 10

SY Syrah

TN Touriga Nacional

CS Cabernet Sauvignon

Trinc Trincadeira

4EC 4-Ethylcatechol

4EG 4-Ethylguaiacol

4EP 4-Ethylphenol

4VC 4-Vinylcatechol

4VG 4-Vinylguaiacol

4VP 4-Vinylphenol

RPM Rotation per minute

PYCC Portugal Yeast Culture Collection

Spp Species

SO2 Sulfur dioxide

mL Milliliters

mg Milligrams

µg Micrograms

µL Microliters

ND no detected

Rational and Thesis aims

The work presented in this thesis is about one of the most important microbiological challenges of the modern winemaking the production of volatile phenols by *Brettanomyces/Dekkera* yeasts. These microorganisms are considered the major cause of microbial wine spoilage worldwide leading to significant economic losses to the wine industry. Several studies have been carried out on this subject over the last 20 years mainly focusing on the origins/sources of *Brettanomyces/Dekkera*, the conditions required to develop in wine, the nutritional requirements, detection and identification methods and on strategies to control the problem. But, scientific data concerning the survival and the metabolism of hydroxycinnamic acids by these organisms in monovarietal wines is extremely scarce and several contradictions are found on the little work that exists. The work is in the scope of a research line aiming to study the relationship between the grape varieties and the production of volatile phenols.

Main objective

The overall aim of this work was to characterise the survival and metabolic behaviour of *Dekkera bruxellensis* in monovarietal wines and to evaluate whether certain grape varieties originate wines more susceptible to develop volatile phenols. In this way, we intend to provide winemakers with scientific data that allows them to make informed decisions on how to manage the risk of contamination of wines by *Brettanomyces*/*Dekkera* according to the grape varieties used.

Thesis outline

This thesis is divided into 6 Chapters. Chapter 1 is the general introduction where a literature survey is done about the occurrence of *Brettanomyces/Dekkera* in wines and the impact on wine quality. The work done on Chapter 2 envisaged the isolation of *Brettanomyces/Dekkera* strains from naturally contaminated wines and the selection of wine strains to perform the studies carried out on Chapters 4 and 5. Chapter 3 dealt with the characterisation of wines from different grape varieties focusing on the volatile phenols and on the respective precursor compounds. Chapter 4 evaluates the survival and metabolism of hydroxycinnamic acids by *Dekkera bruxellensis* in monovarietal wines. The results are discussed based on relevant data and findings detailed in Chapter 3. Chapter 5 focuses on the impact of *D. bruxellensis* on the volatile composition of wines. Finally, Chapter 6 integrates the experimental chapters of the thesis and highlights the main conclusions.

The chart below summarizes the structure of the thesis and presents the specific objectives of each chapter.

Chapter	**Specific objectives**
1. General Introduction	• Framework of the work • Give the scope, context, background information and significance of the topic
2. Isolation of *Brettanomyces/Dekkera* strains from wine and characterisation of ethanol resistance	• Identification of wine isolates • Characterisation of ethanol resistance • Selection of wine strains
3. Phenolic composition of monovarietal red wines regarding volatile phenols and its precursors	• Quantification of volatile phenols in wines from different grape varieties • Characterisation of grape varieties regarding the precursor compounds of volatile phenols.
4. Survival and metabolism of hydroxycinnamic acids by *Dekkera bruxellensis* in monovarietal wines	• Evaluation of the survival capacity of *Dekkera bruxellensis* in monovarietal wines. • Relationship between the phenolic composition of grape varieties and the susceptibility for the development of volatile phenols.
5. Activity of *Dekkera bruxellensis* and the effect on the aroma profile of monovarietal wines	• Description of modifications caused by *Dekkera bruxellensis* on the volatile composition of monovarietal wines.
6. Conclusions and future work	• Integration of the different parts of the work • Highlighting of the main conclusions • Motivations for further research

Part of the work presented in this thesis has been published or submitted to publication in international journals, according to the following list:

Chapter 2

Adriana Nunes de Lima, José António Couto

Isolation of *Brettanomyces/Dekkera* strains from wine and characterisation of ethanol resistance. (2019). Submitted to Biotechnology Letters.

Chapter 3

Adriana Lima, Carla Oliveira, Cristina Santos, Francisco M. Campos. José António Couto. 2018. Phenolic composition of monovarietal red wines regarding volatile phenols and its precursors. European Food Research and Technology 244, 1985-1994. https://doi.org/10.1007/s00217-018-3110-8.

Chapter 4

Adriana Lima, Rui Magalhaes, Francisco Campos, José António Couto

Survival and metabolism of hydroxycinnamic acids by *Dekkera bruxellensis* in monovarietal wines. (2019). Submitted to Journal of Applied Microbiology.

Chapter 5 In preparation for publication

Communications in congresses

Lima, A. N.; Oliveira, C.; Santos, C.; Campos, F.; Couto, J. A. **Phenolic composition of monovarietal red wines: volatile phenols and precursor compounds**, 2018. Rioja Fórum, Logroño - La Rioja; International Congress on Grapevine and Wine Sciences; Instituto de Ciências de la Vid y del Vino.

Lima, A. N.; Campos, F.; Couto, J. A. **Survival of *Brettanomyces/Dekkera* in monovarietal wines**, 2018. Centro de Congressos de Lisboa; Lisboa - Portugal; Evento: Encontro Ciência 18.

Lima, A. N.; Campos, F.; Couto, J. A. **Survival and activity of *Brettanomyces/Dekkera* in monovarietal wines**, 2017. Local: Universidade Católica Portuguesa; Porto; Evento: Micro Biotech 2017.

CHAPTER 1

General Introduction

Chapter 1

The activity of *Brettanomyces/Dekkera* and the accumulation of volatile phenols in wine are responsible for serious economic losses in the wine industry, thus being considered a key point on the control of wine quality. It is considered a global issue affecting the quality of wines from all major wine producing regions. A great deal of research has focused on this subject, but the extensive information which has been collected has not been sufficient to eradicate the contamination of wine by these spoilage yeasts and to control the negative impact on wine organoleptical characteristics.

1.1. Taxonomy of *Brettanomyces/Dekkera* spp

The *Brettanomyces* genus was described for the first time in 1904 when N.H. Claussen isolated yeasts from a slow secondary fermentation of an old English stock beer (Franson, 2001). At that time the flavours produced by this yeast have become characteristic of British beers (Loureiro & Malfeito-Ferreira, 2006; Oelofse *et al.,* 2008).

Yeasts belonging to the *Brettanomyces* genus were later isolated by Kufferath *et al.,* (1921) with the resultant creation of the genus in 1952 by Lodder *et al.,* (1952). The connection of *Brettanomyces* with red wine spoilage was made only much later by Chatonnet *et al.,* (1995). The *Brettanomyces* genus has undergone many revisions and reclassifications in the past. *Dekkera* was classified taxonomically by van der Walt in 1964 after it was observed that certain strains were able to produce spores (Van der Walt, 1984).

Currently five species belonging to the genus *Brettanomyces* are classified*: B. custersianus, B. naardensis, B. nanus, B. anomalus and B. bruxellensis.* The *Brettanomyces* genus refers to the anamorph state (asexual reproduction), while *Dekkera* genus is its teleomorph state (sexual reproduction) and they are represented in the teleomorph form by *D. anomala* and *D. bruxellensis* (Loureiro & Malfeito-Ferreira, 2006; Oelofse *et al.,* 2008) (Table 1.1).

Table 1.1. Anamorphic and Teleomorphic species of *Brettanomyces* and *Dekkera*

Anamorph (non-sporulating)	**Teleomorph (sporulating)**
Brettanomyces anomalus	*Dekkera anomala*
Brettanomyces bruxellensis	*Dekkera bruxellensis*
Brettanomyces naardenensis	Not known
Brettanomyces nanus	Not known
Brettanomyces custersianus	Not known

1.2. Isolation, Identification and quantification of *Brettanomyces/Dekkera* from wine

The detection of *Brettanomyces/Dekkera* in wine has proven to be difficult due to its comparatively slow growing nature. *Brettanomyces/Dekkera* are often overgrown by other yeasts that are present. Current procedures for the detection and isolation involve the use of selective agents capable of inhibiting the growth of other organisms. Rodrigues *et al.,* (2001) developed a selective and differential solid medium able to recover *Brettanomyces/Dekkera* from wine and related environments, thus giving an important step forward for the elaboration of simpler methods for the detection of these organisms. A selective enrichment broth was developed to differentially promote the growth and activity of *Brettanomyces/Dekkera* (Couto *et al.,* 2005. The precursor of 4-ethyl-phenol was incorporated in a concentration high enough to flavors as strong an aromatic signal as possible. The use of sensory detection, as the primary method of attributing positive results, is the major innovation, this being particularly compatible

with the practices of winemaking, even in those cases which do not have microbiology laboratory facilities. A validation test was made by comparing the performance of the methodology proposed in this work with that of Rodrigues *et al.,* (2001), assumed, for this purpose, as a reference method. A good agreement was found between the two methodologies. Thus a methodology that can be easily adopted by the wine industry and that it can be used on a routine basis for the detection of *Brettanomyces/Dekkera* in wine and wine contact surfaces is available.

Molecular methods have already been studied to detect and identify *Brettanomyces/Dekkera* in wine, for example, the development of a PCR method using a genomic DNA fragment of an isolated *Dekkera* strain (Ibeas *et al.,*1996), the development of a fluorescence *in situ* hybridization method using a peptide nucleic acid probe (Stender, 2000), and the development of a method based on a polymorphism in the rRNA internal transcribed spacer region (Egli and Henick-Kling, 2001). A chemical composition approach, based on the differentiation between fermenting and spoilage yeasts in wine, by total free fatty acid analysis was also studied (Rozès *et al.,*1992). These methods all represent advantages in specific applications, but all require a level of laboratory sophistication which is rarely available to most wine producers, certainly in routine laboratories.

1.3. Occurrence of *Brettanomyces/Dekkera* in wines

While most contamination problems by *Brettanomyces*/*Dekkera* yeasts are predominantly in wines, there are indications that they have been isolated from other fermentative practices, a severe occurrence of contamination in the industrial production of ethanol can be mentioned here (Silva, 1994). The contamination of wines with *Brettanomyces*/*Dekkera* and the impact on the wine quality is considered a global problem (Wedral *et al.,* 2010), with several lines of research concerning this matter ongoing in major wine producing regions in the world (Conterno *et al.,* 2006). Several authors concluded that the control of volatile phenol formation is the most important challenge of modern wine microbiology, since it is responsible for significant

economic losses throughout the world (Boulton *et al.,*1996; Fugelsang, 1997). This is an extremely important issue for the wine industry. A Portuguese study revealed that more than 25% of red wines from various countries contained volatile phenols levels above 620 µg/L, value from which consumers reject the product (Chatonnet *et al.,*1992; Loureiro & Malfeito-Ferreira, 2003). Another study in Pinot noir wine in Burgundy showed that about 50% of maturing wine and about 25% of the bottles are contaminated with these compounds (Gerbaux *et al.,* 2000).

The origin of these yeasts in the winery was not completely understood for quite a long time, but through the development of an enrichment media specifically for growth of *B. bruxellensis,* detection on grape berries and the vineyard origin was inferred (Renouf and Lovaud-Funel, 2007). In fact, *Brettanomyces*/*Dekkera* yeasts are more easily found in wineries than on the surface of grapes due to the low cell numbers and to the complex microbial ecosystems in grapes where other yeasts and bacteria clearly dominate (Fugelsang, 1997). Once present in the winery, *D. bruxellensis* can become established in any area with which the wines may come in contact. Even though *D. bruxellensis* can be found throughout the winery, and has been isolated as early in the winemaking process as the completion of alcoholic fermentation, these organisms have been mostly found in the later stages of the winemaking process: wine ageing in barrels but also in the bottle (Barata *et al.,* 2008; Agnolucci *et al*., 2009). In fact, the ageing period, specially in used barrels, together with the malalolactic fermentation (MLF) (low free SO2 and yeast autolysis), have been recognised as the most critical stages of wine production for *Brettanomyces/Dekkera* proliferation (Renouf *et al.,* 2006; Suárez *et al.,* 2007). Oak barrels seem to be beneficial to *Brettanomyces/Dekkera* due to the porous microstructure of these containers, which, by allowing the influx of oxygen favour the growth and development of these contaminants (Loureiro & Malfeito-Ferreira, 2006; Oelofse *et al.,* 2008). Difficulties related to the sanitation of old barrels contribute to the establishments of *Brettanomyces/Dekkera* populations on the inner surfaces thus promoting contamination of wine (Oelofse *et al.,* 2008; Yap *et al.,* 2007).

1.4. Wine spoilage characteristics and impact on wine quality

1.4.1. Production of volatile phenols

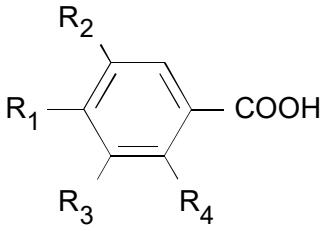
Yeasts of the genera *Brettanomyces/Dekkera* are the main producers of volatile phenols in wines. In fact, the transformation of hydroxycinnamic acids in volatile phenols is predominantly associated with the activity of the genus *Brettanomyces* and its sexual form (spore production) classified as *Dekkera* (Heresztyn, 1986; Edlin *et al.,*1995; Chatonnet *et al.,*1997). The activity and impact of *Brettanomyces/Dekkera* is strongly connected with the phenolic composition of wines. In this introduction, emphasis will be given to phenolic acids, as the precursors, and volatile phenols as the products of *Brettanomyces/Dekkera* metabolism.

Phenolic compounds can be divided broadly into two groups, according to their chemical structure: flavonoids, which possess a distinctive flavan (C_6-C_3-C_6) structure (and include anthocyanins, procyanidins and condensed tannins) and the non-flavonoids which do not have this feature (and include phenolic acids, aldehydes, and alcohols and other compounds such as coumarins, stilbenes or soluble tannins). Phenolic acids have a carboxylic functional group attached to the aromatic ring (either directly or on a side-chain). These molecules are widespread in plants (including fruits), being critical intermediates in the biosynthetic pathways of more complex phenols (Ramos-Nino *et al.,*1996).

The precursors for volatile phenols (vinylphenols and ethylphenols) are phenolic acids. These are natural constituents of grapes and wines and do not have any strong or particular odour. Phenolic acids are normally classified in two different groups according to their chemical structure: hydroxybenzoic acids, phenols with a carboxylic group attached to the benzene ring, and hydroxycinnamic acids, phenols which have a propenoic side chain. In grapes and wines, phenolic acids exist mostly in combined forms: hydroxybenzoic acids are normally present as components of hydrolysable (or soluble) tannins (gallotannins or ellagitannins) while hydroxycinnamic acids exist as esters of tartaric acid (cinnamoyl-tartaric acids). However, a small proportion of phenolic acids exist in free form in wines and during the winemaking process their concentration may increase due to a partial hydrolysis of the combined forms.

The total concentration of phenolic acids in wines ranges from 10 to 20 mg/L in white wines and 100 to 200 mg/L in red wines, depending on grape variety and vinification process (Reguant *et al.,* 2000; Ribéreau-Gayon *et al.,* 2006).

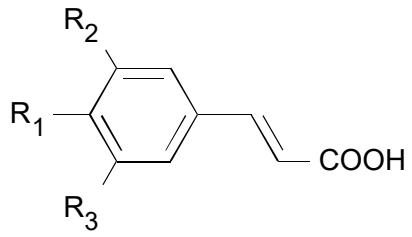
Hydroxybenzoic acids are derivatives from benzoic acid with one or more hydroxyl substituents. Five of these acids are commonly found in grapes and wines: *p*-hydroxybenzoic, protocatechuic, gallic, vanillic and syringic acids.



Name	R1	R2	R3	R4
p-Hydroxybenzoic acid	OH	H	H	H
Protocatechuic acid	OH	OH	H	H
Vanillic acid	OH	OCH_3	H	H
Gallic acid	OH	OH	OH	H
Syringic acid	OH	OCH_3	OCH_3	H

Figure 1.1. Structural formulae of hydroxybenzoic acids commonly found in grapes and wines

Hydroxycinnamic acids differ from hydroxybenzoic acids by having a propenoic side-chain attached to the aromatic ring instead of a carboxylic group. The three hydroxycinnamic acids commonly found in grapes and wines are: *p*-coumaric, caffeic, and ferulic acids. These acids exist predominantly as tartaric esters and, to a less extent, as glucose esters or ethyl esters or in free form (Macheix *et al.,* 1990; Monagas *et al.,* 2005; Hixson *et al.,* 2012).

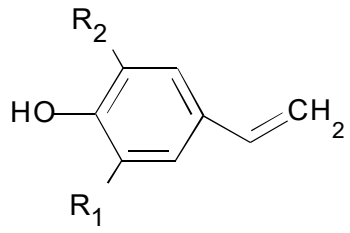


Name	R1	R2	R3
p-Coumaric acid	OH	H	H
Caffeic acid	OH	OH	H
Ferulic acid	OH	OCH_3	H

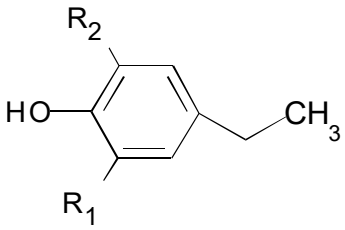
Figure 1.2. Structural formulae of hydroxycinnamic acids commonly found in grapes and wines.

The most abundant tartaric esters of hydroxycinnamic acids in grapes and wines are caffeoyl-tartaric acid (caftaric acid) and *p*-coumaroyl-tartaric acid (coutaric acid), with feruoyl-tartatic acid (fertaric acid) present at lower concentrations (Ong and Nagel, 1978). Grape juice may contain up to 300 mg/L of caftaric acid while the average content of free *p*-coumaric acid is reported to be 1.1 ± 6.7 mg/L in red wines (Clifford, 2000). Besides being esterified with tartaric acid, hydroxycinnamic acids also occur in wines as cinnamoyl esters of anthocyanin glucosides, forming acylated anthocyanins by esterification of hydroxycinnamic acids with the glucose residue (Ribéreau-Gayon *et al.,* 2006).

Phenolic acids can be transformed into volatile phenols - the ethylphenols: 4-ethylphenol (4EP), 4-ethylguaiacol (4EG) and 4-ethylcatechol (4EC) and the vinylphenols: 4-vinylphenol (4VP), 4-vinylguaiacol (4VG) and 4-vinylcatechol (4VC). These compounds arise from the reductive decarboxylation of the corresponding hydroxycinnamic acids: *p*-coumaric acid, ferulic acid, caffeic acid, respectively. The conversion can be carried out by certain yeast and bacteria species.



Name	R1	R2
4-Vinylphenol	H	H
4-Vinylguaiacol	H	OCH_3
4-Vinylcatechol	H	OH



Name	R1	R2
4-Ethylphenol	H	H
4-Ethylguaiacol	H	OCH_3
4-Ethylcatechol	H	OH

Figure 1.3. Structural formulae of volatile phenols commonly found in wines

4EP has a distinctive aroma often described as “horse sweat”, “leather” or “animal-like” while 4EG aroma is generally described as “clove-like” or “medicinal”. Depending on the concentration levels found in wines and on personal preferences, these compounds may be considered as faults. Ethylphenols are frequently found in red wines contaminated with *Brettanomyces*/*Dekkera* but are not normally found in white wines. 4-Ethylcatechol has an aroma described as “horsey” or phenolic (Larcher *et al.,* 2008). Despite the fact that some species of *Brettanomyces* are able to metabolize caffeic acid to 4EC (Edlin *et al.,*1995; Harris *et al.,* 2008), this compound has only been reported recently in contaminated wines with *Brettanomyces/Dekkera.* Table 1.2 provides information about odour and levels found in wines.

Table 1.2. Threshold detection levels of off-flavours and their sensorial impact. Adapted from Chatonnet *et al.,* (1992), Curtin (2005) and Oelofse *et al.,* (2008).

Compound	**Precursor**	**Concentration in red wine (µg/L)**	**Odour**	**Odour threshold (µg/L)**
4-Vinyl-phenol	p-Coumaric acid	4.3-8.8	Medicinal	440*/600**
4-Vinyl-guaiacol	Ferulic acid	0.2–15	Clove-like	33*/110**
4-Vinyl-catechol	caffeic acid	unknown	Phenol	unknown
4-Ethyl-phenol	4-Vinyl-phenol	118–3696	Horse sweat, leader	440***/600**
4-Ethyl-guaiacol	4-Vinyl-guaiacol	1–432	Clove, spice	20***
4-Ethyl-catechol	4-Vinyl-catechol	27–427	Medicinal	10*

*model wine, **red wine, ***water.

The detection threshold of ethylphenols has been reported to be 400-500 µg/L for a 10:1 mixture of 4-ethylphenol and 4-ethylguaiacol (Chatonnet *et al.,*1992). However, the threshold concentrations of these compounds can vary substantially, the perception being greatly influenced by the wine style, the grape variety used and the consumer's perceptive ability (Ribéreau-Gayon *et al*., 2000, Phister and Mills 2004, Fugelsang and Edwards 2007, Oelofse *et al.*, 2008).

The transformation of these compounds involves two enzymatic reactions in sequence. Firstly, the hydroxycinnamic acid is decarboxylated in the corresponding derivative vinyl (4-vinylphenol from p-coumaric acid, or 4-vinylguaiacol from ferulic acid) and in the second reaction, a reductase converts the vinyl in the corresponding ethyl compound (4-ethylphenol and 4-ethylguaiacol) (Heresztyn, 1986). The hydroxycinnamic acids can also be converted into ethylphenol by an alternative biochemical pathway described by Carr and Whiting (1959), which involves a reduction reaction (catalyzed by a phenolic acid reductase), followed by a decarboxylation of the resulting hydroxyphenolpropyonic acid. This route has been found in some lactic acid bacteria (Whiting&Carr, 1959; Barthelmebs *et al.,* 2000; Chesson *et al.,*1999).

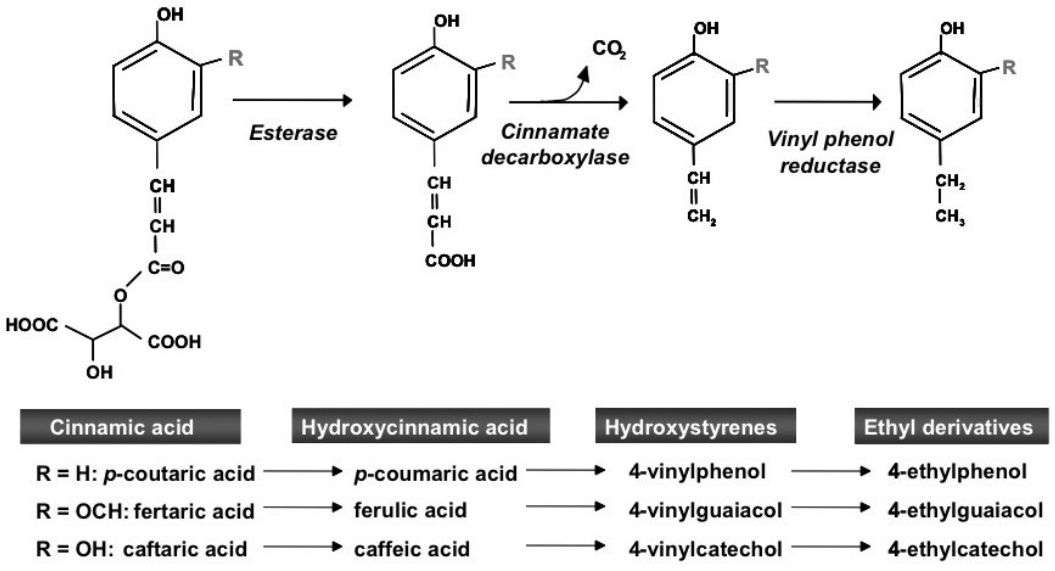


Figure 1.4. Mechanism of production of volatile phenols

1.4.2. Production of acetic acid

Besides the volatile phenols, *Brettanomyces*/*Dekkera* can also produce other compounds with negative impact on wine quality. These include the production of excessive amounts of acetic acid leading to the increase of the volatile acidity (Harris *et al.,* 2008). The formation of acetic acid is stimulated by the presence of oxygen during the winemaking process (Ciani *et al.,* 2003).

1.4.3. Production of volatile fatty acids

Brettanomyces/*Dekkera* may produce a variety of short and medium chain fatty acids (C3-C14), which contribute to the aroma profile of wine (Licker *et al.,* 2008). One of the most prominent of these compounds is isovaleric acid, obtained by the breakdown of L-leucine (Renouf and Lonvaud-Funel, 2007). It has a negative effect on wine smelling sweaty and rancid.

1.4.4. Production of compounds responsible for the mousy off-flavor

The mousy off-flavor in wine was initially connected to the activity of lactic acid bacteria. Later, *Brettanomyces*/*Dekkera* were also involved in the formation of the compounds responsible for this fault: tetrahydropyridins (Snowdon *et al.,* 2006). The main precursor of tetrahydropyridins is the amino acid L-Lysine. Oxygen has been indicated to have a stimulatory effect on the production of tetrahydropyridins but this is probably due to higher biomass development of *Brettanomyces/Dekkera* strains under aerobic conditions (Oelofse *et al.,* 2008).

1.5. Factors contributing to *Brettanomyces/Dekkera* development and wine spoilage

1.5.1. Temperature, sulphur dioxide and ethanol

The growth and survival of *Brettanomyces/Dekkera* in wine are influenced by several factors. The presence of residual sugars and the pH of wine have not a significant effect on the production of volatile phenols (Suarez *et al.,* 2007) but the temperature was found to be a relevant factor (Barata *et al.,*2008), with relatively low temperatures (16˚C) significantly lowering the production rate of volatile phenols comparison to 30˚C. The effect of sulphur dioxide (SO_2) on *D. bruxellensis* was investigated by several research groups (Du Toit *et al.,* 2005; Suarez *et al.,* 2007; Serpaggi *et al.,*2012, Agnolucci *et al.,* 2014; Longin *et al.,* 2016). In spite of the strain variability found in terms of sensitivity to this antimicrobial compound, a consensus exists on the relevance of this factor to control the growth of *Brettanomyces/Dekkera* in wines.

Regarding the effect of ethanol, Benito *et al*., (2009) found that increasing concentrations of ethanol up to 10% v/v lowered the growth and the production rates of volatile phenols but the total production was not affected. Dias *et al.,*(2003) observed that growth and production were significantly lower at 12% v/v and a total inhibition at 13% v/v. Medawar *et al*., (2003) found an increase of the lag phase and a reduction of the specific growth rate and on the final biomass with increasing levels of ethanol and a total growth inhibition at 11.5% v/v. Bassi *et al.,* (2013) found a complete growth inhibition of the 3 *D. bruxellensis* strains tested at 11% v/v in rich medium. Conterno *et al.,* (2006) were able to see that all the studied isolates of *B. bruxellensis* were tolerant to at least 10%. Barata *et al.,* (2008) found higher tolerant strains, capable of growing in the presence of 14.5% v/v.

1.5.2. Grape variety and volatile phenol production

Empirical results obtained by some wine producers indicate that the wines made of particular grape varieties are more susceptible to decay by the production of volatile phenols by *Brettanomyces*/*Dekkera*. There are some international studies in which the authors tried to find a correlation between volatile phenols and grape varieties. Pöllnitz *et al.,* (2000) compared varietal Australian wines regarding their volatile phenols and found a relationship between the two factors with Cabernet Sauvignon and Merlot giving higher values than Shiraz and Pinot Noir. This may be related to the relatively high concentrations of p-coumaric acid found in Cabernet Sauvignon and Merlot as observed by Goldberg *et al.,* (1998). On the other hand, other authors (Pour Nikfardjam, 2009) analyzed the volatile phenols on varietal wines of the Wurttemberg region and found no strong correlation between the two factors, however, they found that the wines made from grape varieties known to have higher levels of hydroxycinnamic acids showed higher concentration of volatile phenol values. A work focusing on three grape varieties (Touriga Nacional, Carbenet Sauvignon and Syrah) suggests that they are equally susceptible to the deterioration by *Brettanomyces*/*Dekkera* (Chandra *et al.,* 2015).

Portugal has a large variety of native grape varieties, many of which have not been well characterized in terms of their chemical composition. Most of the existing studies have focused on the aromatic composition of wines (Oliveira *et al.,* 2004; Falqué *et al.,* 2004; Rocha *et al.,* 2000) and few have been published on non-volatile components, eg. phenolic compounds. The first study known about the composition of phenolic acids of native Portuguese grape varieties was published in 1998 by Ribeiro de Lima and Cabanis. In this study, the authors found differences in the content of p-coumaric acid between varieties with higher values obtained from the Touriga Franca (in red varieties) and Verdelho (in white varieties). However, the authors did not look in detail to other phenolic compounds such as anthocyanins. Another study (Ramos *et al.,*1999) also showed differences in the levels of p-coumaric acid in white wine varieties. Other authors focused on the anthocyanin profile comparison of certain varieties of grapes from certain wine regions. Mateus *et al.,* (2002) found differences in the anthocyanin profile (including e-coumaroyl esters) of Touriga Nacional and Touriga Franca in the Douro region. Dopico-Garcia, (2008) compared anthocyanins and phenolic profile of grape varieties used to produce "vinho verde" wines of

different subregions. Similar results were obtained in a recent work with Alentejo wines made from indigenous Portuguese grape varieties Cabrita *et al.,* (2012).

The results obtained indicate that there are at least a relative consistency to anthocyanin profiles among varieties, and also that some varieties are richer in potential volatile precursors phenols (phenolic acids and their tartaric esters) than others. However, in none of these works, the authors tried to correlate the phenolic profile of varietal wines with the production of volatile phenols.

1.5.3. Other factors

Besides factors affecting the phenolic composition, microbiological factors may also influence volatile phenol production. For instance, the capacity to form those compounds from their precursors varies depending on the strain responsible for spoilage (Shinohara *et al.,*2000; Suárez *et al.,* 2007; Valentao, *et al.,* 2007). This may be explained by differences in enzymatic specificity, activity and the metabolism of phenolic acids between species (Edlin *et al.,*1995). Furthermore, a high correlation between *Brettanomyces/Dekkera* population size and conversion time of *p*-coumaric acid into volatile phenols was found by Benito *et al*., (2009) suggesting that the amount of odorous product formed is greatly influenced by the extent of contamination with the spoilage organism (Benito *et al.,* 2008).

A decrease of volatile phenols formed may also be due to a decrease of phenolic precursors caused by their adsorption to yeast cell walls (Salameh *et al*., 2008). Both Salameh *et al.,* (2008) and Cabrita *et al.,* (2012) have found the initial concentration of *p*-coumaric as well as ferulic and caffeic acid to be decreased immediately after inoculation with *Brettanomyces/Dekkera* yeast in both wine and synthetic medium and attributed the major part of this loss to cell adsorption. Besides adsorption to cell walls smaller amounts of acids may be lost due to esterification with ethanol or instability at high temperatures (Salameh *et al.*, 2008; Cabrita *et al.,* 2012). Although this and other chemical reactions can decrease the quantity of *p*-coumaric acid available for bioconversion, other reactions can cause its increase. This effect was described by Dugelay *et al.,* (1993) who observed the hydrolysis of *p*-coumaroyltartaric acid facilitated by hydrolytic enzymes including pectinase, (hemi-)cellulase and cinnamate esterase releasing *p*-coumaric acid in must. Such

enzyme preparations may be used for clarification improvement purposes, colour extraction or flavour enhancement (Dugelay *et al*., 1993).

1.6. Management and control of *Brettanomyces/Dekkera* and volatile phenols

The origin and the sensory impact of volatile phenols, as well as the characterisation, detection and control of *Brettanomyces*/*Dekkera* have been well studied over the past 20 years. Nevertheless, the extensive information that has been accumulated on this subject has not been sufficient to enable producers to eradicate the contamination by spoilage yeasts and the subsequent development of spoilage characteristics. Thus, defence strategies based on the prevention of contamination, the control of microbial growth or the elimination of microorganisms from wine, whilst contributing greatly to the reduction in the incidence of spoilage, cannot always be counted on to achieve the desired effect.

The strategies to control the production of volatile phenols can be broadly categorized into four approaches - sanitation during pre-production, using physical and chemical methods to control microbial growth, limiting the precursors and the elimination of the volatile phenols. The most common approach is the addition of SO2. Yet, the concentration and addition of SO2 needs to be constantly monitored over the winemaking process and sensitivity to its effect is strain dependent. Moreover, the trend is to use less SO2 to alleviate concerns about health implications (Šućur *et al.,* 2016). Certain alternative strategies include the usage of ozone (5mg/L) (Guzzon *et al*., 2013), dimethyldicarbonate (DMDC) (5 g/L) (Delfini *et al.,* 2002) and chitosan (6 g/L) (Ferreira *et al.,* 2015) to control the growth of *B. bruxellensis.* Novel methods such as High Hydrostatic Pressure (HHP) (Morata *et al.,* 2012), Pulsed Electric Field (PEF) (Puertolas *et al.,* 2009) and Microwave treatment were also tested (Gonzalez-Arenzana *et al.,* 2013). Polyvinylpolypyrrolidone (PVPP) and pyranoanthocyanins are strategies which use polymers to reduce the availability of the precursors of volatile phenols Donovan *et al.,* 1999; Benito *et al.,* 2009). Apart from the methods described above, certain steps can be considered to eliminate the volatile phenols from the wines - adsorption by yeast lees (Chassagne *et al.,* 2004) and treatments with cellulose acetate propionate (CAP) and cellulose propionate (CP) (Larcher *et al.,*2012).

The effects of these strategies on wine quality have to be validated at a larger scale for a more conclusive answer.

Nevertheless the extensive information which has been accumulated on this subject has not been sufficient to enable producers to systematically eradicate the contamination by spoilage yeasts and the subsequent development of the spoilage characteristics. Prevention strategies, whilst contributing greatly to the reduction in the incidence of spoilage, cannot always be counted on to achieve the desired effect. Although the formation of volatile phenols in wines is responsible for significant economic losses worldwide (Boulton *et al.,*1996 and Fugelsang, 1997) and various authors have concluded that controlling growth and activity of *Brettanomyces/Dekkera* is the most important microbiological challenge of modern winemaking (Wedral., *et al*., 2010; Kheir *et al.,*2013; Morata *et al*., 2013; Sturm *et al.,* 2015; Rubio *et al.,* 2015; Smith and Divol, 2016), efficient methods to achieve this goal have, however, not been found yet.

Alternatively, curative measures based on the removal of the odour-active compounds and/or on the reduction of the sensory effect by masking agents may provide methods for the recovering of tainted wines to acceptable quality levels.

Some studies have demonstrated that yeast cell walls have a good capacity to adsorb phenolic compounds, specifically anthocyanins and compounds responsible for the browning of white wines (Razmkhab *et al*., 2002, Morata *et al*., 2003 and 2005). Differences in anthocyanin adsorption capacity have been observed between the different yeast strains tested, possibly due to variation in the composition of yeast cell walls. Chassagne *et al*. ,(2005) studied the capacity of *Saccharomyces cerevisiae* yeast lees to sorb 4-ethylguaiacol and 4-ethylphenol.

Active dry yeast was found to be more effective when volatile phenols were diluted in red wine than in synthetic medium. Wine yeast lees showed a higher affinity for volatile phenols than active dry yeast. The effect of yeast lees on volatile phenol sorption was found to be sensitive to yeast autolysis level and to physicochemical parameters, such as ethanol content, temperature and pH. The clarification of wines with casein or potassium caseinate was also mentioned as capable of reducing the concentration of volatile phenols (Ruiz-Hernández 2003). We note that this study has based its conclusions on sensory analysis only and has used wines with low levels of ethylphenols. More recently, Ugarte et al. (2005) used a two-step process that uses reverse osmosis and an ion-exchange treatment column to

remove *Brettanomyces*-induced odour-active compounds. Wines subjected to this treatment showed a 50-70% reduction in the concentration of 4-ethylphenol and 4-ethylguaiacol. The colour, body and ethanol concentration of the wines did not change significantly, but a significant reduction in the concentration of certain aromatic compounds (higher alcohols, esters, acids, and phenolic compounds) was detected. Thus, caution should be taken due to the risk of stripping the wine of desirable flavour/aromatic components.

1.7. References

Agnolucci, M.; Vigentini, I.; Capurso, G.; Merico, A.; Tirelli, A.; Compagno, C.; Foschino, R.; Nuti, M. 2009. Genetic diversity and physiological traits of Brettanomyces bruxellensis strains isolated from Tuscan Sangiovese wines. International. Journal. *Food Microbiology*, **130, (3),** 238-244.

Barata, A.; Caldeira, J.; Botelheiro, R.; Pagliara, D.; Malfeito-Ferreira, M.; Loureiro V., 2008, Survival patterns of *Dekkera bruxellensis* in wines and inhibitory effect of sulphur dioxide. *International. Journal. Food Microbiology*, **121: (2),** 201-207.

Barata A, Malfeito-Ferreira M., Loureiro, V., 2012. The microbial ecology of wine grape berries. *International journal of food microbiology* **153**:243–59.

Barrera-Garcia, V.D., Gougeon, R.D., Karbowiak, T., Voilley, A. and Chassagne D. 2008. Role of wood macromolecules on selective sorption of phenolic compounds by wood. *Journal of Agricultural and Food Chemistry* **56**: 8498-8506.

Barthelmebs, L., Diviès, C., Cavin, J. F. 2000. Knockout of the p-coumarate decarboxylase gene from *Lactobacillus plantarum* reveals the existence of two other inducible enzymatic activities involved in phenolic acid metabolism. *Journal of Applied Microbiology*, **66**: 3368–3375.

Benito, S., Palomero, F., Morata, A., Uthurry, C., Suarez-Lepe, J.A. 2008. A method for estimating *Dekkera/Brettanomyces* populations in wines. *Journal of Applied Microbiology.* - **Vol. 106**. - pp. 1743-1751.

Benito, S., Palomero, F., Morata, A., Uthurry, C., Suarez-Lepe, J. A. 2009. Minimization of ethylphenol precursors in red wines via the formation of pyranoanthocyanins by selected yeasts. *International Journal Food Microbiology,* **132**:145–152

Bertrand, A. 1981. Formation des substances volatiles au cours de la fermentation alcoolique. Incidence sur la qualité du vin. In: *Colloque de la Société Française de Microbiologie*, Reims, pp. 251-267

Bertrand, A., Canal-Llaubères, R.-M., Feuillat, M., Hardy, G., Lamadon, F., Lonvaud-Funel, A., Pellerin, P. and Vivas, N. 2000. Chapitre VIII: Produits de correction, clarification et stabilisation. In: Produits de traitement et auxiliaires d'élaboration des moûts et des vins. Éditions Féret, Bordeaux, pp. 98-124

Brendan, D. S. and Benoit, D. 2016. *Brettanomyces bruxellensis*, a survivalist prepared for the wine apocalypse and other beverages. Review. *Food Microbiology*, **Volume 59**:161-175.

Cabrita, M. J, Aires de Sousa, J., Gomes da Silva, M. D. R. 2012. Multivariate statistical approaches for wine classification based on low molecular weight phenolic compounds. *Australian Journal Grape Wine Research,* **18**:138–146. doi: 10.1111/j.1755-0238.2012.00182.x

Cabrtia, M. J., Palma, V., Patão, R., Freitas AM. 2012. Conversion of hydroxycinnamic acids into volatile phenols in a synthetic medium and in red wine by *Dekkera bruxellensis. Ciência e Tecnologia dos Alimentos*. **32(1)**:1-6.

Chatonnet, P., Dubordieu, D., Boidron, J.N. 1989. Incidence de certains facteurs sur la decarboxylation des acids phenol par la levure. *Connaissence Vigne* vin. 23: 59-62.

Chatonnet, P., Dubourdieu, D., Boidron, J. N., Pons, M. 1992. The origin of ethylphenols in wines. *Journal Science Food Agriculture* **60**:165–178.

Chatonnet, P., Dubordieu, D., Boidron J. N. 1995. The influence of *Brettanomyces*/*Dekkera* sp. Yeasts and lactic acid bacteria o the ethylphenol content of red wines. *American Journal of Enology and Viticulture* **46**: 463-468.

Chatonnet, P., Viala, C., Dubourdieu, D. 1997 Influence of polyphenolic components of red wines on the microbial synthesis of volatile phenols. *American Journal Enology Viticulture* **48**: 443–448.

Chesson, A., Provan G.J., Russell, W.R. 1999 Hydroxycinnamic acids in the digestive tract of livestock and humans. *Journal Science Food Agriculture* **79**:373–378.

Edlin, D.A.N., Narbad, A., Dickinson, J.R. and Lloyd, D. 1995. The biotransformation of simple phenolic compounds by *Brettanomyces anomalus*. *FEMS Microbiology Letters* **125**: 311-316.

Falqué, E., Ferreira, A.C., Hogg, T., Guedes-Pinho, P. 2004 Determination of aromatic descriptors of Touriga Nacional wines by sensory descriptive analysis. *Flavour and Fragrance Journal*. **19**: 298–302. doi: 10.1002/ffj.1355

Food Standards Agency. 2002. McCance and Widdowson's The Composition of Foods, Sixth Summary Edition. Cambridge: Royal Society of Chemistry.

Fugelsang, K. C. and Edwards, C. 2007. Wine Microbiology: Practical Applications and Procedures, Second Edition. The Chapman & Hall Enology Library, New York.

Fugelsang, K.C., 1997. Wine Microbiology. The Chapman & Hall Enology Library, New York.

Gerbaux, V., Jeudy, S., Monamy, C. 2000. Étude des phénols volatiles dans les vins de Pinot noir en Bourgogne. *Bulletin de l'OIV* 835-**836**: 581–599.

Goldberg, D.M., Tsang, E., Karumanchiri, A., Soleas, G. J. 1998. Quercetin and p-coumaric acid concentrations in commercial wines. *American Journal Enology Viticulture*, **49**:142–151.

Gomez-Rivas, L., Escudero-Abarca, B., Aguilar-Uscanga, M. G., Hayward-Jones, P., Mendoza, P., Ramı´rez, M. 2004. Selective antimicrobial action of chitosan against spoilage yeasts in mixed culture fermentations. *Journal of Industrial Microbiology Biotechnology*, **31**:16–22.

Gonzalez-Arenzana, L., Santamarıa, P., Lopez, R., Garijo, P., Gutierrez, A. R., Garde-Cerdan, T., Lopez-Alfaro, I. 2013. Microwave technology as a new tool to improve microbiological control of oak barrels: a preliminary study. *Food Control*, **30**: 536–539.

González, J. A., Suárez-Lepe. 2013. Reduction of 4-ethylphenol production in red wines using HCDC+ yeasts and cinnamyl esterases. *Enzyme and Microbial Technology* **52**: 99–104.

Guzzon, R., Nardin, T. 2013. Antimicrobial activity of ozone. Effectiveness against the main wine spoilage microorganisms and evaluation of impact on simple phenols in wine. *Australian Journal of Grape and Wine Research,* **19**:180–188.

Harris, V., Christopher, M., Jiranek, V. and Grbin, P. 2008. *Dekkera* and *Brettanomyces* growth and utilization of hydroxycinnamic acids in synthetic media. *Appl Microbiology Biotechnology* **78**: 997–1006

Heresztyn, T. 1986. Metabolism of volatile phenolic compouds from hydroxycinnamic acids by *Brettanomyces* yeast. *Archives of Microbiology*. **146**: 96-98.

Hixson, J. L., Sleep, N. R., Capone, D. L. 2012. Hydroxycinnamic acid ethyl esters as precursors to ethylphenols in wine. *Journal Agriculture Food Chem* **60**: 2293–2298. doi: 10.1021/jf204908s.

Jackson, R.S. 2000. Wine Science: Principles and Applications, 3nd Edition, Academic Press.

Loureiro, V., 2000. Spoilage yeasts in food and beverages: characterisation and ecology for improved diagnosis and control. *Food Research. International,* **33**: 247-256.

Loureiro, V., Malfeito-Ferreira, M., 2006. *Dekkera/Brettanomyces* spp., in: Blackburn, C. (Ed.), *Food Spoilage Microorganisms*. Woodhead Publishers, pp. 354–398.

Macheix, J. J., Fleuriet, A., Billot, J. 1990. Fruit phenolics. CRC Press, Boca Raton, FL.

Martins, G., Miot-Sertier, C., Lauga, B., Claisse, O., Lonvaud-Funel, A., Soulas, G., Masneuf-Pomarède, I., 2012. Grape berry bacterial microbiota: impact of the ripening process and the farming system. *International Journal of Food Microbiology* **158**: 93–100.

Mateus, N., Machado, J. M., De Freitas, V. C. 2002. Development changes of anthocyanins in *Vitis vinifera* grapes grown in the Douro valley and concentration in respective wines. *Journal Science Food Agriculture*, **82**:1689–1695. doi: 10.1002/jsfa.1237.

Mills, D. A., Rawsthorne, H., Parker, C., Tamir, D., Makarova, K. 2005. Genomic analysis of Oenococcus oeni PSU-1 and its relevance to winemaking. *FEMS Microbiol Rev*. **229 (3**): 465-75.

Morata, A., Gómez-Cordovés, M.C., Suberviola, J., Bartolomé, B., Colomo, B. and Suárez, J.A. 2003. Adsorption of anthocyanins by yeast cell walls during the fermentation of red wines. *Journal of Agricultural and Food Chemistry* **51**: 4084-4088.

Morata, A., Gómez-Cordovés, M.C., Colomo, B. and Suárez, J.A. 2005. Cell wall anthocyanin adsorption by different *Saccharomyces* strains during the fermentation of *Vitis vinifera* L. cv Graciano grapes. *European Food Research and Technology*, **220**: 341-346.

Morata, A., Benito, S., Gonzalez, M. C., Palomero, F., Tesfaye, W., Suarez-Lepe, J. A. 2012. Cold pasteurisation of red wines with high hydrostatic pressure to control *Dekkera*/*Brettanomyces*: effect on both aromatic and chromatic quality of wine. *Eur Food Res Technol*, **235**:147–154.

Oelofse, A., Pretorius, I., Toit, M. Du, 2008. Significance of *Brettanomyces* and *Dekkera* during winemaking: a synoptic review. South African *Journal of Enology and Viticulture* **29**: 128-144.

Oliveira, J. M., Araújo, I. M., Pereira, Ó. M. 2004. Characterization and differentiation of five "Vinhos Verdes" grape varieties on the basis of monoterpenic compounds. *Anal Chim Acta* **513**: 269–275. doi: 10.1016/j.aca.2003.10.020.

Ong, B.Y., Nagel, C. W. 1978. Hydroxycinnamic acid tartaric acid ester content in mature grapes and during maturation of white Riesling grapes. *American. Journal Enology and Viticulture*. **29**: 277–281.

Phister, T.G. and Mills, D.A. 2004. Novel methods to detect *Brettanomyces* (*Dekkera*) in wine. In: Technical abstracts, 55th Annual Meeting, San Diego, CA. Davis, CA: *American Society for Enology and Viticulture,* p. 30.

Pollnitz, A. P., Pardon, K. H., Sefton, M. A. 2000. Quantitative analysis of 4-ethylphenol and 4-ethylguaiacol in red wine. *Journal of Chromatography A.* **874**:101–109.

Pour Nikfardjam, M., May, B., Tschiersch, C. 2009. 4-Ethylphenol and 4-ethylguaiacol contents in bottled wines from the German "Württemberg" region. *Eur Food Res Technol* 230: 333–341. doi: 10.1007/s00217-009-1174-1.

Prakash Kumar Sarangi, Hara Prasad Sahoo. 2009. Standardization of cultural conditions for maximum vanillin production through ferulic acid degradation. *Report and Opinion*, 2009; 1(**5**): 49-51.

Puertolas, E., Lo´pez, N., Condo´n, S., Raso, J. A. lvarez, I. 2009. Pulsed electric fields inactivation of wine spoilage yeast and bacteria. *International Journal Food Microbiol*, 130: 49–55.

Ramos, R., Andrade, P. B., Seabra, R. M. 1999. A preliminary study of non-coloured phenolics in wines of varietal white grapes (códega, gouveio and malvasia fina): effects of grape variety, grape maturation and technology of winemaking. *Food Chemistry,* **67**: 39–44.

Razmkhab, S., Lopez-Toledano, A., Ortega, J.M., Mayen, M., Merida, J. and Medina, M. 2002. Adsorption of phenolic compounds and browning products in white wines by yeasts and their cell walls. *Journal of Agricultural and Food Chemistry,* **50**: 7432-7437.

Renouf, V., Claisse, O., Lonvaud-Funel, A., 2005. Understanding the microbial ecosystem on the grape berry surface through numeration and identification of yeast and bacteria. *Australian Journal of Grape and Wine Research,* **11**: 316–327.

Renouf, V.; Lonvaud-Funel, A. 2007. Development of an enrichment medium to detect *Dekkera/Brettanomyces bruxellensis*, a spoilage yeast, on the surface of grape berries. *Microbiological Research,* 162 (**2**): 154-167.

Ribeiro de Lima, M. T., Cabanis, J. C. 1998. The phenolic acid content of different varieties and vintages of wines in continental Portugal and the Azores islands. *Bulletin de l'OIV* 813-814:1012–1027.

Ribéreau-Gayon, P., Glories, Y., Maujean, A. and Dubourdieu, D. 2006. Handbook of Enology Volume II, The Chemistry of Wine: Stabilization and Treatments, Second Edition. John Wiley & Sons Ltd, UK.

Rocha, S., Coutinho, P., Barros A, Coimbra, M. A., Delgadilho, I., Cardoso, A. D., (2000). Aroma Potential of Two Bairrada White Grape Varieties: Maria Gomes and Bical. *Journal Agriculture Food Chem.* **48**:4802–4807. doi: 10.1021/jf000175s.

Ruiz-Hernández, M. 2003. Casein for correction of defects caused by *Brettanomyces* and *Dekkera*. *Semana Vitivinícola.* **58**, 1462-1463.

Rubio, P., P. Garijo, P. Santamaría, R. Lopez, J. Martínez, A.R. Gutierrez. 2015. Influence of oak origin and ageing conditions on wine spoilage by *Brettanomyces* yeasts. *Food Control*. **54:** 176 e 180

Salameh, D., Brandam, C., Medawar, W., Lteif, R., Strehaiano, P. Highlight on the problems generated by p-coumaric acid analysis in wine fermentations. *Food Chemistry*. - 2008. - **Vol. 107**. - pp. 1661-1667.

Silva, R. B. O. 1994. Leveduras contaminantes na produção de etanol industrial por processo contínuo: quantificação e identificação. 145p. Dissertação (Mestrado em Microbiologia Aplicada) – Instituto de Biociências, Universidade Estadual Paulista "Julia de Mesquita Filho", Rio Claro.

Shinohara, T, Kubodera, S and Ynagida, F. 2000. Distribution of phenolic yeasts and production of phenolic off-flavors in wine fermentation. *Journal of Bioscience and Bioengineering*, **90**: 90-97.

Sturm, M.A., M. Assof, M. Fanzone, C. Martinez, M.A. Ganga, V. Jofré, M.L. Ramirez, M. Combina. 2015. Relation between coumarate decarboxylase and vinylphenol reductase activity with regard to the production of volatile phenols by native *Dekkera bruxellensis* strains under 'wine-like' conditions. *International Journal of Food Microbiology* **206**: 51–55.

Suárez R., Suárez-Lepe J. A., Morata A., Calderón, F. 2007. The production of ethylphenols in wine by yeasts of the genera *Brettanomyces* and *Dekkera*: A review. *Food Chemistry*. **Vol. 102**:10-21.

Šućur, S., Čadež, N., and Košmerl, T. 2016. Volatile phenols in wine: Control measures of *Brettanomyces*/*Dekkera* yeasts. *Acta agriculturae Slovenica*, **107**:453 – 472.

Ugarte, P., Agosin, E., Bordeu, E. and Villalobos, J.I. 2005. Reduction of 4-ethylphenol and 4-ethylguaiacol concentration in red wines using reverse osmosis and adsorption. *American Journal of Enology and Viticulture,* **56:** 30-36.

Valentao, P., Seabra, M. R., Lopes, G., Silva, L. R., Martins, V., Trujilo, E. M., Velásquez,E., Andrade, P.B. Influence of *Dekkera bruxellensis* on the contents of anthocyanins, organic acids and volatile phenols of Dao red wine. *Food Chemistry*. 2007. **Vol. 100**: 64-70.

Van Beek, S., Priest, F. G. 2000. Decarboxylation of substituted cinnamic acids by lactic acid bacteria isolated during malt whiskey fermentation. *Applied and Environmental Microbiology*. **66**: 5322-5328.

Wedral, D, Shewfelt, R, Frank, J., 2010. The challenge od Brettanomyces in wine. *LWT-Food Science Technology.* 43:1474-1479. Doi:10.1016/j.lwt.2010.06.10.

Whiting GC, Carr JG (1959) Metabolism of cinnamic acid and hydroxycinnamic acids by *Lactobacillus pastorianus* var. *quinicus*. *Nature*. **184**:1427–1428.

CHAPTER 2

Isolation of *Brettanomyces/Dekkera* strains from wine and characterisation of ethanol resistance

Abstract

The objective of this work was to isolate and identify *Brettanomyces/Dekkera* from wine and to characterise the ethanol resistance of the isolated strains. An enrichment broth and a solid medium, DBDM (*Dekkera/Brettanomyces* Differential Medium), were used to selectively and differentially promote the growth of *Dekkera*/*Brettanomyces*. Both mediums contained chloramphenicol and cycloheximide to eliminate bacteria and non-*Brettanomyces/Dekkera* yeasts, respectively. The isolated strains were identified to species level by PCR amplification of fungal ITS1 and ITS2 rDNA gene regions and DNA sequencing of the PCR products. The percentage of contaminated wines was 37% and all strains were identified as *Dekkera bruxellensis*. Phenotypic variation was found among the strains studied regarding the tolerance to ethanol. The limiting ethanol concentration for growth of the most tolerant strains was 14% v/v and the wine isolates were, in general, more tolerant than the reference strain *D. bruxellensis* PYCC 4801. Growth conditions affected the survival of *D. bruxellensis* PYCC 4801 in wine. Cultures grown in the presence of ethanol (6 to 10%) were more resistant to wine conditions than those grown in the absence of ethanol. The pre-adaptation of cells by shock exposure to ethanol did not produce the same effect. This study allowed the selection of strains, from the most tolerant group, to perform survival and metabolic studies in real wine conditions in future work.

Isolation of *Brettanomyces/Dekkera* strains from wine and characterisation of ethanol resistance

2.1.Introduction

It has been extensively reported that volatile phenols, mainly 4-ethylphenol and 4-ethylguaiacol, affect the quality of wines, being responsible for aroma defects described as "animal", “barnyard”, "leather" and “horse sweat" (Wedral *et al*., 2010; Kheir *et al*., 2013). Certain non-flavonoids, namely the hydroxycinnamic acids p-coumaric and ferulic acids, are the precursors of volatile phenols (Heresztyn 1986; Chatonnet *et al*., 1995), taking part of metabolic pathways carried out by *Brettanomyces* and its sporulating form *Dekkera* (Heresztyn 1986, Chatonnet *et al*., 1997, Edlin *et al*., 1995). The aroma threshold of volatile phenols is 425 µg/L for a 1:10 ratio of 4-ethylguaiacol/4-ethylphenol in red wines (Chatonnet *et al*., 1990). Since wine is a very complex and variable matrix, this threshold value may not be equally applicable to all wines.

Accurate detection procedures are essential to control the proliferation of *Brettanomyces/Dekkera* in wines and wine environments. Cultural methods are available based on selective and differential media able to recover *Brettanomyces/Dekkera* from wine and related environments (Rodrigues *et al*., 2001; Couto *et al*., 2005; Morneau *et al*., 2011). Several molecular biology techniques have also been proposed such as a method based on a polymorphism in the rRNA internal transcribed spacer region (Egli and Henick-Kling 2001), real time PCR assay (Phister and Mills, 2003), PCR restriction enzyme analysis (Cocolin *et al*., 2004), mitochondrial DNA (mtDNA) restriction analysis (Martorell *et al*., 2006), random amplified polymorphism DNA (RAPD) PCR (Miot-Sertier and Lonvaud-Funel 2007) and amplified fragment length polymorphism (AFLP) analysis (Curtin *et al*., 2007).

The survival and metabolic activity of *Brettanomyces/Dekkera* in wine are influenced by several factors. The presence of residual sugars and the pH of wine have not a significant effect on the production of volatile phenols (Suarez *et al.,* 2007) but the temperature was found to be a relevant factor (Barata *et al.,* 2008). While the final concentration of volatile phenols was the same at 16 and 30˚C, the production rate at 16˚C was significantly slower. The effect of sulphur dioxide (SO_2) on *D. bruxellensis* was investigated by several research groups (Du Toit *et al.,* 2005; Suarez *et al.,* 2007; Curtin *et al.,* 2012; Serpaggi *et al.,* 2012,

Agnolucci *et al.,* 2014; Longin *et al.,* 2016), leading to the conclusion that it is a very important factor to control the growth of *Brettanomyces/Dekkera* in wines. The effect of ethanol on the growth of these yeasts is a less studied matter and the scientific available results are somehow inconsistent. Benito *et al.,* (2009) found that increasing concentrations of ethanol up to 10% v/v lowered the growth and the production rates of volatile phenols but the total production was not affected. Conterno *et al.,* (2006) were able to see that all the studied isolates of *B. bruxellensis* were tolerant to at least 10%. Bassi *et al.,* (2013) obtained a complete growth inhibition of the 3 *D. bruxellensis* strains tested at 11% v/v in rich medium. Medawar *et al.,*(2003) found an increase of the lag phase and a reduction of the specific growth rate and on the final biomass with increasing levels of ethanol and a total growth inhibition at 11.5% v/v. Dias *et al.,* (2003) observed that growth and production were significantly lower at 12% and a total inhibition at 13% v/v by the single *D. bruxellensis* strain studied. Barata *et al.,* (2008) found higher resistant strains. Except for Conterno *et al.,* (2006) and Barata *et al.,* (2008), the above studies used a relatively small number of strains for the ethanol tolerance characterisation of *Brettanomyces/Dekkera*. The aim of this work was to assess the prevalence and to isolate strains of *Brettanomyces/Dekkera* from wines and to characterize these strains with regard to ethanol resistance. Moreover, it was also studied whether the exposition of cells to ethanol would render these cells more resistant to wine real conditions.

2.2.Material and Methods

2.2.1. Wine samples and isolation of *Brettanomyces/Dekkera*

Seventy-five wine samples from the 2014 and 2015 harvestings obtained from 5 wineries of wine producing demarcated regions in Portugal were used in this study. All samples were collected before bottling. A selective enrichment broth was used for the differential promotion of the growth and activity of *Brettanomyces/Dekkera* (Couto *et al.,* 2005). The medium contained glucose (10 g/L) as carbon and energy source, cycloheximide (20 mg/L) to prevent growth of *Saccharomyces*, chloramphenicol (200 mg/L) to prevent growth of bacteria and p-coumaric acid (20 mg/L) as the precursor for the production of 4-ethyl-phenol. 20 ml of this medium in 100 ml flasks were inoculated with 20 ml of each wine sample. After the inoculation, the medium was monitored by visual inspection of turbidity and by periodic olfactive analysis of the 4-ethylphenol off-odour. Then, the solid medium DBDM (*Dekkera/Brettanomyces* Differential Medium) (Rodrigues *et al.,* 2001) was used for the isolation of strains. DBDM consisted of 6.7 mg /L of Yeast Nitrogen Base (Difco, Detroit, USA), 6% v/v ethanol, 10 mg/L cycloheximide, 100 mg/L p-coumaric acid, 22 mg/L bromocresol green, and 18 g /L agar. All yeasts were maintained on Yeast Mold (YM) slants (Difco) (pH 6) prepared with 18 g/L agar (Lab M, Bury, UK) and kept at 4°C. For long-term storage, strains were maintained at -80 °C in YM broth with 20% v/v glycerol.

2.2.2. Yeast identification

The identification of the isolated strains was confirmed and further fulfilled to species level by PCR amplification of fungal ITS1 and ITS2 rDNA gene regions using the universal primers ITS1F (forward) (5'- CTT GGT CAT TTA GAG GAA GTA A -3') and ITS4 (reverse) (5'- TCC TCC GCT TAT TGA TAT GC -3') (Egli and Henick-Kling, 2001).

The purification of the PCR products was made using the commercial kit EzWay™ PCR Clean-Up (Koma Biotech, Seoul, South Korea). Purified PCR products were subjected to DNA sequencing (Macrogen, Seoul, South Korea) and the resulting sequences were analysed using BLASTN software and GenBank NCBI reference sequences.

2.2.3. Medium and growth conditions for the characterisation of ethanol tolerance

Eighteen strains previously isolated from naturally contaminated wine samples and *D. bruxellensis* PYCC 4801 (Portuguese Yeast Culture Collection, Portugal) were used. Growth at different concentrations of ethanol was determined in YM medium at pH 4.5 to which filter sterilised ethanol was added to the cooled sterilised medium to yield the desired concentrations (10, 12, 13 and 14% v/v). Growth, at 25°C, was monitored spectrophotometrically by measuring the increase in the optical density at 650 nm (OD_{650}), against a medium blank, in a UV/VIS Unicam 8620 spectrophotometer (Unicam, Cambridge, UK) using optical cells of 1 cm path length. Samples showing an OD_{650} higher than 0.8 were diluted with sterile medium before measurement to maintain linearity of absorbance and cell mass.

2.2.4. Viability assays-ethanol shock response

For the ethanol shock response experiments, *D. bruxellensis* PYCC 4801 was grown at 25°C in YM medium with pH adjusted to 4.5 to a density of $5x10^8$ cells ml^{-1} (initial stage of stationary phase) in the absence of added ethanol. The culture was then divided in 10 ml aliquots, each receiving a different addition of ethanol to final concentration of 6, 10 and 12% v/v. These shock cultures were held at 25°C during 4 h before being challenged at wine (red wine, 13% v/v ethanol, pH 3.6, total acidity 6 g/L, residual sugar 2.4 g/L) for 170 h. Cells grown in YM broth at different concentrations of ethanol (0, 6, 10 and 12% v/v) were also challenged.

Samples were taken at regular intervals during the challenge period and serially diluted from 10^{-1} to 10^{-6} (decimal dilutions in sterile 9 ml Ringer solution [Lab M, Bury, UK]). The diluted samples were plated in duplicate on YM agar, using the drop count technique (Miles & Misra, 1938) for the determination of colony forming units per mL (CFU ml/L). Counts were done after the incubation of YM plates at 25° C for 72 h.

2.2.5. Statistical analysis

Data were analyzed according to ANOVA procedures to test significant differences. Normality and homoscedasticity of data were tested using Shapiro Wilk and Levene's tests, respectively. When these requirements were not verified, non-parametric tests were carried out instead: Kruskal–Wallis test (alternative to one-way ANOVA) and Mann–Whitney (alternative to post-hoc pairwise comparisons). The significance level assumed was 5% in all situations. IBM SPSS Statistics 25 for Windows® (SPSS Inc., Chicago, USA) was used in all data analyses.

2.3 Results and Discussion

Twenty-eight out of seventy-five wine samples gave positive result on the detection analysis of *Brettanomyces/Dekkera* using the selective enrichment broth for the differential promotion of the growth and activity of *Brettanomyces/Dekkera* described by Couto *et al.,* (2005). Visible turbidity was developed in the medium inoculated with these wines and the 4-ethylphenol off-odour was easily detected by smelling. Yeast strains were then isolated from the selective enrichment broth using the solid medium DBDM (*Dekkera/Brettanomyces* Differential Medium) (Rodrigues *et al.,* 2001). Presumptive *Dekkera/Brettanomyces* strains were further identified to species level by PCR amplification of fungal ITS1 and ITS2 rDNA gene regions and were all found to belong to *D. bruxellensis*.

Yeasts of the genus *Brettanomyces* and of its ascoporogenous sexual form *Dekkera* are generally considered the main wine spoilage microorganisms. Currently, five species are recognized as members of the genus *Brettanomyces*: *Brettanomyces custersianus, Brettanomyces naardenensis, Brettanomyces nanus, Brettanomyces bruxellensis* and *Brettanomyces anomalus* (Cocolin *et al.,* 2004; Zuehlke *et al.,* 2013). Teleomorphs are known for the last two species, *Dekkera bruxellensis* and *Dekkera anomala* (Kurtzman and Fell, 2000; Kurtzman *et al.,* 2011). Our results are in accordance to the attempts made to isolate and identify grape, wine and winery isolates, in which only the species *D. bruxellensis* has been found (Egli and Henick-Kling, 2001; Cocolin *et al.,* 2004; Conterno *et al.,* 2006; Curtin *et al.,* 2007). A discussion still exists on wether *D. anomala* is a significant spoilage organism in wine (Stender *et al.,* 2001; Phister and Mills, 2003). Our results clearly support the view that *D. bruxellens*is is the main, or most probable the only yeast species responsible for the production of volatile phenols in wines.

The percentage of contaminated wines found in this study was 37%. A study that analysed the level volatile phenols in wines from different countries found that more than 25% of red wines had 4-ethylphenol levels higher than the preferred threshold of 620 µg/L (Loureiro and Malfeito-Ferreira, 2003). Another study demonstrated that *Brettanomyces/Dekkera* is present in about 50% of the wines undergoing maturation and about 25% in bottled wines (Gerbaux *et al.,* 2000). Garijo *et al.,* (2015) also found a prevalence of 25% in recently bottled red wines. The high percentage of positive results in our study might be related to the fact that the samples were taken before bottling and so have not yet been subjected to final treatments, such as SO_2 adjustments. This work also shows that the cultural methodology followed was able to selectively isolate *Brettanomyces/Dekkera*, thus evidencing the suitability of the media used for the detection of these organisms in wine environments.

*D. bruxellens*is is considered well adapted to the winemaking conditions due to the relatively high tolerance to SO_2 and ethanol (Oelofse *et al.,* 2008). But, scientific data reporting the growth and survival of this species in the presence of ethanol is scarce; particularly, there is a lack of information on the quantification of the limiting ethanol concentrations for growth.

Experiments have been conducted to characterise the tolerance to ethanol of 18 of the isolated strains of *D. bruxellens*is and the reference strain *D. bruxellens*is PYCC4801.

Culture media (YM, pH 4.5) were prepared with ethanol concentrations varying from10 to 14% v/v. Phenotypic variation was found among the strains studied regarding the tolerance to ethanol. It is possible to establish three groups: Group I - less tolerant (limiting ethanol concentration for growth of 13%), Group II - intermediate tolerance (limiting ethanol concentration for growth of 14% and maximum OD_{650} attained at this concentration lower than 2) and Group III - more tolerant (limiting ethanol concentration for growth of 14% and maximum OD_{650}>2).

Figure 2.1 shows the growth curves at different ethanol concentrations of a representative strain of each of the 3 groups.

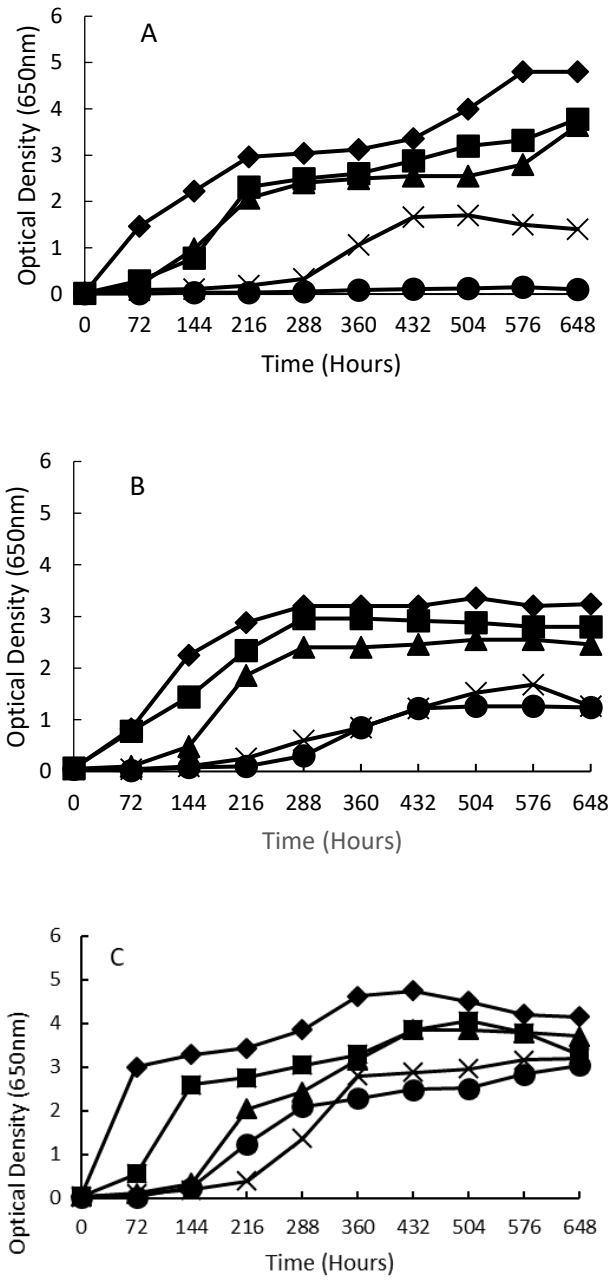


Figure 2.1 – Growth of *Dekkera bruxellensis* strains representative of Group I (A), Group II (B) and Group III (C) at different concentrations of ethanol in YM broth at 25°C. Concentrations of ethanol in media: 0 (◆), 10 (■), 12 (▲), 13 (**x**) and 14% v/v (●). The values are the means of two independent experiments, the relative standard deviation of log CFU never varied more than 12% of the mean value in each time point.

Growth parameters are summarised in Table 2.1. The lag phase was not determined since it was difficult to accurately establish the boundaries of this phase from the growth curves obtained. The growth rates (μ_m) obtained were not significantly different. The maximum OD_{650} was strongly affected by the ethanol concentration. Significant higher values were found in Group III at 13 and 14% v/v ethanol in comparison with the other groups. Our results are not in total agreement with Loureiro and Malfeito-Ferreira (2006) who stated that 14 to 14.5% v/v ethanol might inhibit growth of *Dekkera/Brettanomyces* and Bassi *et al.,* (2013) who found a complete growth inhibition at 11%. The results are more in accordance with Barata *et al.,* (2008) who found that most of the strains were capable of growing at 14.5% and the most sensitive in the presence of 13.5% v/v. A few strains were found to grow at 15% ethanol by Barata *et al.,* (2008) and Sturm *et al.,* (2014). Several studies have demonstrated that the phenotypic traits associated with spoilage activity of *D. bruxellensis* are strain dependent (Renouf *et al.,* 2009; Curtin *et al.,* 2012; Divol *et al.,* 2012; Agnolucci *et al.,* 2014; Di Toro *et al.,* 2015).

Table 2.1. Growth parameters of the three groups of *Dekkera bruxellensis* strains grown in YM broth (25°C) at different concentrations of ethanol. Different lowercase letters indicate statistically significant differences between the ethanol concentrations. Different capital letters indicate statistically significant differences between groups.

	Group I (n=3)		Group II (n=12)		Group III (n=4)	
Ethanol concentration (% v/v)	μ_m (h^{-1})	OD_{650} máx	μ_m (h^{-1})	OD_{650} máx	μ_m (h^{-1})	OD_{650} máx
0	0.0057	4.28aA	0.0043	4.01aA	0.0218	4.29aA
10	0.0122	3.36aAB	0.0064	2.48bB	0.0115	3.60bA
12	0.0152	2.59aA	0.0073	2.19bA	0.0151	3.37bcA
13	0.0089	1.65aA	0.0056	1.62cA	0.0071	3.03bcB
14	ng*	ng	0.0065	1.35dA	0.0077	2.77cB

*ng: no growth

We noted that, in most cases, laboratory cultures fail to survive when put back in their natural habitat (wine). Knowing that the growth conditions may affect the survival of microorganisms to stressful environments, the effect of growth at different concentrations of ethanol (0, 6, 10 and 12% (v/v) on the survival of *D. bruxellens*is PYCC 4801 in a commercial wine was evaluated. It was possible to observe that cells grown in the presence of 6 and especially 10% ethanol survived better in the wine than those grown in the absence of ethanol (Figure 2.2). Growth in the presence of ethanol, until 10% v/v, adapted the cells to the stress conditions of the wine. The kinetics of inactivation was also evaluated in cells grown in the absence of ethanol and shocked with 6, 10 and 12% ethanol for 4 h. It was found that none of the ethanol shocks tested was effective in increasing the survival of the yeast to the stress conditions (Figure 2.3). Thus, shock exposure to sub-lethal concentrations of ethanol didn't increase the protection of cells in wine. The practical interest of these pre-adaptation experiments is to ascertain the growth conditions toward an efficient inoculum preparation to ensure survival in real wine conditions. Strains selected from the most tolerant group will be grown in the presence of 6 to 10% v/v ethanol to perform survival and metabolic studies in wine.

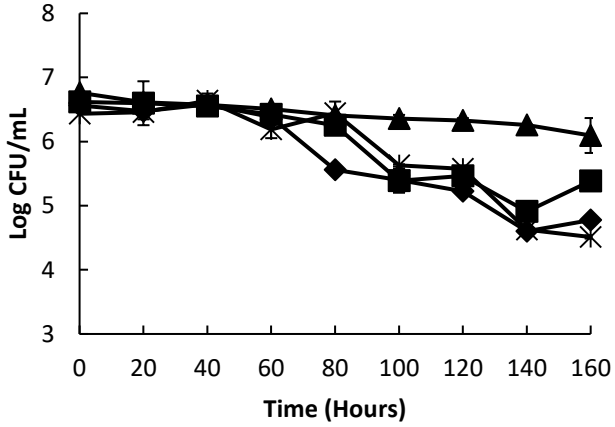


Figure 2.2 .The effect of growth at different concentrations of ethanol [0 (◆), 6 (■), 10 (▲) and 12% v/v (**x**)] in YM broth at 25°C on the survival of *Dekkera bruxellensis* PYCC 4801 in a red wine (13% v/v ethanol, pH 3.6, total acidity 6 g/L, residual sugar 2.4 g/L). The values are the means of two independent experiments.

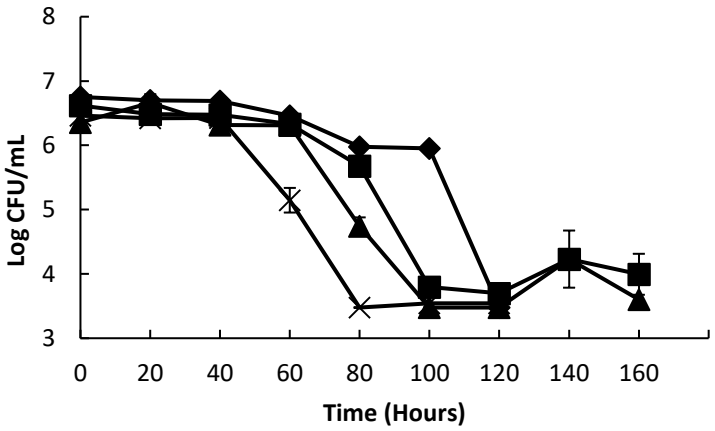


Figure 2.3. The effect of ethanol shock on the survival of *Dekkera bruxellensis* PYCC 4801 in a red wine (13% v/v ethanol, pH 3.6, total acidity 6 g/L, residual sugar 2.4 g/L). Cells were grown at 0% (◆) and then shifted to 6 (■), 10 (▲) and 12 % v/v ethanol (**x**) for 4 h before being exposed to the wine. The values are the means of two independent experiments.

2.4. References

Agnolucci, M., Cristani, C., Maggini, S., Rea, F., Cossu, A., Tirelli, A., Nuti, M. 2014. Impact of sulphur dioxide on the viability, culturability, and volatile phenol production of *Dekkera bruxellensis* in wine. *Ann Microbiology*, **64***,* 653-659.

Barata, A., Pagliara, D., Piccininno, T., Tarantino, F., Ciardulli, W., Malfeito-Ferreira, M., Loureiro, V. 2008. The effect of sugar concentration and temperature on growth and volatile phenol production by *Dekkera bruxellensis* in wine. *FEMS Yeast Res,* **8**: 1097-1102.

Bassi AP, da Silva JC, Reis VR, Ceccato-Antonini SR (2013) Effects of single and combined cell treatments based on low pH and high concentrations of ethanol on the growth and fermentation of *Dekkera bruxellensis* and *Saccharomyces cerevisiae*. *World Journal Microbiology Biotechnology*, **29**: 1661-76.

Benito S, Palomero F, Morata A, Calderón F, Suárez –Lepe JA (2009). Factors Affecting the Hydroxycinnamate Decarboxylase/Vinylphenol Reductase Activity of *Dekkera/Brettanomyces*: Application for *Dekkera/Brettanomyces* Control in Red Wine Making. *Journal Food Science,* **74**: M15-M22.

Chatonnet, P., Boidron, J. N., Pons, M. 1990. Elevage des vins rouges en fûts de chéne: evolution de certains composés volatils et de leur impact aromatique. *Science Aliments* **10**: 565-587.

Chatonnet, P., Dubordieu, D., Boidron, J. 1995. The influence of *Brettanomyces*/*Dekkera* sp. and lactic acid bacteria on the ethylphenol content of red wines. *American Journal Enology Viticulture,* **46***:* 463-468.

Chatonnet, P., Viala, C., Dubordieu, D. 1997. Influence of polyphenolic components of red wines on the microbial synthesis of volatile phenols. *American Journal Enology Viticulture* **48**: 443-448.

Cocolin L, Rantsiou K, Iacumin L, Zironi R, Comi G (2004) Molecular detection and identification of *Brettanomyces*/*Dekkera bruxellensis* and *Brettanomyces*/*Dekkera anomalus* in spoiled wines. *Applied Environmental Microbiology* **70**: 1347–1355.

Conterno, L., Joseph, C. M. L., Arvik, T. J., Henick-Kling, T., Bisson, L. F. 2006. Genetic and physiological characterization of *Brettanomyces bruxellensis* strains isolated from wines. *American Journal Enology Viticulture*, **57**: 139-147.

Couto, J. A., Barbosa, A., Hogg, T. 2005. A simple cultural method for the presumptive detection of *Brettanomyces*/*Dekkera* in wines. *Letters Applied Microbiol*ogy, **41**: 505-510.

Curtin, C. D., Bellon, J. R., Henschke, P. A., Godden, P. W., de Barros Lopes, M. A. 2007. Genetic diversity of *Dekkera bruxellensis* yeasts isolated from Australian wineries. *FEMS Yeast Res* **7**: 471-481.

Curtin, C. D., Langhans, G., Henschke, P. A., Grbin, P. R. 2013. Impact of Australian *Dekkera bruxellensis* strains grown under oxygen-limited conditions on model wine composition and aroma. *Food Microbiology*, **36**: 241-247.

Curtin, C., Kennedy, E., Henschke, P. A. 2012 Genotype-dependent sulphite tolerance of Australian *Dekkera (Brettanomyces) bruxellensis* wine isolates. *Letters Applied Microbiology, **55***: 56-61.

Di Toro, M. R., Capozzi, V., Beneduce, L., Alexandre, H., Tristezza, M., Durante, M., Tufariello, M., Grieco, F., Spano, G. 2015. Intraspecific biodiversity and 'spoilage potential' of *Brettanomyces bruxellensis* in Apulian wines. *LWT - Food Science Technology,* **60**: 102-108.

Dias, L., Pereira-da-Silva, S., Tavares, M., Malfeito-Ferreira, M., Loureiro, V. 2003. Factors affecting the production of 4-ethylphenol by the yeast *Dekkera bruxellensis* in enological conditions. *Food Microbiology*, **20**: 377-384.

Divol, B., Du Toit, M., Duckitt, E. 2012. Surviving in the presence of sulphur dioxide: strategies developed by wine yeasts. *Applied Microbiology Biotechnology*, **95**: 601-13.

Du Toit, W. J., Pretorius, I. S., Lonvaud-Funel, A. 2005. The effect of sulphur dioxide and oxygen on the viability and culturability of a strain of *Acetobacter pasteurianus* and a strain of *Brettanomyces bruxellensis* isolated from wine. *Journal Applied Microbiology,* **98**: 862-871.

Edlin, D. A.N., Narbad, A., Dickinson, J. R., Lloyd, D. 1995. The biotransformation of simple phenolic compounds by *Brettanomyces anomalus*. *FEMS Microbiology Letters*, **125**: 311-315.

Egli, C. M., Henick-Kling, T. 2001. Identification of *Brettanomyces*/*Dekkera* Species Based on Polymorphism in the rRNA Internal Transcribed Spacer Region. *American Journal Enology Viticulture*, **52**: 241-247.

Garijo, P., González-Arenza, L., López-Alfaro, I., Garde-Cerdán, T., López, R., Santamaría, P., Gutiérrez, A. R. 2015. Analysis of grapes and the first stages of the vinification process in wine contamination with *Brettanomyces bruxellensis*. *European Food Research Technology*, **240** :525–532.

Gerbaux, V., Naudin, R., Meurgues, O., Monamy, C. 2000. Etude des phénols volatils dans les vins de Pinot noir en Bourgogne. *Bull del´OIV*, **73**: (835–836):581–599.

Heresztyn, T. 1986. Metabolism of volatile phenolic compounds from hydroxycinnamic acids by *Brettanomyces* yeast. *Archive Microbiology*, **146**, 96-98.

Kheir, J., Salameh, D., Strehaiano, P., Brandam, C., Lteif, R. 2013. Impact of volatile phenols and their precursors on wine quality and control measures of *Brettanomyces/Dekkera* yeasts. *European Food Research Technology*, **237**, 655-671

Kurtzman, C.P., Fell, J. W. 2000. Yeasts: A Taxonomic Study (4th ed revised). Elsevier Science Publisher BV, Amsterdam, The Netherlands.

Kurtzman, C., Fell, J., Boekhout, T. 2011. Yeasts: A Taxonomic Study, Vols. 1–3, 5th ed. Elsevier Science Publisher BV, Amsterdam The Netherlands.

Longin, C., Degueurce, C., Julliat, F., Guilloux-Benatier, M., Rousseaux, S., Alexandre, H. 2016. Efficiency of population-dependent sulfite against *Brettanomyces bruxellensis* in red wine. *Food Research International*, **89**: 620-630.

Loureiro, V., Malfeito-Ferreira, M. 2003. Spoilage yeasts in the wine industry. *International Journal Food Microbiology*, **86**: 23–50.

Loureiro, V., Malfeito-Ferreira, M. 2006. Spoilage activities of *Dekkera*/*Brettanomyces* spp. In: Blackburn, editor. Food spoilage microorganisms. Cambridge, U.K.: Woodhead Publishers. p 354–98.

Martorell P, Barata A, Malfeito-Ferreira M, Fernandez-Espinar MT, Loureiro V, Querol A (2006) Molecular typing of the yeast species *Dekkera bruxellensis* and *Pichia guilliermondii* recovered from wine related sources. *International Journal Food Microbiology*, **106**:79–84

Medawar, W., Strehaiano, P., Delia, M. L. 2003. Yeast growth: Lag phase modelling in alcoholic media. *Food Microbiology*, **20**: 527-532.

Miles, A., Mishra, S. 1938. The estimation of the bactericidal power of blood. J Hyg-Cambrige, **38**: 732-749.

Miot-Sertier, C., Lonvaud-Funel, A. 2007. Development of a molecular method for the typing of *Brettanomyces bruxellensis* (*Dekkera bruxellensis*) at the strain level. *Journal Applied Microbiology*, **102**, 555-562.

Morneau, A. D., Zuehlke, J. M., Edwards, C. G. 2011. Comparison of media formulations used to selectively cultivate *Dekkera/Brettanomyces*. *Letters Applied Microbiology*, **53**, 460–465

Oelofse, A., Pretorius, I.S., du Toit, M. 2008. Significance of *Brettanomyces* and *Dekkera* during Winemaking: A Synoptic Review. *South African Journal of Enology and Viticulture*, **29**: 128-144.

Phister, T. G., Mills, D. A. 2003. Real-time PCR assay for detection and enumeration of *Dekkera bruxellensis* in wine. *Applied Environmental Microbiology*, **69**: 7430-7434.

Renouf, V., Miot-Sertier, C., Perello, M., de Revel, G., Lonvaud-Funel, A. 2009. Evidence for differences between *B. bruxellensis* strains originating from an enological environment. *International Journal Wine Research*, **1**: 95–100.

Rodrigues, N., Gonçalves, G., Pereira-da-Silva, S., Malfeito-Ferreira, M., Loureiro, V. 2001. Development and use of a new medium to detect yeasts of the genera *Brettanomyces*/*Dekkera* sp. *Journal Applied Microbiology*, **90**: 588-599.

Serpaggi, V., Remize, F., Recorbet, G., Gaudot-Dumas, E., Grand, A. S-Le., Alexandre, H. 2012. Characterization of the "viable but nonculturable" (VBNC) state in the wine spoilage yeast *Brettanomyces*. *Food Microbiology*, **30**: 438-447.

Stender, H., Kurtzman, C., Hyldig-Nielsen, J. J., Sørensen, D., Broomer, A., Oliveira, K., Perry-O'Keefe, H., Sage, A., Young, B., Coull, J. 2001. Identification of *Dekkera bruxellensis* (*Brettanomyces*) from Wine by Fluorescence In Situ Hybridization Using Peptide Nucleic Acid Probes. *Applied Environmental Microbiology*, **67**: 938-941.

Sturm, M. E., Arroyo-López, F. N., Garrido-Fernández, A., Querol, A., Mercado, L. A., Ramirez, M. L., Combina, M. 2014. Probabilistic model for the spoilage wine yeast *Dekkera bruxellensis* as a function of pH, ethanol and free SO_2 using time as a dummy variable. *International Journal Food Microbiology,* **170**: 83-90.

Suárez, R., Suárez-Lepe, J. A., Morata, A., Calderón, F. 2007. The production of ethylphenols in wine by yeasts of the genera *Brettanomyces* and *Dekkera*: A review. *Food Chemistry*, **102**:10–21.

Wedral, D., Shewfelt, R., Frank, J. 2010. The challenge of *Brettanomyces* in wine. *LWT Food Science Technology*, **43**, 1474-1479.

Zuehlke, J. M., Petrova, B., Edwards, C. G. 2013. Advances in the control of wine spoilage by *Zygosaccharomyces* and *Dekkera*/*Brettanomyces*. *Annual Review Food Science Technology*, **4** (1). DOI: 10.1146/annurev-food-030212-182533.

CHAPTER 3

Phenolic composition of monovarietal red wines regarding volatile phenols and its precursors

Abstract

The aim of this study was to characterise and compare wines from different grape varieties focusing on the volatile phenols and on the respective precursor compounds, both on the free form (p-coumaric, ferulic and caffeic acids) and as tartaric esters of hydroxycinnamic acids (caftaric, coutaric and fertaric acids). Fifty-eight commercial monovarietal red wines from eight selected grape varieties were used: Cabernet Sauvignon, Syrah, Aragonez, Castelão, Touriga Franca, Touriga Nacional, Trincadeira and Vinhão (Sousão). It was found that volatile phenols precursors exist mostly as esters of tartaric acid, with caftaric acid as the most abundant cinnamate (16.6-111.0 mg/L), followed by coutaric and fertaric acids. The predominant hydroxycinnamic acid was p-coumaric acid, the highest concentrations being found in Syrah and Touriga Franca (6-7 mg/L) and the lowest in Touriga Nacional and Trincadeira (2-3 mg/L). Touriga Nacional exhibits the highest difference between bound and free forms. Malvidin-3-O-(6-p-coumaroyl)-glucoside, a potential source of p-coumaric acid, was found in most of the wines with average values varying between 1 and 5 mg/L. Twenty two % of the wines analysed presented levels of volatile phenols above the perception threshold. Ethylphenols were highest in Vinhão and Trincadeira, showing an average value well above the perception threshold. The concentrations found in Cabernet Sauvignon and Syrah wines were around 10 times lower than those reported in previous works. The results show relevant differences among grape varieties but the availability of the precursors in meaningful amounts may not be the only factor explaining the formation of volatile phenols in wines.

Phenolic composition of monovarietal red wines regarding volatile phenols and its precursors

3.1.Introduction

Volatile phenols (mainly 4-ethylphenol [4EP] and 4-ethylguaiacol [4EG]) are aromatic compounds which, above certain concentrations, affect the quality of the wine imparting aroma defects normally described as "horse sweat", "animal", "leather", “medicinal”, etc (Chatonnet *et al.,* 1995; Fugelsang and Edwards, 2007; Ribéreau-Gayon *et al.,* 2000) . The control of volatile phenols formation is one of the most important microbiological challenges in the modern wine production, since it is responsible for significant economic losses throughout the world (Wedral *et al.,* 2010).

The precursors of volatile phenols are natural constituents of grapes and wines - the hydroxycinnamic acids, p-coumaric and ferulic acids (Heresztyn, 1986; Chatonnet *et al.,*1995). These phenolic acids can exist in wine in the form of tartaric esters (caftaric, coutaric and fertaric acids) but can also be found in the free form or esterified with anthocyanins (Macheix *et al.,*1990) and ethanol (Hixson., 2012). The transformation of these compounds involves two sequential enzymatic reactions. Firstly, the hydroxycinnamic acid is decarboxylated in the corresponding vinyl derivative (4-vinylphenol from p-coumaric acid, or 4-vinylguaiacol from ferulic acid) and in the second reaction, a reductase converts the vinylphenol in the corresponding ethyl compound (4-ethylphenol and 4-ethylguaiacol) (Heresztyn, 1986). The transformation of hydroxycinnamic acids into volatile phenols is predominantly associated with the activity of yeasts from the genus *Brettanomyces* and its sexual form (spore production) classified as *Dekkera* (Heresztyn, 1986; Edlin *et al.,*1995; Chatonnet *et al.,*1997, Loureiro and Malfeito-Ferreira, 2006).

Empirical data obtained by some wine producers indicate that wines made from particular grape varieties are more susceptible to decay due to the production of volatile phenols by *Brettanomyces*/*Dekkera* (Personnal communication).

There are some studies in which the authors tried to find a correlation between volatile phenols and grape varieties. Pöllnitz *et al.,* (2000) compared varietal Australian wines regarding their volatile phenols and found higher values in Cabernet Sauvignon and Merlot than in Syrah and Pinot Noir. This could be related to the relatively high

concentrations of p-coumaric acid found in Cabernet Sauvignon and Merlot as observed by Goldberg *et al.,* (1998). A greater potential for volatile phenols production may arise from higher levels of precursors (ferulic and p–coumaric acids) (Shinohara *et al.,* 2000). There is some evidence that grapevine varieties differ in the concentration of hydroxycinnamic acids, both on the free and bound forms. Nagel and Wulf (1979), reported that the average concentrations of caftaric, p-coutaric, and fertaric acids in Merlot grapes from Washington were 59.2, 16.9, and 3.2 mg/L, respectively. Other grapevine varieties have been identified to have higher amounts of hydroxycinnamic acids, for example wines from Grenache variety contain between 270 and 460 mg/L of caftaric acid (Morel-Salmi *et al.,* 2006). On the other hand, Nikfardjam *et al*., (2009) analyzed the volatile phenols on varietal wines of the Wurttemberg region (Germany) and found no strong correlation between grape varieties and the volatile phenols.

While data is already published about the phenolic content in wines from different regions of the world, there is still a lack of data regarding Portuguese native grape varieties and wines. Thereofore the aim of the present study was to identify and quantify the precursor compounds of volatile phenols in wines from six of the most exploited *V. vinifera* grape varieties in Portugal and two international grape varieties (Cabernet Sauvignon and Syrah) and to relate with the levels of volatile phenols. The ultimate aim of this research line is to evaluate whether certain grape varieties originate, potentially, wines more susceptible to develop volatile phenols.

3.2. Materials and Methods

3.2.1. Wine samples

Fifty eight commercial monovarietal red wines representing eight different grape varieties were used in this study: Aragonez (9), Cabernet Sauvignon (9), Castelão (4), Syrah (6), Touriga Franca (2), Touriga Nacional (17), Trincadeira (5) and Vinhão (Sousão) (6), most of them from wine demarcated regions in Portugal.

3.2.2. Quantification of the precursors of volatile phenols

The wine samples were filtered directly to vials using 0.45 μm syringe filters with polyester membrane (Chromafil Pet – Macherey-Nagel, Germany). The following molecules were purchased to Sigma-Aldrich (Portugal): hydroxycinnamic acids: caffeic acid (C0625) (98% w/w); *p*-coumaric acid (C9008) (98% w/w); ferulic acid (128708) (99% w/w); and hydroxycinnamic esters: *trans*-caftaric acid (15029) (97% w/w).

Phenolics identification was conducted by HPLC-DAD on a Beckman model 126 quaternary solvent system (Beckman Coulter, Fullerton, CA, USA), equipped with a System 32 Karat 5.0 software and a 168 rapid scanning UV-visible photodiode array detector (Beckman Coulter, Fullerton, CA, USA). The absorption spectra were recorded between 212 and 600 nm. A Kromasil (AkzoNobel, Bohus, Sweden) 100-5-C18 HPLC column (with dimensions 250 x 4.6 mm and a particle diameter or 5.0 μm) was used for the chromatographic analysis. The mobile phases used were: Solvent A: acetonitrile (100%) (Merck pure grade) with 0.2% TFA; Solvent B: acetonitrile/water (5:95 v/v) (Merck pure grade and pure water) with 0.2% TFA (Sigma-Aldrich, Germany) at a flow rate of 1 mL/min. The following solvent gradient was employed: 0-1 min (100% B); 1-30 min (100 to 79% B); 30-42 min (79 to 73% B); 42-55 min (73 to 42% B); 55-60 min (42 to 100% B); and 60-61 min (100% B). Hydroxycinnamic acids and their esters were detected at 320 nm wavelength while Malvidin-3-*O*-coumaroylglucoside was detected at 528 nm, using the sme methodology of a previous work (Oliveira *et al.,*2015).

Two independent samples were filtered using 0.45 μm syringe filters with a polyester membrane (Chromafil ®, Germany) and analyzed directly. Table 3.1 represents the identification of the 10 compounds separated on the HPLC chromatographic runs, based on available standards and/or UV-vis spectra of the monovarietal red wines.

The peak assignment for hydroxycinnamic acids and for *trans*-caftaric acid was made on the basis on available standards while for the hydroxycinnamic esters: *cis*-caftaric acid, *cis*-coutaric acid, *trans*-coutaric acid, *trans*-fertaric acid, and *cis*-GRP was made based on their UV-vis spectra as shown in Table 3.1. The results are in agreement with previous reports: maximum at 308 nm for *cis*-caftaric acid, maximum at 312 nm and a shoulder at 286 nm for *cis*-coutaric acid, maximum at 314 nm and a shoulder at 296 nm for *trans*-coutaric acid, maximum at 328 nm and a shoulder at 300 nm for *trans*-fertaric acid and

maximum at 316 nm for *cis*-GRP (Pati *et al.,* 2014; Cejudo-Bastante *et al.,* 2010; Oliveira *et al.,* 2015). The peak assignment for Malvidin-3-*O*-coumaroylglucoside was made on the basis of the UV-vis spectra present in Table 3.1 and available published data (Guerrero *et al.,* 2009; Oliveira *et al.,* 2015). Malvidin-3-*O*-coumaroylglucoside has a maximum at 282 and 534 nm, and a shoulder at 312 nm.

Calibration curves were obtained by the injection of the hydroxycinnamic acids (caffeic acid, *p*-coumaric acid, and ferulic acid) and the hydroxycinnamic ester *trans*-caftaric acid standard solutions, with the corresponding analytical parameters presented in Table 3.2. The hydroxycinnamic esters: *cis*-caftaric acid, *cis*-coutaric acid, *trans*-coutaric acid, *trans*-fertaric acid, and *cis*-GRP were quantified using as equivalent concentrations of the corresponding hydroxycinnamic acid: *cis/trans*-coutaric acid - *p*-coumaric acid; *trans*-fertaric acid - ferulic acid; *cis*-GRP - *trans*-caftaric acid; and for *cis*-caftaric acid the isomer *trans*-caftaric acid was used. Malvidin-3-*O*-coumaroylglucoside was quantified as an equivalent concentration of *p*-coumaric acid.

LC-ESI-UHR-QqTOF-MS analysis was performed to quantify free ferulic acid, on an UltiMate 3000 Dionex UHPLC (Thermo Scientific), coupled to an ultrahigh-resolution Qq-time-of-flight (UHR-QqTOF) mass spectrometer with 50,000 full-sensitivity resolution (FSR) (Impact II, Bruker Daltonics, Bremen, Germany).

An Acclaim RSLC 120 C18 column (Bruker Daltonics, Bremen, Germany) with dimensions 100 x 2.1 mm (particle diameter of 2.2 μm) was used at 35 °C. The mobile phase used was prepared as following: Solvent A - water with 0.1% formic acid (Sigma-Aldrich, Germany) and Solvent B - acetonitrile ("LC-MS grade" from Fischer Chemical) with 0.1% formic acid (Sigma-Aldrich, Germany); the flow rate used was 0.25 mL/min. The following gradient was employed: 0-0.4 min (5% B); 0.4-0.5 min (5 to 20% B); 0.5-1 min (20% B); 1-3 min (30 to 40% B); 3-5 min (40 to 50% B); 5-7 min (50-100% B); 7-12 min (100% B); 12-12.1 min (100-5% B); and 12.1-15 min (5% B). The sample injection volume was 5.0 μL. Parameters for MS analysis were set using negative ionization mode with spectra acquired over a range from m/z 20 to 1000 using the following parameters: capillary voltage, 2.5 kV; drying gas temperature, 200 °C; drying gas flow, 8.0 L/min; nebulizing gas pressure, 2 bar; collision RF, 300 Vpp; transfer time, 120 μs; and prepulse storage, 4 μs.

Post acquisition internal mass calibration used sodium formate clusters with the sodium formate delivered by a syringe pump at the start of each chromatographic analysis (100 μL/h). Ferulic acid was identified by MS mode full scan and confirmed by broadband CID: Observed m/z $[M-H]^-$ = 193.0578; and fragment ions (relative intensity %): 178.0266 (45), and 134.0362 (100).

Table 3.1. Identification of phenolic compounds based on UV-vis spectra

Nº	Retention time (min)	λ_{max1}	λ_{max2}	λ_{max3}	Compound	Identification
1	17.5		308		*cis*-Caftaric acid	[Pati *et al.,* 2014]
2	18.3	(296)	328		*trans*-Caftaric acid	Available standard
3	20.4	(286)	312		*cis*-Coutaric acid	[Pati *et al.,* 2014]
4	21.6	(296)	322		Caffeic acid	Available standard
5	21.9		316		*cis*-*GRP*	[Cejudo-Bastante *et al.,* 2010]
6	22.6	(296)	314		*trans*-Coutaric acid	[Pati *et al.,* 2014, Oliveira *et al.,* 2015]
7	24.0	(300)	328		*trans*-Fertaric acid	[Pati *et al.,* 2014, Oliveira *et al.,* 2015]
8	27.6	(296)	308		*p*-Coumaric acid	Available standard
9	30.3	(278)	320		Ferulic acid	Available standard
10	40.7	282	(312)	534	Malvidin-3-*O*-coumaroylglucoside	[Oliveira *et al.,* 2015, Guerrero *et al.,* 2009]

Table 3.2. Analytical parameters used for the HPLC quantification of *trans*-caftaric, caffeic, *p*-coumaric and ferulic acids.

	Trans-Caftaric acid	Caffeic acid	*p*-Coumaric acid	Ferulic acid
Linear concentration range[a]	0.2-100	0.2-100	0.2-100	0.2-100
Linear regression equation[b]	y = 60453.6x+17508	y = 114701.5x+3874	y = 121501.9x-16087.3	y = 109896.8x-26288.1
R^2	0.995	0.997	0.998	0.999
LOD[a]	0.1	0.05	0.05	0.05
LOQ[a]	0.3	0.2	0.2	0.2

[a] (mg/L); [b] n=7

3.2.3. Quantification of volatile phenols

The volatile phenols analysis was performed according to the protocol described by Bertrand (1981). A 50:50 (v/v) solution of diethyl ether/ n-hexane (Honeywell – EUA) / Carlo Erba Reagents – IT, respectilly) was prepared. A 3-octanol solution at a concentration of 500 mg/L was prepared using methanol (Carlo Erba Reagents – IT) as solvent and was used as the internal standard. A sample volume of 50.00 ml was transferred to a 100.0 mL volumetric flask, and 50 μL of the internal standard solution was added. The samples were successively extracted using the above-described diethyl ether and n-hexane mixture. Three extractions were performed with addition of 4.0 mL, 2.0 mL and 2.0 mL of the extraction solvent mixture. The sample was stirred for 5 min after the addition of the extraction mixture.

The organic phase was collected after three extractions, dehydrated using Na_2SO_4 (Merck) and transferred to a 1.50 mL vial, where it was concentrated under a nitrogen stream prior to the instrumental analysis on the GC-FID chromatograph.

Fifty μL of octan-3-ol at 500 mg/L (internal standard) was added to 50 mL of wine. This mixture was successively extracted with 4, 2 and 2 mL of ether/hexane (1:1) by stirring for 5 min. The organic phases were collected, mixed and concentrated under a stream of nitrogen to approximately half of the initial volume. One microliter of the extract was injected into a Varian 3900 GC-FID (Walnut Creek, CA, USA). The column employed was a FFAP (50 m x 0.25 mm x 0.2 μm) from SGE (Austin, Texas). The injector (split/splitless) was heated to 220 °C with a split flow of 30 ml/min and a splitless time of 0.5 min. The carrier gas flow was adjusted to 1 mL/min. The temperature of the oven was maintained at 4°C for 1 min and was then increased at a rate of 2°C/min up to 220 °C. This temperature was then maintained for 30 min. Calibration curves were obtained by the injection of the volatile phenols standard solutions extracted in the same wine conditions, with the corresponding analytical parameters presented in Table 3.3. The methodology applied was adapted from a previous work (Isa *et al.,*2011). Volatile phenols eluted in the former order: 4-ethylguaiacol (56.6 min); 4-ethylphenol (63.4 min); 4-vinylguaiacol (64.2 min); and 4-vinylphenol (72.6 min).

Table 3.3. Analytical parameters used for the GC-FID quantification of 4-ethylguaiacol, 4-ethylphenol, 4-vinylguaiacol and 4-vinylphenol.

	4-ethylguaiacol	4-ethylphenol	4-vinylguaiacol	4-vinylphenol
Linear concentration range[a]	50-700	50-700	50-700	50-700
Linear regression equation[b]	y = 0.0013x+0.013	y = 0.0021x+0.0146	y = 0.0020x-0.0069	y = 0.0021x-0.0043
R^2	0.998	0.997	0.999	0.997
LOD[a]	20	10	10	10
LOQ[a]	45	30	30	30

[a] (µg/L); [b] n=7

3.2.4. Statistical analysis

Data was analysed according to ANOVA procedures to test significance differences between the concentrations of target components in wine varieties. Multiple pairwise differences of means were posteriorly evaluated using Duncan's test. Normality and homoscedasticity of data was tested using Kolmogorov–Smirnov and Levene's tests, respectively. When these requirements were not verified, non-parametric tests were carried out instead: Kruskal-Wallis test (alternative to one-way ANOVA) and Mann-Whitney (alternative to post-hoc pairwise comparisons). The significance level assumed was 5% in all situations. IBM SPSS Statistics 24 for Windows® (SPSS Inc., Chicago, USA) was used in all data analyses.

3.3. Results

Fifty eight commercial monovarietal red wines representing 8 different grape varieties were used in this study: Aragonez, Castelão, Touriga Franca, Touriga Nacional, Trincadeira and Vinhão (Sousão), which are among the most widely used grape varieties in Portugal, and Carbernet Sauvignon and Syrah, two of the most planted grape varieties in the world. The most relevant phenolic compounds, both precursors and final products, involved on the "Brett character" defect of wines were analysed. Figure 3.1 shows the average values obtained for the precursors of volatile phenols in the free form, the hydroxycinnamic acids p-coumaric and caffeic. The predominant phenolic acid detected was p-coumaric acid, the highest concentrations being found in Syrah and Touriga Franca with average values of 6.3 and 6.7 mg/L, respectively, and the lowest in Touriga Nacional and Trincadeira with average values of 2.4 and 2.3 mg/L, respectively. Statistical significant differences were found between Trincadeira and the richest grape varieties (Touriga Franca and Syrah) and between Syrah and the poorest varieties (Touriga Nacional and Trincadeira) ($p<0.05$). Caffeic acid is present in considerable amounts but showing similar average values among the grape varieties, from 0.52 in Trincadeira to 1.49 mg/L in Vinhão.

A statistical significant difference was only found between Cabernet Sauvignon and Trincadeira ($p<0.05$). Since ferulic acid could not be detected by HPLC-DAD, a LC-MS method was used as described in materials and methods. The concentrations of ferulic acid

were generally lower than caffeic and p-coumaric acids. The highest concentration found was 0.16 mg/L in a Touriga Nacional wine while it has not been detected (below the detection limit: 50 µg/L) on the majority of the wines (95%).

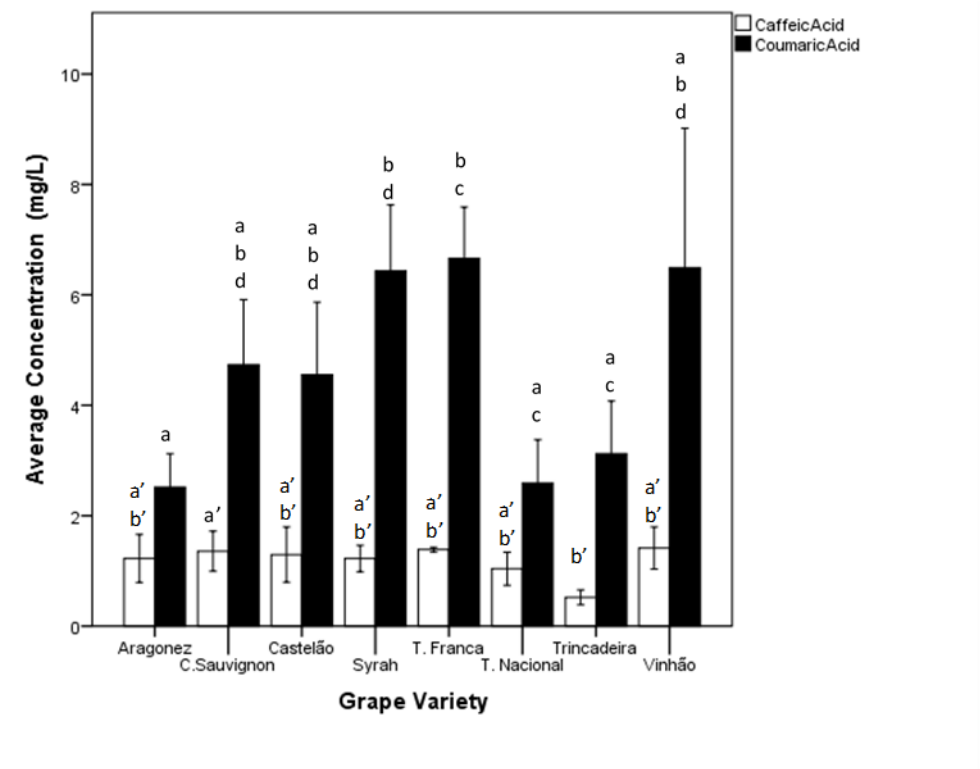


Figure. 3.1. Average values of precursors of volatile phenols (p-coumaric and caffeic acids) in the free form. Error bars indicate standard errors. Different letters indicate statistically significant differences between grape varieties

Figure 3.2 shows the average values obtained for the tartaric esters of hydroxycinnamic acids (caftaric, coutaric and fertaric acids). Data correspond to the total *cis* + *trans* isomers of caftaric and coutaric acids and *trans* isomer of fertaric acid. Caftaric acid was found to be the predominant hydroxycinnamic acid tartaric ester in all grape varieties except for Touriga Franca. Touriga Nacional showed the highest average value (*trans*+*cis*: 106.2+4.8 mg/L), significantly different from the other varieties except for Syrah and Touriga Franca ($p<0.05$). The lowest level was for Trincadeira (*trans*+*cis*: 11.7+4.8 mg/L), significantly different from Aragonez, Syrah, Touriga Nacional and Vinhão ($p<0.05$). For the coutaric acid, the

highest concentration was found in Touriga Franca (*trans*: 22.6 mg/L), but only significantly different from Cabernet Sauvignon ($p<0.05$). The lowest level was for Trincadeira (*trans*+*cis*: 3.49+3.40 mg/L), significantly different from Aragonez, Syrah, Touriga Nacional and Vinhão ($p<0.05$). Touriga Franca and Touriga Nacional exhibit the highest levels of fertaric acid (*trans*: 2.9 and 2.8 mg/L, respectively), but only significantly different from Syrah and Trincadeira ($p<0.05$).

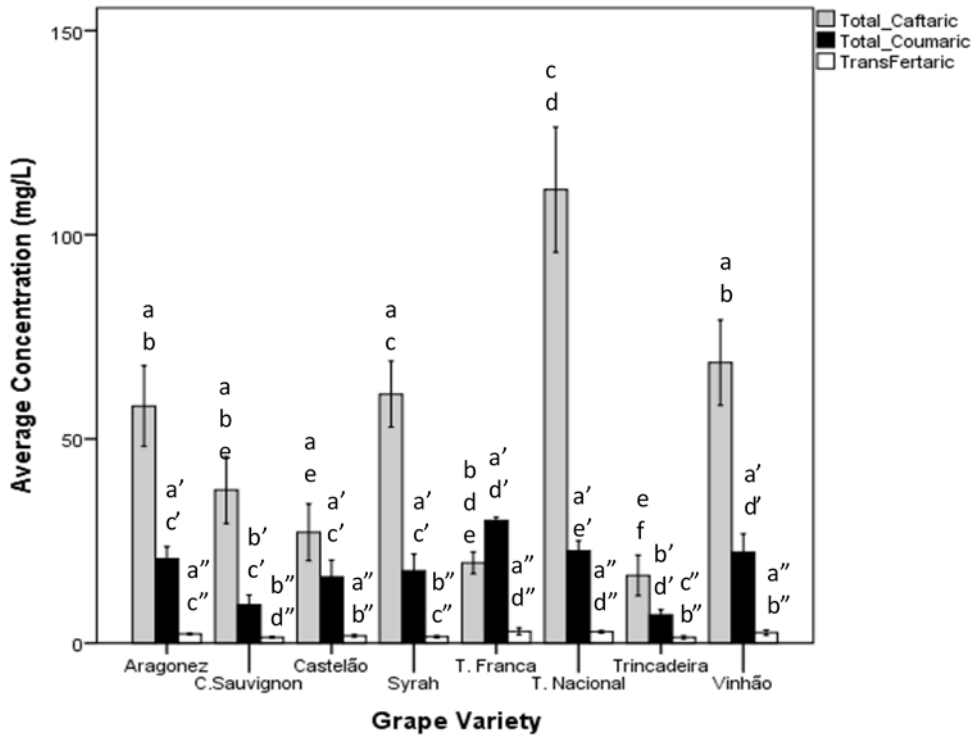


Figure 3.2. Average values of tartaric esters of hydroxycinnamic acids (caftaric, coutaric and fertaric acids). Error bars indicate standard errors. Different letters indicate statistically significant differences between grape varieties.

Table 3.4 shows the bound/free molar ratios of the precursors of volatile phenols. The values reflect the predominance of the bound forms, as esters of tartaric acid, over the free forms.

Table 3.4 - Bound/free molar ratios of the precursors of volatile phenols calculated from the mean values

Grape variety	Caftaric/Caffeic acids	Coutaric/p-coumaric acids
Aragonez	27.34	4.61
Cabernet Sauvignon	15.95	1.15
Castelão	12.11	2.11
Syrah	28.71	1.55
Touriga Franca	8.18	2.50
Touriga Nacional	61.68	5.33
Trincadeira	18.36	1.58
Vinhão	20.03	2.33

Clearly, the difference between bound and free forms is higher for the pair caftaric/caffeic acids than for coutaric/p-coumaric acids. Touriga Nacional exhibits the highest difference between bound and free forms, the esterified form being 5.33 and 61.68 times higher than the free hydroxycinnamic acids precursor for p-coumaric acid and caffeic acid, respectively.

We also analysed another form of bound hydroxycinnamic acids, the malvidin-3-O-(6-p-coumaroyl)-glucoside which varied from undetected to 13.5 mg/L. The highest average values were found in Touriga Nacional, Touriga Franca and Aragonez, but significant differences were only found between Touriga Nacional and the varieties that presented the lowest values (Cabernet Sauvignon and Syrah) ($p<0.05$) (Figure 3.3).

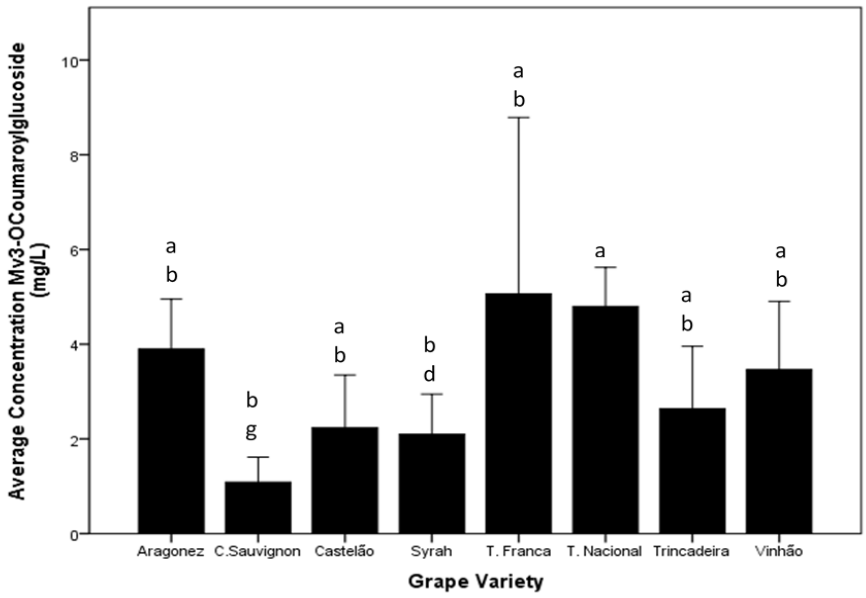


Figure. 3.3 Average values of malvidin-3-O-(6-p-coumaroyl) glucoside. Error bars indicate standard errors. Different letters indicate statistically significant differences between grape varieties

The wines were also characterised regarding the volatile phenols. Values of 4-ethyphenol varied from undetected to a maximum of 3950 µg/L in a Vinhão wine. The highest concentration of 4-ethylguaicol (445 µg/L) was found in the same Vinhão wine. The levels of 4-vinylguaiacol and 4-vinylphenol were much lower than the 4-ethyl compounds, not exceeding 90 µg/L.

Figure 3.4 shows the average values found in the different grape varieties. Ethylphenols clearly predominate over vinylphenols, due to the significant contribution of 4-ethyphenol. This volatile compound stands out in Vinhão and Trincadeira in comparison with the other grape varieties, showing an average value well above the perception.

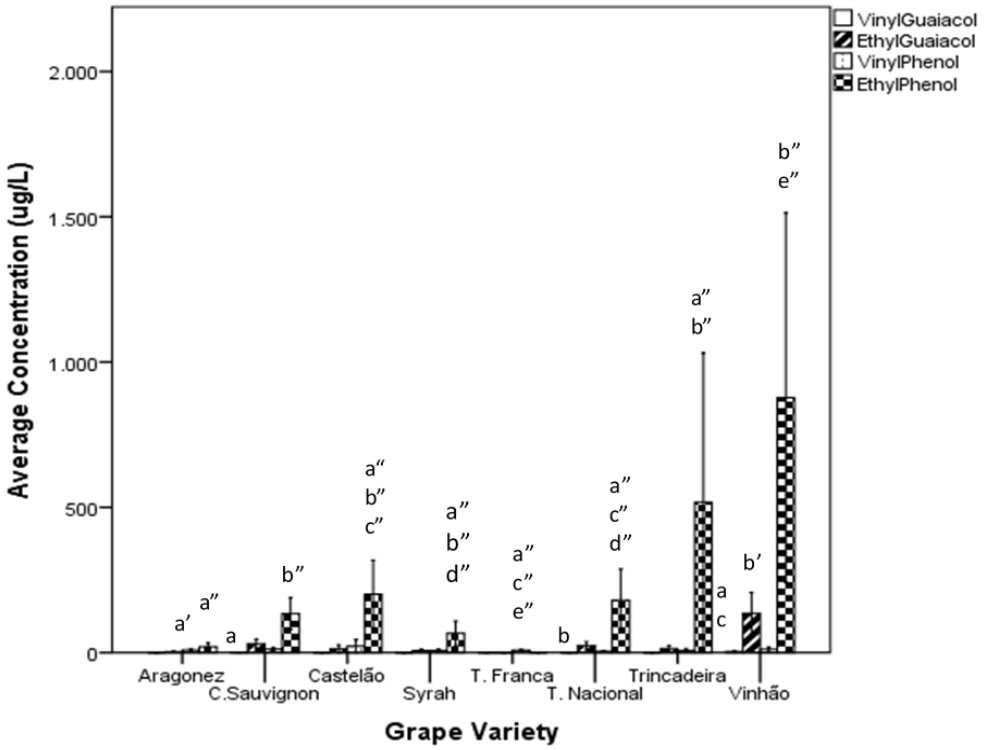


Figure 3.4. Average values of volatile phenols. Error bars indicate standard errors. Different letters indicate statistically significant differences between grape varieties.

Figure 3.5 shows the estimated initial concentrations of (total) p-coumaric of each grape variety calculated by adding the equivalent mass concentration of p-coumaric acid necessary to produce the concentrations of 4-vinylphenol and 4-ethylphenol (assuming a 1:1 molar stoichiometry of the conversion) observed in the wines to the measured p-coumaric acid values, thus generating the possible profile of wines before the precursor was metabolized. In comparison to Figure 3.1, the estimated initial concentration of p–coumaric acid increased for Vinhão and Trincadeira.

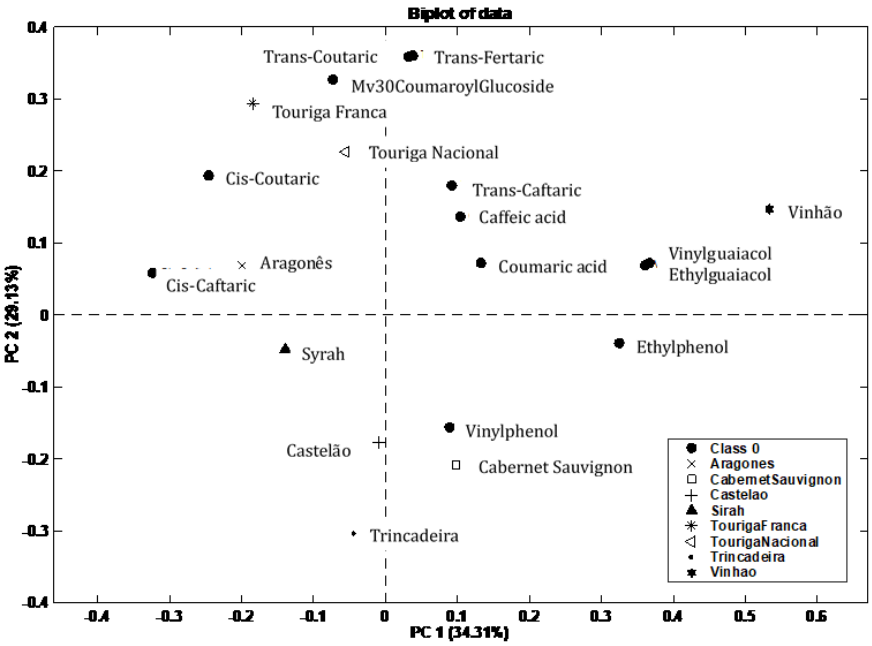


Figure. 3.5. PCA analysis for phenolic compounds and grape varieties

3.4. Discussion

The results of the precursors of volatile phenols in the free form, the hydroxycinnamic acids p-coumaric ferulic and caffeic, are not in accordance with studies performed on Canadian, Greek and Hungarian wines that found caffeic acid as the predominant phenolic acid (4.5 – 30 mg/L) while p-coumaric was present at lower concentrations (3.0 – 9.5 mg/L) (Soleas *et al.,* 1996, Kallithraka *et al.,* 2006, Avar *et al.,* 2007). It is known that caffeic acid is not as easily metabolised by *Brettanomyces/Dekkera* as the other hydroxycinnamic acids. The conversion rates of caffeic acid into 4-ethylcatechol found by Cabrita *et al.,* (2012) in a synthetic medium and red wine was smaller than 20%, while rates above 90% were obtained for the conversion of p-coumaric and ferulic acids into 4-ethylphenol and 4-ethylguaicol, respectively. Thus, our study shows that, given the low concentrations of ferulic acid and considering the low microbial conversion rates of caffeic acid, the production of volatile phenols in these wines would mostly depend on the presence of p-coumaric acid.

As for the tartaric esters of hydroxycinnamic acids (caftaric, coutaric and fertaric acids), the *trans* isomers clearly predominate over the *cis* isomers, probably due to the higher stability of the *trans* forms (Rentzsch *et al.,* 2009). Singleton *et al*., (1978) assumed that the *trans* configuration of caftaric and coutaric acids is a natural phenomenon whereas the *cis* form is the product of UV-induced isomerisation. Caftaric acid was found to be the predominant hydroxycinnamic acid tartaric ester in all grape varieties except for Touriga Franca, which is in accordance to what has been previously reported for *Vitis vinifera* grapes. Hydroxycinnamic acids exist mostly as esters of tartaric acid, with *trans*-caftaric acid as the most abundant cinnamate, followed by *trans*-coutaric acid and fertaric acid (Waterhouse, 2002). Ginjom *et al.,* (2011) also found caftaric and coutaric acids as the major hydroxycinnamic acids in Cabernet and Syrah wines. Nagel and Wulf (1979) detected caftaric acid (average concentration of 59.2 mg/L) as the predominant tartaric ester of hydroxycinnamic acids in Merlot wines, followed by coutaric and fertaric acids (16.9 and 3.2 mg/L, respectively).

The calculation of the bound/free molar ratios of the precursors of volatile phenols shows a predominance of the bound forms in all wines. Touriga Nacional exhibits the highest difference between bound and free forms, while other grape varieties such as Cabernet Sauvignon, Castelão and Trincadeira exhibit lower differences, which is more in accordance to Ginjom *et al.,* (2011) who found ratios of coutaric/p-coumaric acids to vary from 0.6 to 2.1.

The colour of red wines is largely attributed to the presence of anthocyanins, occurring as glycosides in which specific hydroxyl functions combine with sugar residues. Malvidin-3-O-g1ucoside is the major anthocyanin in red wines representing usually 40% or more and it apparently makes up the majority of those red pigments that are acylated with acetic, caffeic or p-coumaric acid (Zoecklein *et al.,* 1995). The level of malvidin-3-O-g1ucoside found in a young Cabernet Sauvignon was 144.6 mg/L, the acylated form often being a smaller fraction (Bolton *et al.,* 1996). Arnous and Meyer (2010) found that p-coumaric acid was released during the enzyme catalyzed degradation of acylated anthocyanins, most probably as a result of cinnamate esterase activity. This is the result of a sequential hydrolysis of malvidin-3-(6-p-coumaroyl)-D-glucose by cinnamate esterase and then by beta-glucosidases yielding the p-coumaric acid, as a first step, and then glucose and the aglycone form of anthocyanins. These findings indicate that malvidin-3-O-(6-p-

coumaroyl)-glucoside may be a source of free p-coumaric acid, hence increasing the concentration of an important volatile phenol precursor. The conditions and factors that influence the release of p-coumaric acid from malvidin-3-O-(6-p-coumaroyl)-glucoside are not yet fully known. This is, to the best of our knowledge, the first time that a p-coumaroylated anthocyanin is discussed as a potential precursor (bound form) of volatile phenols.

The results of volatile phenols obtained for Vinhão are in accordance with the empirical evidence that wines from this grape variety show a consistent high susceptibility to develop volatile phenols (personal communication from winemakers). A similar judgment about Touriga Nacional was not confirmed by our results, but further studies with more wine samples would be desirable for a final conclusion. The average values of 4-ethyphenol found for Cabernet Sauvignon and Syrah wines (134 and 66 µg/L, respectively) were around 10 times lower than those found by Pollnitz *et al.,* (2000) (1250 and 605 µg/L, respectively). Twenty-two % of the wines analysed had volatile phenols in concentrations above the perception threshold in red wines (4-ethylguaicol+4-ethyphenol: 41+328 µg/L; Chatonnet *et al.,* 1992). The ratio 4-ethylphenol/4-ethylguaicol was calculated in these wines and it was found to vary greatly, from 1.5:1 to 47:1. Even within the same grape variety the variation was found to be high, e.g. Vinhão which ranged from 1.5:1 to 9:1. 4-ethylguaicol was not detected in a Castelão wine with 414 µg/L of 4-ethyphenol, which is quite unusual. Although these variations are not yet fully understood, they might be related with different concentrations of the volatile phenols precursors in wines and also to metabolic strain variability of *Brettanomyces/Dekkera*. The ratio of 4-ethylphenol to 4-ethylguaiacol found by Pollnitz *et al.,* (2000) also varied from wine to wine. The average ratio was approximately 10:1 for Cabernet Sauvignon, 9:1 for Syrah, 8:1 for Merlot and 3.5:1 for Pinot Noir. Our results showed a ratio of approximately 5:1 for Cabernet Sauvignon and 10:1 for Syrah. Castelão and Trincadeira stood out for high ratios, 15:1 and 37:1 respectively.

Assuming the initial availability of the precursors (p-coumaric acid in particular) as the main factor influencing the production of volatile phenols in wines, the grape varieties Syrah, Touriga Franca and Vinhão should have a higher potential for the development of volatile phenols than the other grape varieties. This is confirmed for Vinhão but not for Syrah, suggesting that the availability of the precursors in meaningful amounts may not be the only factor explaining the formation of volatile phenols in wines. Factors such as the

exposition to microbial contamination, *Brettanomyces/Dekkera* survival capacity, physiochemical properties of the wines and storage conditions may also play an important role.

Further studies are needed to describe correctly the correlation between the level of precursors and the production of volatile phenols in wines and the relationship with the different grape varieties, considering the diversity of existing precursors and the complexity of factors involved.

3.5. References

Arnous, A., Meyer, A. S. 2001. Discriminated release of phenolic substances from red wine grape skins (*Vitis vinifera* L.) by multicomponent enzymes treatment. *Biochem Eng Journal* **49**: 68–77.

Avar, P., Nikfardjam, M. S. P., Kunsági-Máté, S., Montskó, G., Szabó, Z., Böddi, K., Ohmacht, R., Márk, L. 2007. Investigation of phenolic components of Hungarian wines. *International Journal Molecular Science,* **8** (10): 1028-1038.

Boulton, R. B., Singleton, V. L., Bisson, L. F., Kunkee, R. E. 1996. Principles and Practices of Winemaking. Chapman & Hall, New York.

Cabrita, M. J, Torres, M., Palma, V., Alves, E., Patão, R., Costa Freitas, A. M. 2008. Impact of malolactic fermentation on low molecular weight phenolic compounds. Talanta **74**:1281-1286.

Cejudo-Bastante, M. J., Pérez-Coello, M. S., Hermosín-Gutiérrez, I. 2010. Identification of new derivatives of 2-S-glutathionylcaftaric acid in aged white wines by HPLC DAD-ESI-MS. *Journal Agriculture Food Chemistry*, **58**: 11483-11492.

Chatonnet, P., Viala, C., Dubourdieu, D. 1997. Influence of polyphenolic components of red wines on the microbial synthesis of volatile phenols. *American Journal Enology and Viticulture,* **48**:443–448.

Chatonnet, P., Dubourdieu, D., Boidron, J. N. 1995. The influence of *Brettanomyces/Dekkera* sp. yeasts and lactic acid bacteria on the ethylphenol content of red wines. *American Journal Enology and Viticulture,* **46**: 463–468.

Chatonnet, P., Dubourdieu, D., Boidron, J. N., Pons, M. 1992. The origin of ethylphenols in wines. *Journal Science Food Agriculture,* **60**: 165−178.

Edlin, D., Narbad, A., Dickinson, J. R., Lloyd, D. 1995. The biotransformation of simple phenolic compounds by *Brettanomyces anomalus*. *FEMS Microbiology Letters*, **125**: 311−315.

Fugelsang, K., Edwards, C. 2007. Wine Microbiology, Practical Applications and Procedures, second ed. The Chapman & Hall Enology Library, UK

Ginjom, I., D'Arcy, B., Caffin, N., Gidley, M. 2011. Phenolic compound profiles in selected Queensland red wines at all stages of the wine-making process. *Food Chemistry*, **125**: 823–834.

Goldberg, D. M., Tsang, E., Karumanchiri, A., Soleas, G. J. 1998. Quercetin and *p*-Coumaric Acid Concentrations in Commercial Wines. *American Journal Enology Viticulture*, **49**: 142-151.

Guerrero, R. F., Liazid, A., Palma, M., Puertas, B., González-Barrio, R., Gil-Izquierdo, A., García-Barroso, C., Cantos-Villar, E. 2009. Phenolic characterisation of red grapes autochthonous to Andalusia. *Food Chemistry*, **112**: 949-955.

Heresztyn, T. 1986. Metabolism of volatile phenolic compounds from hydroxycinnamic acids by *Brettanomyces* yeast. *Archives Microbiology*, **146**: 96–98..

Hixson, J. L., Sleep, N. R., Capone, D. L. 2012. Hydroxycinnamic acid ethyl esters as precursors to ethylphenols in wine. *Journal Agriculture Food Chemistry*, **60**: 2293–2298.

Kallithraka, S. E., Tsoutsouras, E., Tzourou, E., Lanaridis, P. (2006). Principal phenolic compounds in Greek red wines. *Food Chemistry,* **99**: 784–793.

Loureiro, V., Malfeito-Ferreira, M. 2006. Spoilage yeasts: *Dekkera/ Brettanomyces* spp. In: Blackburn, C. (Ed.). Food spoilage microorganisms. Woodhead Publishing Limited: Cambridge, p. 354-398.

Macheix, J. J., Fleuriet, A., Billot, J. 1990. Fruit phenolics. CRC Press, Boca Raton, F.L.

Morel-Salmi C, Souquet J-M, Bes M., Cheynier, V. 2006. Effect of Flash Release Treatment on Phenolic Extraction and Wine Composition. *Journal Agriculture Food Chemistry*, **54** (12): 4270–4276.

Nagel, C.W., Wulf, L. W. 1979. Changes in the anthocyanins, flavonoids and hydroxycinnamic acid esters during fermentation and aging of Merlot and Cabernet Sauvignon. *American Journal Enology and Viticulture*, **30**: 111–116.

Nikfardjam, M. S.P., May B., Tschiersch, C. 2009. 4-Ethylphenol and 4-ethylguaiacol contents in bottled wines from the German 'Württemberg' region. *European Food Research Technology*, **230** (2): 333-341.

Oliveira, C. M, Barros, A. S, Silva Ferreira, A. C, Silva, A. M. S. 2015. Influence of the temperature and oxygen exposure in red Port wine: A kinetic approach. *Food Research International*, **75**: 337-347.

Pati, S., Crupi, P., Benucci, I., Antonacci, D., Di Luccia, A., Esti, M. 2014. HPLC-DAD–MS/MS characterization of phenolic compounds in white wine stored without added sulfite. *Food Research International*, **66**: 207-215.

Pollnitz, A. P., Pardon, K. H., Sefton, M. A. 2000. Quantitative analysis of 4-ethylphenol and 4-ethylguaiacol in red wine. *Journal of Chromatography A*, **874**: 101–109.

Rentzsch, M., Wilkens, A., Winterhalter, P. 2009. Non-flavonoid phenolic compounds. In: Moreno-Arribas MV, Polo C (eds) Wine chemistry and biochemistry. Springer, New York, pp 509–527.

Ribéreu-Gayon, P., Dubourdieu, D., Donéche, B., Lonvaud, A. 2000. Handbook of Enology Volume II, The Chemistry of Wine and Stabilization and Treatments, second ed. John Wiley & Sons Ltd, UK.

Shinohara, T., Kubodera, S., Yanagida, F. 2000. Distribution of phenolic yeasts and production of phenolic off-flavors in wine fermentation. *Journal Bioscience Bioengineering*, **90**: 90–97.

Singleton, V. L., Timberlake, C. F., Lea, A. G. 1978. The phenolic cinnamates of white grapes and wines. *Journal Science Food Agriculture*, **29**: 403–410.

Soleas, G. J., Dam, J., Carey, M., Goldeberg, D. M. 1997. Toward the Fingerprinting of Wines: Cultivar-Related Patterns of Polyphenolic Constituents in Ontario Wines, *Journal Agriculture Food Chemistry*, **45**: 3871−3880.

Waterhouse, A. L. 2002. Wine phenolics. In D. K. Das (Ed.), &F. Ursini, Alcohol and Wine in Health and Disease, vol. 957 (pp. 21–36). New York: Annals of the New York Academy of Sciences.

Wedral, D., Shewfelt, R., Frank, J. 2010. The challenge of *Brettanomyces* in wine. *LWT-Food Science Technology*, **43**:1474–1479.

Zoecklein, B.W, Fugelsang, K. C, Gump, B. H, Nury, S. F. 1995. Wine Analysis and Production Springer US.

CHAPTER 4

Survival and metabolism of hydroxycinnamic acids by *Dekkera bruxellensis* in monovarietal wines

Abstract

The aim of this work was to evaluate the survival of *Dekkera bruxellensis* in monovarietal wines and its metabolism of hydroxycinnamic acids. Fifteen wines, five from each of the grape varieties Touriga Nacional, Cabernet Sauvignon and Syrah, with pH and ethanol concentration adjusted to 3.8 and 13% (v/v), were inoculated with a strain of *D. bruxellensis*. Yeast growth and survival were monitored by viable counting in solid culture medium and by flow cytometry using the fluorescent dyes propidium iodide and Syto™ 9. Yeast culturable populations of 10^7 CFU/mL were dramatically reduced to undetectable numbers (<$3x10^3$ CFU/mL) in 24 h in all wines as revealed by the plate count method. Plate viable counts of 10^4-10^6 CFU/mL were, however, detected after 48 h in most of the Touriga Nacional and Cabernet Sauvignon wines. Plate counts of around 10^6 CFU/mL were also found in the Syrah wines but later than in the other grape varieties. Viability measurement by flow cytometry showed, however, the presence of viable cells at levels between 10^5 and 10^7 cells/mL in populations not detected by the plate count method, suggesting that a significant part of the populations was in a viable-but-not-culturable state (VBNC). Yeasts were able to attain the culturable state in most of the wines but the time required for the recovery was highly dependent on the wine, being longer on the Syrah wines. Since the molecular SO_2 concentration was lower than 0.8 mg/L, the results suggest that the VBNC physiological state might has been triggered by other wine stress factors. Cells were found to be metabolically active (production of volatile phenols), but the results suggest that the VNBC state favors the accumulation of vinylphenols rather than ethylphenols, independently of the grape variety. The concentration of ethylphenols in the Touriga Nacional wines was more than twice the levels found in the other grape varieties. The flow cytometry methodology also showed a higher survival capacity of *D. bruxellensis* in Touriga Nacional wines than in the other grape varieties. The influence of the phenolic composition, specifically the bound/free molar ratios of hydroxycinnamic acids, on the susceptibility of wines to *Brettanomyces*/*Dekkera* spoilage is discussed.

Survival and metabolism of hydroxycinnamic acids by *Dekkera bruxellensis* in monovarietal wines

4.1 Introduction

The production of volatile phenols in wines and the consequent perception of unpleasant aromas described as "horse sweat", "animal", "leather" and “medicinal”, greatly affects the wine quality (Chatonnet *et al.,*1995; Fugelsang and Edwards, 2007; Ribéreau-Gayon *et al.,* 2000). The precursors of volatile phenols are natural constituents of grapes and wines - the hydroxycinnamic acids, p-coumaric and ferulic acids (Heresztyn, 1986; Chatonnet *et al.,*1995). The transformation of hydroxycinnamic acids into volatile phenols is predominantly associated with the activity of yeasts from the genus *Brettanomyces* and its sexual form (teleomorph) classified as *Dekkera* (Edlin *et al.,*1995; Chatonnet *et al.,* 1997, Loureiro and Malfeito-Ferreira, 2006; Kheir *et al.,* 2013; Di Toro *et al.,* 2015). In fact, these microorganisms are considered the major cause of microbial wine spoilage worldwide leading to significant economic losses to the wine industry (Wedral *et al.,* 2010). The transformation of hydroxycinnamic acids involves two sequential enzymatic reactions. Firstly, the hydroxycinnamic acid is decarboxylated in the corresponding vinyl derivative (4-vinylphenol from p-coumaric acid or 4-vinylguaiacol from ferulic acid) and then a reductase converts the vinylphenol in the corresponding ethyl compound (4-ethylphenol and 4-ethylguaiacol) (Heresztyn, 1986).

Monitoring and controlling *Brettanomyces/Dekkera* remains a problem for most wine cellars. Although a large number of culture-based techniques are currently available for assessing the presence of this undesirable spoilage yeast during the winemaking processes, *Brettanomyces/Dekkera* is not detected in numerous cases. However, volatile phenols are found in these wines later on, which could be explained by the ability of *Brettanomyces/Dekkera* to enter a viable but nonculturable (VBNC) state (Agnolucci *et al.,* 2010; Divol *et al.,* 2012, Chandra *et al.,* 2016). Plating methods detect only cells capable of forming colonies under the conditions of the medium, ignoring the presence of stressed non-colonizing living cells, which may be metabolically active or not.

The laboratory culture media rarely resemble natural environmental conditions, so the traditional plating results may not reflect the reality. Despite the VNBC state on eukaryotic cells has received much less attention than on bacteria, the ability of *Brettanomyces* to enter such a state has been previously demonstrated (Agnolucci *et al.,* 2010, Serpaggi *et al.,* 2012, Agnolucci *et al.,* 2014; Capozzi *et al.,* 2016). This may explain the poor correlation often observed between the concentration of volatile phenols and the quantification of *Brettanomyces* biomass (Wedral *et al.,* 2010). Wines giving a negative result for the analysis of *Brettanomyces* by culture-based methods, nonetheless developed unpleasant aromas associated with the "Brett character" (Agnolucci *et al.,* 2010). The induction of the VBNC state on *Brettanomyces* has been often associated with the presence of SO_2, an antimicrobial agent commonly used in food preservation (Agnolucci *et al.,* 2010, Divol *et al.,* 2012; Serpaggi *et al.,* 2012, Agnolucci *et al.,* 2014). But other chemical and environmental factors have been reported as inducers, including nutrient starvation, extreme temperatures, osmotic pressure and oxygen, in other microorganisms (Oliver, 2010).

Flow cytometry has been used as a technique to quantify microorganisms in foods, including wine, becoming a valuable tool to the food microbiology field (Herrero *et al.,*2006; Comas-Riu and Rius, 2010; Serpaggi *et al.,* 2010). As cells differ in their metabolic or physiological states, this technique allows not only to detect a particular cell type but also to find different subpopulations according to their structural or physiological parameters. The shape and size of the organisms are determined by scattering of light while the measurement with fluorescent light provides information on cell viability (Jepras *et al.,* 1995; Shapiro, 1995; Caron *et al.,* 1998). Flow cytometry allows the distinction between viable, metabolically active and dead cells, which is of great importance in food development and food spoilage. More recently, flow cytometry has been applied in the wine industry to quantify wine yeast and bacteria viability (Comas-Riu and Rius; 2009). Fluorescent dyes can be used in association with flow cytometry. SYTO 9™ (Invitrogen Molecular Probes, Eugene, OR) and propidium iodide have been mostly used for bacterial analysis, however Zhang and Fang (2004) demonstrated the applicability of these nucleic acid dyes for the determination of yeast cell viability. SYTO 9™ stains all cells regardless of their viability whereas propidium iodide stains non-viable cells with damaged membrane integrity.

When a mixture of the two dyes is used, propidium iodide competes with SYTO 9™ for nucleic acid binding sites in non-viable cells. Propidium iodide was also used by Quirós *et al.,* (2007) in association with flow cytometry to assess the physiological states of *Saccharomyces cerevisiae* and to develop kinetic models in pure culture fermentations experiments. Malacrinó *et al.,* (2001) used flow cytometry for the rapid detection of viable wine bacteria (*Oenococcus oeni*) and yeasts (*S. cerevisiae* and *S. bayanus*).

Since there is evidence that grapevine varieties differ in the composition of phenolic compounds, specifically of hydroxycinnamic acids, both on the free and bound forms (Avar *et al.,* 2007; Ginjom *et al.,* 2011; Lima *et al.,* 2018), the aim of this work was to evaluate the survival and activity (production of volatile phenols) of *Dekkera bruxellensis* in monovarietal wines and to estimate the influence of the grape variety.

4.2. Materials and Methods

4.2.1. Wine samples

Fifteen commercial monovarietal red wines, five of each of the grape varieties Touriga Nacional (from wine demarcated regions in Portugal), Cabernet Sauvignon and Syrah (international wines) were used. The pH of all wines was adjusted to 3.8 with a 1 M NaOH solution. The ethanol concentration was adjusted to 13% (v/v) by diluting the wines with distilled water. The wines were pasteurized (50°C, 5 min) before inoculation. The pasteurization regime was chosen in accordance with Couto *et al.,* (2005a), who have previously established time and temperature parameters for the thermal inactivation of yeasts in wine, specifically *Brettanomyces*/*Dekkera*.

4.2.2. Strains and culture conditions

One strain of *Brettanomyces/Dekkera* selected from a group of 28 strains isolated from naturally contaminated wines was used in this study. Strains were isolated using a selective enrichment broth to differentially promote the growth and activity of *Brettanomyces/Dekkera* (Couto *et al.,* 2005b) and a solid medium - DBDM (*Dekkera/Brettanomyces* Differential Medium) (Rodrigues *et al.,* 2001). Both mediums

contain chloramphenicol and cycloheximide to eliminate bacteria and non-*Brettanomyces/Dekkera* yeasts, respectively. The identification of the strains was confirmed and further fulfilled to species level by PCR amplification of fungal ITS1 and ITS2 rDNA gene regions using the primers ITS1F (forward) (5'- CTT GGT CAT TTA GAG GAA GTA A -3') and ITS4 (reverse) (5'- TCC TCC GCT TAT TGA TAT GC -3'). PCR products were subjected to DNA sequencing (Macrogen, Korea) and the resulting sequences were analyzed using BLASTN software and GenBank NCBI reference sequences. All strains were identified as *Dekkera bruxellensis*. The resistance of these strains to ethanol was characterized (data not shown) and the strain used in this work belongs to the group of the most resistant. For long-term storage, strains were mantained at -80 °C in Yeast Mold (YM) broth (Difco, Detroit, USA) with glycerol 20% (v/v).

For the preparation of the inoculum, yeasts were grown on 300 mL of YM broth with 5% (v/v) ethanol, at a temperature of 25° C without agitation, to late exponential phase (4 days). The culture was centrifuged at 8000 rpm for 10 min and the pellet was resuspended in 3 mL of sterile distilled water. 300 mL of each wine in 500 mL flasks were inoculated to an initial inoculum of approximately $1x10^7$ CFU/mL. After inoculation the wines were incubated at 25 °C for a period of 18 days. The experiment was performed in duplicate, to obtain an independent reproduction of the experimental conditions.

4.2.3. Plate counting

Samples were taken at regular intervals during the incubation period of 18 days and serially diluted from 10^{-1} to 10^{-6} (decimal dilutions in sterile 9 ml Ringer solution [Lab M, Bury, UK]). The diluted samples were plated in duplicate on YM agar (YM broth with 2% agar [Lab M, Bury, UK]), using the drop count technique (Miles and Misra, 1938) for the determination of colony forming units per mL (CFU/mL). Counts were done after the incubation of YM plates at 25° C for 72 h.

4.2.4. Flow Cytometry

Yeast growth and survival were monitored by flow cytometry using the fluorescent dyes propidium iodide and SYTO 9™ for discrimination of live and dead cells. Aliquots of 1 mL of wine were collected at three sampling times (144, 288 and 432 hours) and centrifuged at 8000 g for 3 min. The supernatant was discarded and the pellet was suspended in sterile Ringer solution. The washing procedure was repeated and the cells were finally suspended in 0.5 mL of Ringer solution. SYTO 9™ and propidium iodide dyes were obtained commercially from Invitrogen Molecular Probes (Eugene, Oregon, USA). Equal volumes of the two stains were combined in a microfuge tube and 0.5 µL of this solution was added to 500 µL of the yeast suspensions (the concentration of dyes for cell staining was 1.7 and 10 µmol/L for SYTO 9™ and propidium iodide, respectively). These were mixed thoroughly and incubated at room temperature in the dark for 15 min as recommended by the manufacturer.

Flow cytometry analyses of stained samples were carried out in a BD Accuri C6 (Becton Dickinson, San José, CA), equipped with 2 lasers, blue (488 nm) and red (635 nm), with Setup default, 3 detectors blue and 1 detector red. The yeast populations were detected on the basis of their intrinsic properties of light scattering (signals corresponding to forward and side scatter, FSC and SSC). Sample acquisition was done at a flow rate setting of 12 µl/min. A total of 10 000 events was acquired for each sample. Threshold levels were set to eliminate noise or particles smaller than intact cells. Data analysis was performed using the BD Accuri C6 Software version 1.0.264.21.

4.2.5. Quantification of volatile phenols

Samples of 50 mL were transferred to 100 mL volumetric flasks and 50 µL of 3-octanol at 500 mg/L prepared in methanol (Carlo Erba Reagents – IT) was added as internal standard. The samples were successively extracted using a 50:50 (v/v) solution of diethyl ether/n-hexane (Honeywell – EUA)/Carlo Erba Reagents – IT, respectively).

Three extractions were performed with addition of 4, 2 and 2 mL of the extraction solvent mixture and stirring for 5 min. The organic phase was collected after each extraction, dehydrated using Na_2SO_4 (Merck) and transferred to a 1.5 mL vial where it was concentrated

under a nitrogen stream prior to analysis on the GC-FID chromatograph. One µL of the extract was injected into a Varian 3900 GC-FID (Walnut Creek, CA, USA). The column employed was a FFAP (50 m x 0.25 mm x 0.2 µm) from SGE (Austin, Texas). The injector (split/splitless) was heated to 220 °C with a split flow of 30 mL/min and a splitless time of 0.5 min. The carrier gas flow was adjusted to 1 mL/min. The temperature of the oven was maintained at 4 °C for 1 min and was then increased at a rate of 2 °C/min up to 220 °C. This temperature was then maintained for 30 min. Calibration curves were obtained by the injection of the volatile phenols standard solutions extracted in the same wine conditions. Volatile phenols, obtained from Sigma-Aldrich (Steinheim, Germany), eluted in the order: 4-ethylguaiacol (56.6 min); 4-ethylphenol (63.4 min); 4-vinylguaiacol (64.2 min) and 4-vinylphenol (72.6 min).

4.2.6. Statistical analysis

Data was analysed according to ANOVA procedures to test for significant differences between wine varieties. Multiple pairwise differences of means were subsequently evaluated using Duncan's test. Normality and homoscedasticity of data was tested using Shapiro Wilk and Levene's tests, respectively. When these requirements were not verified, non-parametric tests were carried out instead: Kruskal-Wallis test (alternative to one-way ANOVA) and Mann-Whitney (alternative to post-hoc pairwise comparisons). The significance level assumed was 5% in all situations. IBM SPSS Statistics 24 for Windows® (SPSS Inc., Chicago, USA) was used in all data analyses.

4.3. Results and discussion

Wines from 3 grape varieties were inoculated with a strain of *Dekkera bruxellensis* at an initial population density of approximately 10^7 cells/mL. Yeast growth and survival were monitored by viable counting in solid medium (YM medium) and by flow cytometry using the fluorescent dyes propidium iodide and SYTO^{TM} 9 for discrimination of live and dead cells. Figure 4.1 shows that yeast populations were dramatically reduced to undetectable numbers (<3x10^3 CFU/mL) in 24 h in all wines as revealed by the plate count method. Plate viable counts of 10^4-10^6 CFU/mL were, however, detected after 48 h in 4 Touriga Nacional and 3 Cabernet Sauvignon wines. Populations of around 10^6 CFU/mL were reached in the subsequent sampling times until the end of the experiment (432 h). CFU counts were generally lower in the Syrah wines, with only two wines showing plate counts of around 10^6 CFU/mL from 96 and 144 h onwards (Figure 4.1).

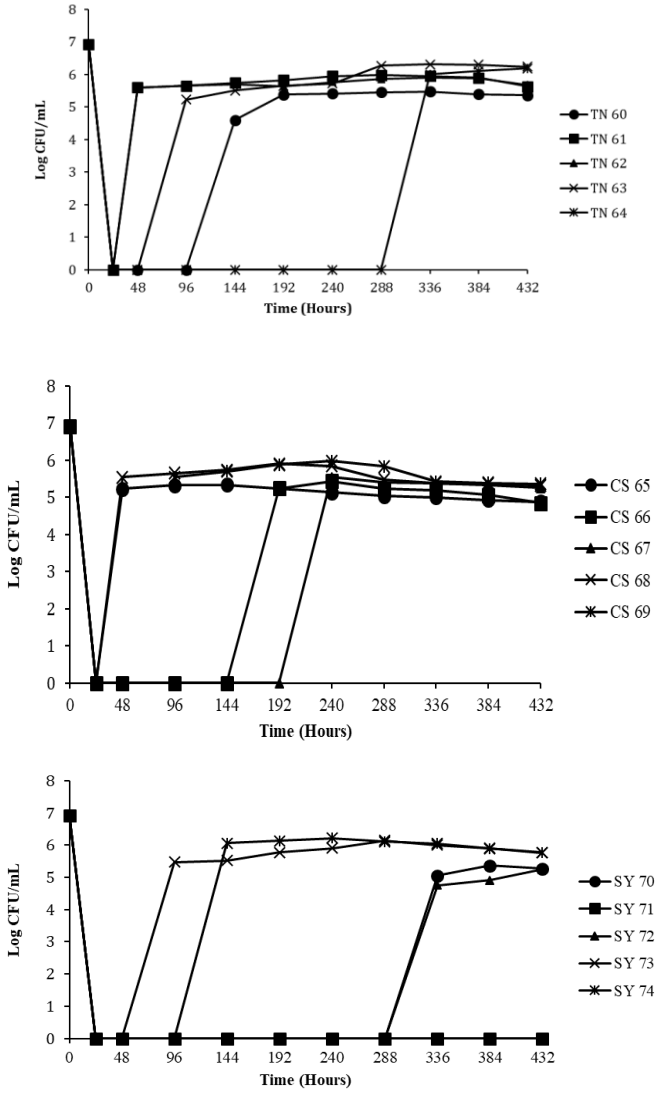
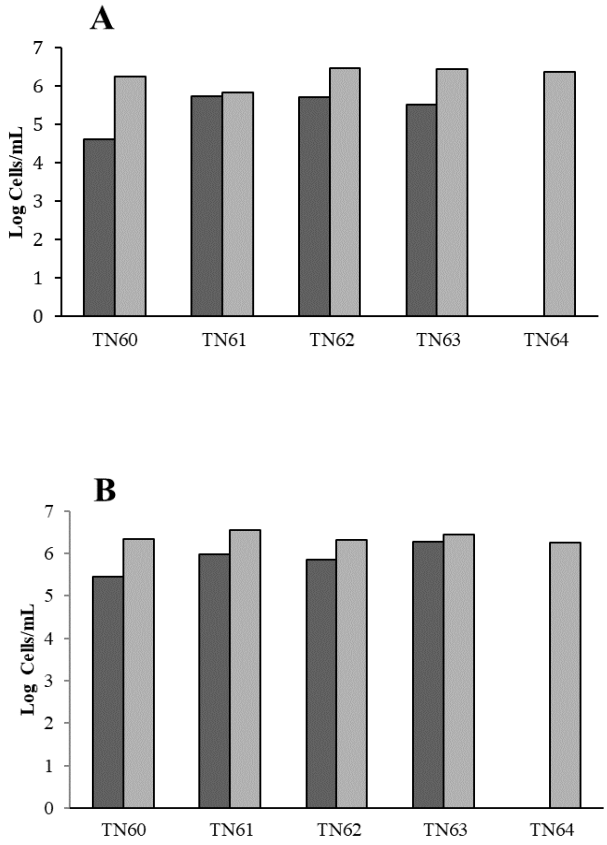


Figure 4.1. Culturable cells of *Dekkera bruxellensis* in Touriga Nacional (A), Cabernet Sauvignon (B) and Syrah wines (C) monitored by plate counts on YM medium. The values are the means of two independent experiments. CFU/mL of 0 (zero) represent values below the detection limit of the method ($3x10^3$ CFU/mL).

Viability was also assessed by flow cytometry in combination with two DNA reactive dyes. This analysis revealed the presence of two yeast subpopulations corresponding to damaged cells (stained with propidium iodide) and cells with intact membranes, which are

impermeable to propidium iodide (stained with SYTO 9™). The variety Cabernet Sauvignon showed the higher average ratio live/dead cells (90:10) at the end of the experimental incubation time, followed by Touriga Nacional (80:20) and Syrah (50:50) (data not shown). Figures 2 to 4 show the results obtained for three sampling times (144, 288 and 432 h) comparing plate counts with flow cytometry viable counts in each wine. No viable cells were encountered in wine TN64 by plate count at 144 h, however the flow cytometry analysis shows the presence of viable cells at 10^6–10^7 cells/mL (Figure 4.2).



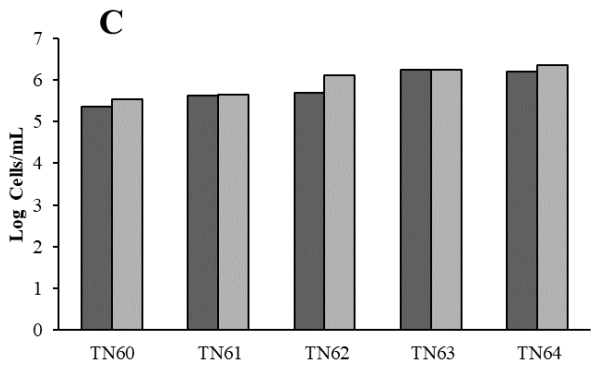


Figure 4.2. Plate culturable counts (dark grey bars) and viable counts monitored by flow cytometry (light grey bars) of Touriga Nacional wines determined at 144 (A), 288 (B) and 432 h (C) sampling times. The values are the means of two independent experiments.

Live populations were detected on TN60 by plate count but at a lower concentration (2 log difference) than by flow cytometry, and also on TN62 and TN63 with a 1 log difference. There is a good correspondence between the two growth monitorization methods for wine TN61. The results at 288 h were similar to those obtained at 144h. At 432h it can be seen that yeast cells in TN64 recovered their culturability and similar counts were found between the two methods for all wines. For the Cabernet Sauvignon wines, the populations of two wines (CS66 and CS67) were not detected by viable plate counts at 144 h (Figure 4.3), however flow cytometry shows the presence of viable cells at levels higher than 10^6 cells/mL. A difference of approximately 1 log was found for CS65 and CS69. CS66 and CS67 recovered their culturable status at 288 h. From this sampling point to 432 h, a decrease of 1 log cycle on the concentration of viable cells was observed in all wines (except for CS66).

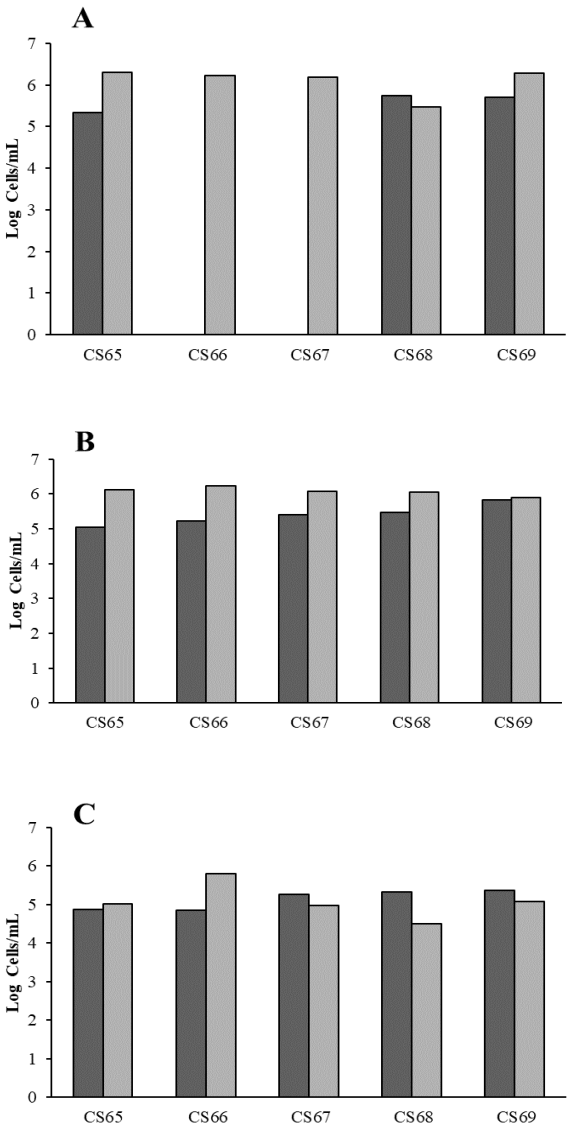


Figure 4.3. Plate culturable counts (dark grey bars) and viable counts monitored by flow cytometry (light grey bars) of Cabernet Sauvignon wines determined at 144 (A), 288 (B) and 432 h (C) sampling times. The values are the means of two independent experiments.

For the Syrah grape variety, three wines (SY70, SY71 and SY72) showed undetectable numbers by plate counts at 144 h (Figure 4.4), however flow cytometry shows the presence of live cells at levels of 10^6 cells/mL. Similar results were obtained at 288 h.

Wines SY70 and SY72 recovered culturability at 432 h, but the yeast population on wine SY71 continues undetectable by plate count, though the flow cytometry analysis reveals the presence of a viable population of almost 10^5 cells/mL.

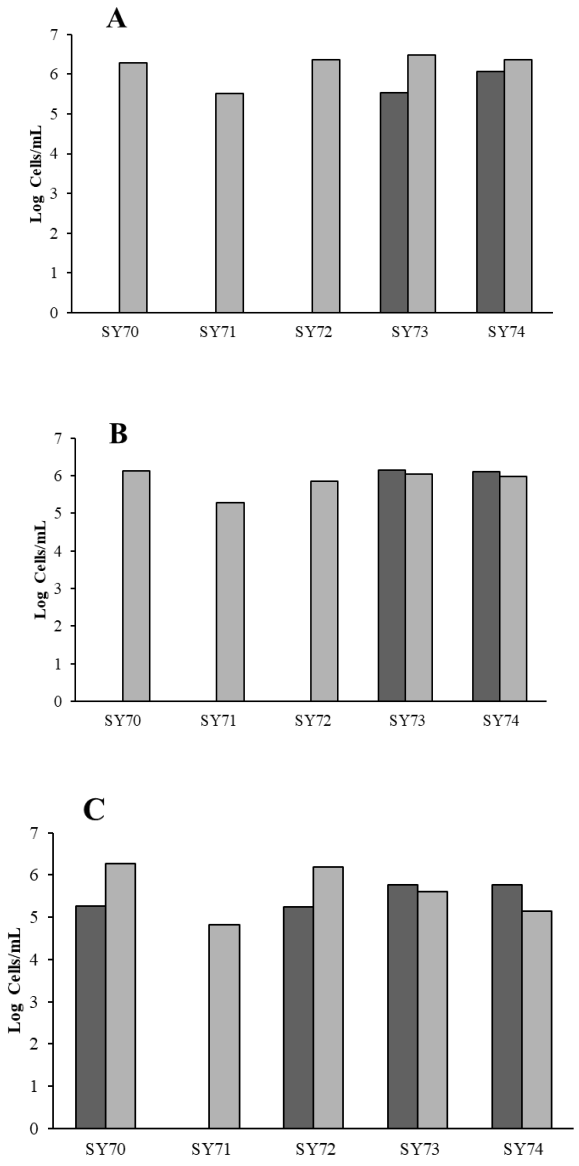


Figure 4.4. Plate culturable counts (dark grey bars) and viable counts monitored by flow cytometry (light grey bars) of Syrah wines determined at 144 (A), 288 (B) and 432 h (C) sampling times. The values are the means of two independent experiments.

Figure 4.5 shows the average of live cells for each grape variety determined by flow cytometry in three sampling points. The number of viable cells in Touriga Nacional wines remained approximately the same over time, while a significant decrease was observed in Cabernet Sauvignon at 432 h ($P<0.05$) and in Syrah wines at 288 and 432 h ($P<0.05$).

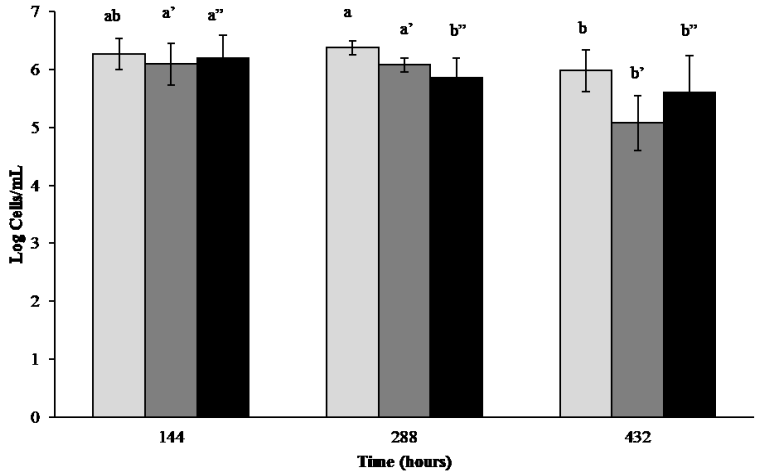


Figure 4.5. Survival of the *Dekkera bruxellensis* as determined by flow cytometry in Touriga Nacional (light grey bars), Cabernet Sauvignon (dark grey bars) and Syrah wines (black bars) in 3 sampling times. The values are the means of 5 different wines of each grape variety. Different letters indicate statistically significant differences for each grape variety over time.

The viability measurement by flow cytometry showed the presence of viable cells at relevant levels in populations not detected by the plate count method, suggesting that a significant part of the population was in a viable-but-not-culturable state (VBNC) for much of the incubation time. The recovery of these populations occurred at a fast rate, thus giving evidence that a true recover from the VBNC state occurred and that it was not due to the multiplication of culturable cells not originally detected by the plating method. Yeasts were able to attain the culturable state in most of the wines, but the recovery time was highly dependent on the wine, being longer in the Syrah wines. The VBNC physiological state has been described in wine yeast populations, and specifically on *Brettanomyces*/*Dekkera*, in

response to stress factors (Du Toit *et al.,* 2005; Laforgue and Lonvaud-Funel, 2012). Phenolic off-flavours and the "Brett character" have been described in wines where *Brettanomyces*/*Dekkera* populations were undetectable (Laforgue and Lonvaud-Funel, 2012) supposedly based on the capacity of these yeasts to enter the VBNC state. The entrance of *Brettanomyces*/*Dekkera* to the VBNC state has been commonly described as being induced by the exposure of cells to SO_2 (Agnolucci *et al.,* 2010; Serpaggi *et al.,* 2012; Capozzi *et al.,* 2016). The pH of the wines used in this study was raised to 3.8 in order to have the same pH value in all wines and to minimize the effect of SO_2 on the survival of the yeasts. At this pH value, SO_2 is almost exclusively in the bisulfite form (HSO_3^-), which has an insignificant antimicrobial activity, while the molecular SO_2 (antimicrobial activity) will be close to zero, certainly at a much lower concentration than the 0.8 mg/L reported to induce a complete loss of culturability in *B./D. bruxellensis* (Agnolucci *et al.,* 2014; Capozzi *et al.,* 2016). At pH 3.8, a concentration of almost 90 mg/L of free SO_2 would be needed to yield 0.8 mg/L in the molecular form, which is unrealistic in commercial wines. Moreover, we may also expect a decrease on the concentration of total SO_2 due to the pasteurization of the wines (Boulton *et al.,* 1996; Scrimgeour *et al.,* 2015). Therefore, the results strongly suggest that the entrance on the VBNC state might have been triggered by stress factors other than SO_2, such as nutrient shortage, high ethanol concentration and exposure to phenolic compounds with antimicrobial activity. Several chemical and environmental factors have been reported to induce the VBNC state in bacteria (Oliver, 2010), all of which would normally be encountered in natural environments. Therefore, we consider that other wine factors should also be studied as potential inducers of the VBNC state on *Brettanomyces*/*Dekkera*. It has been widely reported the effect of oxygen on the growth and metabolic activity of *Brettanomyces*/*Dekkera.* Du Toit *et al.,* (2005) showed that the addition of O_2 supported the growth and survival of *Brettanomyces*. Ciani *et al.,* (2003) and Blomqvist *et al.,* (2012) observed no growth or limited growth of *D. bruxellensis* in medium under complete anaerobiosis. Uscanga *et al.,* (2003) and Curtin *et al.,* (2013) demonstrated that the presence of O_2 accelerated the growth of *D. bruxellensis* and underlined the critical practical importance of excluding O_2 from wine during storage. Anaerobiosis conditions detain the growth of *Brettanomyces* but this can be reverted by the presence of O_2 or by the addition of hydrogen acceptors such as carbonyl compounds.

The micro-oxygenation technique and the naturally oxidizing conditions of barrel aging are known to promote the growth of *Brettanomyces*/*Dekkera* species. The recovery from the VNBC state observed in our study could be due to the inclusion of O_2 due to wine manipulation (opening the flasks to remove samples). Anaerobiosis as a potential inducer condition of the VBNC state deserves further investigation, in separate and in combination with other factors such as ethanol.

The metabolic activity, specifically the production of the volatile phenols 4-ethylguaiacol, 4-ethylphenol, 4-vinylguaiacol and 4-vinylphenol was examined. Only one wine (SY72) presented a sensory relevant value of volatile phenols before inoculation, 645 µg/L of 4-ethylphenol, which increased to 1146 µg/L. Table 4.1 shows that in most wines the concentration of volatile phenols increased, even in the wines where the yeast population remained in VNBC for relatively long periods (TN60, TN64, CS66, CS67, SY70, SY72). For example, TN60 reached 451 and 230 µg/L of 4-vinylphenol and 4-ethylpenol, respectively, and CS67 reached 389 and 1535 µg/L, showing that VNBC populations in these wines were able to produce volatile phenols at relevant levels, well above the perception threshold (Chatonnet *et al.,* 1995). Interestingly, relatively high concentrations of vinylphenols were found, higher than what would be expected from the metabolism of hydroxycinnamic acids by *D. bruxellensis.* This organism is known to undertake the pathway of production of volatile phenols to the final products, leading to the accumulation of ethylphenols rather than vinylphenols. The levels of vinylphenols in naturally contaminated red wines are generally low; the maximum value of 4-vinylphenol found by Chatonnet *et al.,* (1993) and Lima *et al.,* (2018) was 111 and 90 µg/L, respectively. In our study, the concentration of 4-vinylphenol was higher than 4-ethylphenol in eight (out of 15) wines. This is in accordance with Agnolucci *et al.,* (2010), who found that neither 4-ethylphenol nor 4-ethylguaiacol were detected for VNBC cultures of *B. bruxellensis* in a synthetic wine medium and with Laforgue and Lonvaud-Funel (2012), who found hydroxycinnamic acid decarboxylase activity in non culturable cells. The results suggest that the VNBC state seems to favor the accumulation of vinylphenols rather than ethylphenols. Vinylphenols might either be physiologically more favorable to VBNC cells than ethylphenols or the vinylphenol reductase is inhibited in the dormant sate.

Table 4.1. Concentration of volatile phenols (μg/L) before the inoculation of the wines with *Dekkera bruxellensis* (T_0) and after 432 h of incubation (T_{432h}). Values are the means of two independent experiments. TN: Touriga Nacional, CS: Cabernet Sauvignon, SY: Syrah

	4-vinylphenol		4-ethylphenol		4-vinylguaiacol		4-ethylguaiacol	
Wines	T_0	T_{432h}	T_0	T_{432h}	T_0	T_{432h}	T_0	T_{432h}
TN60	nd	452	nd	230	nd	nd	nd	nd
TN61	nd	719	nd	2487	nd	109	nd	239
TN62	nd	1164	82	2354	nd	nd	nd	305
TN63	nd	1127	nd	773	nd	nd	69	408
TN64	nd	301	nd	nd	nd	nd	nd	255
TN mean		753		1169				242
CS65	nd	1018	94	nd	nd	nd	nd	79
CS66	nd	1402	nd	22	nd	nd	nd	59
CS67	nd	389	109	1535	nd	nd	nd	92
CS68	nd	71	nd	45	nd	nd	nd	nd
CS69	nd	522	nd	1085	nd	nd	nd	116
CS mean		680		538				69
SY70	nd	Nd	64	164	nd	nd	nd	33
SY71	nd	45	nd	40	nd	nd	nd	nd
SY72	83	nd	645	1146	59	nd	43	77
SY73	nd	3689	nd	1900	nd	nd	nd	128
SY74	nd	nd	nd	nd	nd	nd	nd	nd
SY mean		747		650				48

[a] nd: not detected (below the detection limit of 10 μg/L)

The formation of vinylphenols derives from the decarboxylation of phenolic acids. Several decarboxylation reactions are known to be electrogenic, being associated with energy metabolism, via an H^+ pump mechanism that enhances the cell proton-motive force driving the ATP synthesis through F0-F1 ATPases (Poolman, 1993). Thus, the synthesis of 4-vinylphenol may represent an advantageous strategy for *D. bruxellensis* for energy production in wine stress conditions.

Touriga Nacional wines showed the highest concentrations of volatile phenols of all tested wines. The mean values of 4-ethylphenol (1169 μg/L) and 4-ethylguaiacol (242 μg/L) were 2 and 4 times higher, respectively, than on Cabernet Sauvignon and Syrah wines (Table 4.1). The results are in accordance with the empirical evidence that wines from this grape variety show a consistent high susceptibility to develop volatile phenols (personal

communication from winemakers). Lima *et al.,* (2018), however, revealed that the concentration of free hydroxycinnamic acids in Touriga Nacional wines is relatively low in comparison with other seven grape varieties. In fact, the precursor compounds of volatile phenols on the Touriga Nacional variety are found mainly in the bound form (coutaric and caftaric acids and malvidin-3-O-(6-p-coumaroyl)-glucoside), exhibiting the highest difference between bound and free forms of the eight grape varieties studied. The coutaric/p-coumaric acids molar ratio was 5.3 for Touriga Nacional while it was 1.6 and 1.2 for Syrah and Cabernet Sauvignon, respectively (Lima *et al.,* 2018). This specific phenolic composition may be related with the observed higher survival capacity of *Brettanomyces*/*Dekkera* on Touriga Nacional wines. Harris *et al.,* (2008, 2010) demonstrated that hydroxycinnamic acids, mainly ferulic acid, exhibit antimicrobial properties against *Dekkera* and suggested the possible application of ferulic acid to control the development of *Dekkera* in wines. Couto *et al.,* (2005a) studied the influence of selected phenolic acids (ferulic, vanillic, caffeic and gallic acids) on the thermal inactivation of *Dekkera* and found that all compounds caused a decrease in the D values. Pastorkova *et al.,* (2013) and Sabel *et al.,* (2017) revealed an antimicrobial effect of p-coumaric and ferulic acids against *D. bruxellensis* and other wine yeasts. Campos *et al.,* (2003, 2009) and Garcia-Ruiz (2011) reported an inhibitory effect of phenolic acids on wine lactic acid bacteria. Relevantly, Salhi *et al.,* (2000) showed an effective activity of ferulic and p-coumaric acids on *Lactobacillus plantarum* but, in contrast, esters of this acid (p-coumaroyl quinic acid) did not affect the growth of this species. Thus, taking into account the evidence that hydroxycinnamic acids are more toxic than cinnamate esters (precursors of volatile phenols in the bound form), the bound/free molar ratio and the total concentration of phenolic acids on the free form seem to be relevant factors to explain the differences on the susceptibility of wines to *Brettanomyces*/*Dekkera* spoilage.

4.4. Conclusion

This work shows that *D. bruxellensis* acquired the VBNC state on all wines of each grape variety studied soon after the inoculation (detected at 24 h). Yeasts were able to recover the culturable state in all wines, except for one Syrah, but the time required for the recovery was highly dependent on the wine, being longer on the Syrah wines. Although the entrance of *Brettanomyces*/*Dekkera* on the VBNC state has been commonly described as being induced by the exposure of cells to SO_2, our results strongly suggest that other stress factors may be involved as potential inducers of the VBNC state. The majority of the wines underwent the production of volatile phenols, even those where the yeast population remained in the VNBC for much of the incubation period. This means that cells in the VBNC sate are metabolically active, thus causing the spoilage of the wine. The metabolism of hydroxycinnamic acids by VBNC cells is, however, different from what it would be expected from the activity of *Brettanomyces*/*Dekkera*, leading to the accumulation of vinylphenols at relatively high levels.

The survival capacity of *D. bruxellensis,* as determined by flow cytometry, was higher on Touriga Nacional wines than in Cabernet Sauvignon and Syrah wines. The different susceptibility of wines to *Brettanomyces*/*Dekkera* spoilage may be correlated with the phenolic composition of wines, specifically the bound/free molar ratio and the total concentration of phenolic acids on the free form. Further studies should be carried out to better characterise the levels of total hydroxycinnamic acids that exert a significant inhibitory action against *Brettanomyces*/*Dekkera* in wine conditions.

4.5. References

Agnolucci, M., Rea, F., Sbrana, C., Cristani, C., Fracassetti, D., Tirelli, A., Nuti, M., 2010. Sulphur dioxide affects culturability and volatile phenol production by *Brettanomyces*/*Dekkera bruxellensis*. *International Journal Food Microbiology*, **143**, 76 - 80. http://dx.doi.org/10.1016/j.ijfoodmicro.2010.07.022.

Agnolucci, M., Cristani, C., Maggini, S., Rea, F., Cossu, A., Tirelli, A., Nuti, M., 2014 Impact of sulphur dioxide on the viability, culturability, and volatile phenol Prodction of *Dekkera bruxellensis* in wine. *Annual Microbiology*, **64**: 653 e 659. http:// dx.doi.org/10.1007/s13213-013-0698-6.

Aguilar, M. G., Uscanga, M. L., Lia, D., Strehaiano, P. 2003. *Brettanomyces bruxellensis*: effect of oxygen on growth and acetic acid production. *Applied Microbiology Biotechnology,* **61**: 157–162.

Almudena García-Ruiz M., Victoria Moreno-Arribas., Pedro, J., Martín-Álvarez, Begoña Bartolomé. 2011. Comparative study of the inhibitory effects of wine polyphenols on the growth of enological lactic acid bacteria. *International Journal of Food Microbiology*, **145:** 426–431.

Avar P, Nikfardjam, MSP, Kunsági-Máté S, Montskó G, Szabó Z, Böddi K, Ohmacht R, Márk L (2007) Investigation of phenolic components of Hungarian wines. *International Journal Molecular Science,* **8***(*10): 1028-1038.

Blomqvist, Johanna., Sànchez Nogué, Violeta., Gorwa-Grauslund, Marie., and Volkmar, Passoth. 2012. Physiological requirements for growth and competitiveness of *Dekkera bruxellensis* under oxygenlimited or anaerobic conditions. *Yeast*, **29**: 265–274.

Boulton, R., Singleton, V. L., Bisson, L. F., Kunkee, R. E. 1996. Principles and practices of winemaking. Kluwer Academic/Plenum, New York.

Boyd, A. R., Gunasekera, S. T., Attfield, V. P., Simic, K., Vicent, F. S., Veal, A. D. 2003. A flow cytometric method for determination of yeast viability and cell number in a brewery. FEMS *Yeast Research***, v. 3**, n. 1, p. 11-16.

Campos, F.M., Couto, J.A., Figueiredo, A.R., Tóth, I.V., Rangel, A.O.S.S., Hogg, T.A. 2009. Cell membrane damage induced by phenolic acids on wine lactic acid bacteria. *International Journal of Food Microbiology*, **135**: 144-151.

Campos, F., Couto, J. A., and Hogg, T. 2003. The influence of phenolic acids on growth and survival of wine lactic acid bacteria. *Journal of Applied Microbiology*, **94**: 167 – 174.

Capozzi, V., Di Toro, M. R., Grieco, F., Michelotti, V., Salma, M., Lamontanara, A., Russo, P., Orrù, L., Herv Alexandre, Spano, G. 2016. Viable But Not Culturable (VBNC) state of *Brettanomyces bruxellensis* in wine: New insights on molecular basis of VBNC behaviour using a transcriptomic approach. *Food Microbiology*, **59**: 196 e 204.

Chatonnet, P., Dubourdieu, D., Boidron, J.N., Lavigne, V. 1993. Synthesis of volatile phenols by *Saccharomyces cerevisiae* in wines. *Journal of the Science of Food and Agriculture*, **62**: 191-202.

Chatonnet, P., Dubordieu, D., Boidron, J., 1995. The influence of *Brettanomyces/Dekkera* sp. and lactic acid bacteria on the ethylphenol content of red wines. *American Journal of Enology and Viticulture,* **46**, 463-468.

Chatonnet, P., Viala, C., Dubourdieu, D. 1997. Influence of polyphenolic components of red wines on the microbial synthesis of volatile phenols. *American Journal of Enology and Viticulture*, **48**: 443-448.

Ciani, M., Maccarelli F., and Fatichenti F. 2003. Growth and fermentation behaviour of *Brettanomyces*/*Dekkera* yeasts under different conditions of aerobiosis. *World Journal of Microbiology & Biotechnology*, **19**: 419–422.

Couto, J. A., Barbosa, C., and Hogg, T. 2005. A simple cultural method for the presumptive detection of *Brettanomyces/Dekkera* in wines. *Letters in Applied Microbiology*. **41**: 505-510.

Couto, J. A., Neves, F., Campos, F. and Hogg, T. 2005. Thermal inactivation of the wine spoilage yeasts *Brettanomyces/Dekkera. International Journal of Food Microbiology*, **104**: 337-344.

Curtin, C. D., Geoffrey Langhans, Paul A., Henschke, Paul R. Grbin. 2013. Impact of Australian *Dekkera bruxellensis* strains grown under oxygen-limited conditions on model wine composition and aroma. *Food Microbiology,* **36**, 241-247

Diaz, M., Herrero, M., Garcia, L. A., Quiros, C. 2010. Application of flow cytometry to industrial microbial bioprocesses. *Biochemical Engineering Journal*, **48**: 385–407.

Di Toro, Maria Rosaria., Capozzi, V., Beneduce, L., Alexandre, H., Tristezza, M., Durante, M., Tufariello, M., Grieco, F., Giuseppe, S. 2017. Intraspecific biodiversity and 'spoilage potential' of *Brettanomyces bruxellensis* in Apulian wines. *LWT - Food Science and Technology*, **60**: 102 e 108.

Divol, B., Du Toit, M., Duckitt, E. 2012. Surviving in the presence of sulphur dioxide: strategies developed by wine yeasts. *Applied Microbiology Biotechnology*, **95**(3): 601-13.

Drysdale, G.S. & Fleet, G.H., 1988. Acetic acid bacteria in winemaking - A Review. *American Journal Enology Viticulture*, **39**: 143-154.

Du Toit, W.J., Pretorius, I.S., Lonvaud-Funel, A., 2005. The effect of sulphur dioxide and oxygen on the viability and culturability of a strain of Acetobacter pasteurianus and a strain of *Brettanomyces bruxellensis* isolated from wine. *Journal Applied Microbiology*, **98**: 862 e 871.

Edlin, D.A.N., Narbad, A., Dickinson, J.R., Lloyd, D. 1995. The biotransformation of simple phenolic compounds by *Brettanomyces anomalus*. *FEMS Microbiology Letters*, **125**: 311-315.

Fugelsang, K.C., 1997. Wine Microbiology. The Chapman & Hall Enology Library, New York.

Ginjom, I., D'Arcy, B., Caffin, N., Gidley, M. 2011. Phenolic compound profiles in selected Queensland red wines at all stages of the wine-making process. *Food Chemistry*, **125**: 823–834.

Herrero, M., Quiros, C., Garcia, L. A., Diaz, M. 2006. Use of flow cytometry to follow the physiological states of microorganisms in cider fermentation processes. *Applied and Environmental Microbiology*, **72**(10):6725–33.

Harris, V., Ford, CM, Jiranek, V, Grbin, PR. 2008. *Dekkera* and *Brettanomyces* growth and utilization of hydroxycinnamic acids in synthetic media. *Applied Microbiology Biotechnology*, **86**: 721–729.

Harris, V., Jiranek, V. Ford, C. M., Grbin, P. R. 2010. Inhibitory effect of hydroxycinnamic acids on *Dekkera* spp. *Applied Microbiology Biotechnology*, **78**: 997–1006.

Heresztyn, T. 1986. Metabolism of volatile phenolic compounds from hydroxycinnamic acids by *Brettanomyces* yeast. *Archives Microbiology*, **146**: 96-98.

Jaume Comas-Riu; Núria Rius. 2009. Flow cytometry applications in the food industry. *Journal Industrial Microbiology Biotechnology*. **36**: 999–1011- DOI 10.1007/s10295-009-0608-x.

Jepras, R.I., Carter, J., Pearson, S.C., Paul, F.E., Wilkinson, M.J., 1995. Development of a robust flow cytometric assay for determining numbers of viable bacteria. *Applied Environmental Microbiology*, **61**: 2696–2701.

Kheir, J., Salameh, D., Strehaiano, P., Brandam, C., Lteif, R. 2013. Impact of volatile phenols and their precursors on wine quality and control measures of *Brettanomyces*/*Dekkera* yeasts. *European Food Research and Technology*, **vol. 237** (5): 655-671.

Laforgue, R., and Lonvaud-Funel, A. 2012. Hydroxycinnamic acid decarboxylase activity of *Brettanomyces bruxellensis* involved in volatile phenol production: Relationship with cell viability. *Food Microbiology*, **32**: 230-234.

Lima, A., Oliveira, C., Santos, C., Campos, F.M., Couto, J.A. 2018. Phenolic composition of monovarietal red wines regarding volatile phenols and its precursors. *European Food Research and Technology*. https://doi.org/10.1007/s00217-018-3110-8.

Loureiro, V., Malfeito-Ferreira, M. 2006. Spoilage activities of *Dekkera*/*Brettanomyces* spp. In: Blackburn, C. (Ed.), Food Spoilage Microorganisms. Woodhead Publishing Limited, Cambridge, England, pp. 354 e 398.

Mahesh Chandra, Inês Madeira, Ana-Rute Coutinho, Helena Albergaria, Manuel Malfeito-Ferreira. 2016. Growth and volatile phenol production by *Brettanomyces bruxellensis* in different grapevine varieties during fermentation and in finished wine. *European Food Research Technology*, **242**: 487–494.

Miles, A., Mishra, S., 1938. The estimation of the bactericidal power of blood. *Journal of Hygiene*, **38**: 732-749.

Nebe-von Caron, G., Stephens, P., Badley, R.A., 1998. Assessment of bacterial viability status by flow cytometry and single cell sorting. *Journal Applied Microbiology*, **84**: 988–998.

Oliver, J.D., 2010. Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiology. Review*, **34**, 415 e 425.

Ormerod, M.G., 1990. Flow Cytometry: A Practical Approach. IRL Press, Oxford, 279 pp.

Paola Malacrino, Giacomo Zapparoli, Sandra Torriani, Franco Dellaglio. 2001. Rapid detection of viable yeasts and bacteria in wine by flow cytometry. Journal of Microbiological Methods 45 Ž2001. 127–134.

Pastorkova E., Zakova T., Landa P., Novakova J., Vadlejch J., Kokoska L.2013. Growth inhibitory effect of grape phenolics against wine spoilage yeasts and acetic acid bacteria *International Journal of Food Microbiology*, **161**: 209-213.

Poolmam, B. 1993. Energy transduction in lactic acid bacteria. *FEMS Microbiology Review,* **12**(1-3):125-47.

Quirós C, Herrero M, García LA, Díaz M. 2007. Application of flow cytometry to segregated kinetic modeling based on the physiological states of microorganisms. *Applied Environmental Microbiology*, **73**(12): 3993-4000.

Ribéreu-Gayon, P., Dubourdieu, D., Donéche, B., Lonvaud-Funel, A., 2000. Handbook of Enology Volume II, The Chemistry of Wine and Stabilization and Treatments, second ed. John Wiley & Sons Ltd, UK.

Rodrigues, N., Gonçalves, G., Pereira-da-Silva, S., Malfeito-Ferreira, M., and Loureiro, V. 2001. Development and use of a new medium to detect yeasts of the genera *Brettanomyces/Dekkera* sp. *Journal. Applied Microbiology,* **90**: 588-599.

Sabel A., Bredefeld, S., Schlander, M., and Claus, H.. 2107. Wine Phenolic Compounds: Antimicrobial Properties against Yeasts, Lactic Acid and Acetic Acid Bacteria. Beverages, 3, 29.

Salih, A. G., Le Quéré, J. M., & Drilleau, J. F. 2000. Action des acides hydroxycinnamiques libres set estérifiés sur la croissance des bacteÅLries lactiques. *Science Aliments*, **20**: 537–560.

Scrimgeour, N., Nordestgaard, S, Lloyd, N.D.R., Wilkes, E.N. 2015. Exploring the effect of elevated storage temperature on wine composition. *Australian Journal of Grape and Wine Research*, **21**: 713-722.

Serpagi V.; Remize F.; Grand A. S-Le. 2010. Specific Identification and Quantification of the Spoilage Microorganism *Brettanomyces* in Wine by Flow Cytometry: A Useful Tool for Winemakers. *Journal of International Society for Advancement of Cytometry*. Cytometry Part A. 77A, 497-499.

Shapiro, H.M., 1995. Pratical Flow Cytometry. 2nd edn. Alan R. Liss, New York, p. 339.

Silva, T. R. A.; Hewitt, C.; Roseiro, J. (2004). "Citometria de fluxo-Funcionalidade celular on-line em bioprocessos**.** "*Boletim de Biotecnologia*", **v. 77**, p. 32-40.

Serpaggi, V., Remize, F., Recorbet, G., Gaudot-Dumas, E., Sequeira-Le Grand, A., Hervé A. 2012. Characterization of the "viable but nonculturable" (VBNC) state in the wine spoilage yeast Brettanomyces. *Food Microbiology*, **30**: 438-447.

Wedral, D., Shewfelt, R., & Frank, J. 2010. The challenge of *Brettanomyces* in wine. *LWT-Food Science and Technology,* **43**: 1474 e 1479.

CHAPTER 5

Activity of *Dekkera bruxellensis* and the effect on the aroma profile of monovarietal wines

Chapter 5

Activity of *Dekkera bruxellensis* and the effect on the aroma profile of monovarietal wines

Abstract

The metabolic profile of two *D. bruxellensis* strains was studied in monovarietal wines of the grape varieties Touriga Nacional and Cabernet Sauvignon, focusing on the volatile composition. The two strains of *D. bruxellensis* involved showed different capacities to produce volatile phenols. Different volatile phenols profiles were obtained depending on the wine and on the yeast strain. The concentration of ethyl esters, including ethyl acetate, was higher in the inoculated wines of both grape varieties than in the control wines. On the contrary, isoamyl acetate was lower in most of the treated wines. The fatty acids isovaleric acid and caprylic acid increased in the inoculated wines, specially on Cabernet Sauvignon wines, and, as also happened for the esters, *D. bruxellensis* PYCC4801 produced higher values than strain 21. The concentration of the three monoterpenes analysed increased in the Cabernet Sauvignon wines but not in Touriga Nacional wines. The grape variety effect observed can be related with different compositions of glycosidically bound terpenes subjected to the ß-glycosidase activity of *D. bruxellensis.* Besides the production of volatile phenols, *D. bruxellensis* was able to modify the composition of wines through the production of metabolites of other chemical families.

5.1. Introduction

The problem of wine contamination by *D. bruxellensis* is mainly due to the production of flavors associated with the vinylphenols and especially ethylphenols. But these yeasts can also produce several other metabolites such as acetic acid, isovaleric acid, 2-methylbutyric acid and isobutyric acid which can influence the quality of the wine and the perception of volatile phenols (Oro *et al*., 2019). A masking effect of isobutyric and isovaleric acids on the detection of ethylphenols aroma was revealed by Romano *et al*., (2009). *Brettanomyces* is also known to produce small amounts of acetaldehyde and ethyl acetate during aerobic fermentation (Ciani *et al.,* 2003). Acetaldehyde has sherry/nutty/bruised apple descriptors, and ethyl acetate contributes nail polish/fruity aromas to wine. The presence of these compounds makes more difficult the sensory perception of volatile phenols.

Substrates other than hydroxycinnamic acids, such as amino acids, can be used to make a wide variety of other aroma-active compounds (Joseph *et al*., 2013). Esterase activity of *Brettanomyces/Dekkera,* responsible for the formation of ethyl esters, such as ethyl acetate and ethyl lactate, along with the hydrolysis of acetate esters, such as isoamyl acetate and phenylethyl acetate, was described to occur in beer (Spaepen and Verachtert, 1982). There is also some scientific information on the production of esters by these yeasts in wines (Romano et al, 2008, Curtin *et al*., 2013). Conterno et al (2013) applied a wide-range metabolomics approach to investigate the *D. bruxellensis* metabolism in low nutrient availability and different ethanol concentrations modelling the wine environment. All the above mentioned compounds may contribute to overall effect of *Brettanomyces/Dekkera* on wines, normally called the “Brett character". Thus, in addition to the phenolic composition, it is very important to make a more complete chemical analysis to demonstrate the complexity of the "Brett character".

This chapter aimed at characterizing the behaviour of two *D. bruxellensis* strains in wines of two grape varieties (Touriga Nacional and Cabernet Sauvignon) focusing on families of aroma compounds such as esters, higher alcohols and volatile fatty acids.

5.2. Materials and Methods

5.2.1. Wine samples

Three commercial monovarietal red wines from two grape varieties, Touriga Nacional (from wine demarcated regions in Portugal) and Cabernet Sauvignon (national and international wines), were used.

Table 5.1. Wines used in this assay. TN- Touriga Nacional; CS - Cabernet Sauvignon

Wine	Harvesting year	Ethanol conc. (%) v/v	pH
TN75	2014	14	3.82
TN76	2016	14.5	3.84
TN77	2012	13.5	3.91
CS78	2015	13.5	3.68
CS79	2016	14	3.47
CS80	2015	13	3.65

The wines were pasteurized (50°C, 5 min) before inoculation. The pasteurization regime was chosen in accordance with Couto *et al*. (2005a), who have previously established time and temperature parameters for the thermal inactivation of yeasts in wine, specifically *Brettanomyces*/*Dekkera*.

5.2.2. Strains and culture conditions

Two strains of *Dekkera bruxellensis* were used: D. *bruxellensis* 21, selected from the group of strains isolated in Chapter 2, and the reference strain D. *bruxellensis* PYCC 4801 (Portuguese Yeast Culture Collection). For the preparation of the inoculum, yeasts were grown on 300 ml of YM broth with 5% (v/v) ethanol, at a temperature of 25°C without agitation, to late exponential phase (4 days). The culture was centrifuged at 8000 rpm for 10 min and the pellet was resuspended in 3 ml of sterile distilled water. 300 ml of each wine in 500 ml flasks were inoculated to an initial inoculum of approximately 10^7 CFU ml/L. After

inoculation the wines were incubated at 25°C for a period of 60 days. The experiment was performed in duplicate, to obtain an independent reproduction of the experimental conditions.

5.2.3. Plate counting and Flow Cytometry

Samples were taken at regular intervals during the incubation period of 18 days and serially diluted from 10^{-1} to 10^{-6} (decimal dilutions in sterile 9 ml Ringer solution [Lab M, Bury, UK]). The diluted samples were plated in duplicate on YM agar (YM broth with 2% agar [Lab M, Bury, UK]), using the drop count technique (Miles and Misra, 1938) for the determination of colony forming units per mL (CFU ml/L). Counts were done after the incubation of YM plates at 25°C for 72 h.

Yeast growth and survival were also monitored by flow cytometry using the fluorescent dyes propidium iodide and SYTO 9™ for discrimination of live and dead cells as described in Chapter 4.

5.2.4. Quantification of volatile phenols

The volatile phenols were quantified as described in Chapter 4.

5.2.5. Higher alcohols, methanol and ethyl acetate quantification

An aliquot of 5 mL of wine was used for the analysis. Sample was mixed and with 0.5 g of sodium sulphate (Na_2S0_4 for dehydration) and used a magnetic stirring bar in a 15 mL glass vial. The sample was incubated in a water bath at 40°C for 20 min under continuous stirring and extracted with a Divinylbenzene / Carboxen / Polydimethylsiloxane (DVB/CAR/PDMS) fiber (Supelco, USA) by exposition to the headspace (HS). After extraction, the fiber was removed from the vial and the sample manually injected for 5 min at 220°C in split mode (1/30). The identification and quantification of most of the higher

alcohols, acetaldehyde and methanol were conducted on a Hewlett Packard 5890 Gas Chromatograph System with flame ionization detector (GC-FID). Chromatographic separations were done by using a CP-Wax 57CB column (Chrompack) with the following characteristics: 50 m length, 0.25 mm intern diameter, 0.2 μm film thickness. The carrier gas was hydrogen at a flow rate of 1.0 mL/min. The column oven temperature program was: initial temperature 40°C for 5 min, 40°C to 80°C at a rate of 3°C/min, 80°C to 200°C at a rate of 15°C/min, and then held for 10 min. The total run time was 36 min. All wines were injected in duplicate. The volatile compounds: 1-propanol (n-propyl alcohol), 1-butanol, isobutanol (2-methyl-1-propanol or isobutyl alcohol), 2-methyl-1-butanol (active amyl alcohol), isoamyl alcohol (3-methyl-1-butanol) and methanol from internal standard and the standard calibration curves.

According to IPAC, (Portugal laboratories accreditation institution OGC002) calibrations were performed for each compound and constants values were adjusted according to linear equation (Y = aX+b; Y=chromatographic area/internal standard area; a - slope; X – concentration; b - intercept) - (Table 5.2).

Table 5.2. Analytical parameters used for the GC-FID quantification of higher alcohols

Compound	Slope	R^2	K (mg/L)	LD (mg/L)	LQ (mg/L)	Linear zone (mg/L)
Methanol	0,0419	0,996	23,8	9,731	10	10-400
1-propanol	0,0351	0,993	28,5	4,754	10	10-400
1-butanol	0,0695	0,991	14,4	5,395	10	10-400
2-methyl-1-propanol	0,0454	0,992	22,0	6,647	10	10-400
2-methyl-1-butanol	0,0506	0,995	19,7	5,291	10	10-400
3-methyl-1-butanol	0,0660	0,993	15,1	5,525	10	10-400

5.2.6. Esters, fatty acids and terpenes quantification

For the analysis of other volatile compounds, 5 mL of wine was mixed with 1 g of Na_2S0_4 (for dehydration) and 20 µl of 3-octanol (Sigma-Aldrich, Germany) at 50 mg/L (internal standard) in a 15 mL glass vial sealed with a PTFE-silicone septum (Supelco, USA). The sample was pre-incubated 5 min at 40°C, and the headspace (HS) extracted by solid phase microextraction (SPME) with a fiber Divinylbenzene / Carboxen / Polydimethylsiloxane (DVB/CAR/PDMS) 50/30 mm from Supelco (Bellefonte PA, USA) for five more minutes at the same temperature. After extraction, the wine samples were injected in split mode (30 s, 1/30) through an automatic infector set at 220°C and analyzed with a Varian CP-450 GC gas chromatograph coupled to a Varian 240 MS mass spectrometer (Agilent Technologies, USA). Chromatographic separations were done by using a FactorFour VF-WAXms column (Agilent, USA) with the following characteristics: 15 m length, 0.15 mm intern diameter, 0.15 µm film thickness. The carrier gas was helium at a flow rate of 1 mL/min. The column oven temperature program was: initial temperature 40°C for 1 min, 40°C to 220°C at a rate of 4°C/min, then held for 2.5 min. The total run time was 48.5 min. Mass spectral data were collected over a range of 33 to 150 m/z in full scan mode. The identification of volatile compounds was achieved on the basis of their mass spectra and linear retention index. Mass spectrometric information of each chromatographic peak was compared to the NIST 14 mass spectral library. The concentrations of the volatile compounds were calculated by relating the peak area of volatiles to that of the internal standard. The analyses were performed in duplicate.

According to IPAC, (Portugal laboratories accreditation institution OGC002) calibrations were performed for each compound and constants values were adjusted according to linear equation (Y = aX+b; Y=chromatographic area/internal standard area; a - slope; X – concentration; b - intercept) - (Table 5.3).

Table. 5.3. Analytical parameters used for the GC-MS quantification of esters, fatty acids and terpenes.

Compounds	slope	r^2	K (mg/L)	LD (mg/L)	LQ (mg/L)	Zona Linear (mg/L)
Ethyl hexanoate	1,051	0,998	0,952	0,043	0,05	0,05-2,0
Ethyl octanoate	1,202	0,991	0,832	0,040	0,05	0,05-2,0
Ethyl decanoate	1,522	0,991	0,657	0,042	0,05	0,05-2,0
Ethyl dodecanoate	2,301	0,991	0,435	0,041	0,05	0,05-2,0
Isoamyl acetate	0,493	0,996	2,030	0,032	0,05	0,05-2,0
2 Furfural	0,132	0,995	7,580	0,043	0,05	0,05-2,2
Isovaleric acid	0,029	0,984	35,005	0,039	0,04	0,04-7,0
Octanoic acid	0,029	0,967	33,900	0,035	0,04	0,04-7,0
Linalool	0,365	0,980	2,740	0,041	0,04	0,04-0,7
α Terpineol	0,800	0,994	1,250	0,020	0,02	0,02-0,7
Citronellol	1,287	0,994	0,777	0,020	0,02	0,02-0,7

5.2.7. Statistical analysis

Data was analysed according to ANOVA procedures to test for significant differences between wine varieties. Multiple pairwise differences of means were subsequently evaluated using Tukey's test. Normality and homoscedasticity of data was tested using Shapiro Wilk and Levene's tests, respectively. When these requirements were not verified, non-parametric tests were carried out instead: Kruskal-Wallis test (alternative to one-way ANOVA) and Mann-Whitney (alternative to post-hoc pairwise comparisons). The significance level assumed was 5% in all situations. IBM SPSS Statistics 25 for Windows® (SPSS Inc., Chicago, USA) was used in all data analyses.

5.3. Results and discussion

Tables 5.4 and 5.5 present the results obtained for the volatile phenols. Significant differences can be observed amongst the two strains of *D. bruxellensis,* thus showing different capacities to produce volatile phenols. Relatively high values are found for 4-vinylphenol (wines TN76, TN77, CS78 and CS79), which agrees with the results of Chapter

4, in coherence with the VBNC physiological state of the cells. Also in this experiment, yeast culturable populations were undetectable in all wines, but viability measurement by flow cytometry confirmed the presence of populations in the VBNC state in at levels of 10^5-10^6 cells/mL (data not shown).

Table 5.4. Volatile phenols (µg/L) of three wines of Touriga Nacional 60 days after inoculation with *D. bruxellensis* 21 or *D. bruxellensis* PYCC4801. The values are the averages of two independent assays. Different letters indicate statistically significant differences between the samples in each wine.

	TN75			TN76			TN77		
V. Phenols (µg/L)	C*	Db21	Db4801	C	Db21	Db4801	C	Db21	Db4801
4VG	22,8a	66,9b	378,3c	19,8a	39,7a	884,5b	58,8a	38,7b	153,2c
4VP	54,3a	658,3b	569,7b	57,6a	2672,9c	1057,1b	39,9a	2238,5c	170,2b
4EG	22,4a	119,1b	77,5b	61,0a	76,2b	78,4b	nd	nd	45,4b
4EP	48,2a	2190,3c	108,4b	nd	137,8b	131,6b	nd	19,5a	297,2b

*C – control, non-inoculated wines stored under the same conditions as treated wines

nd- not detected.

Table 5.5. Volatile phenols (µg/L) of three wines of Cabernet Sauvignon 60 days after inoculation with *D. bruxellensis* 21 or *D. bruxellensis* PYCC4801. The values are the averages of two independent assays. Different letters indicate statistically significant differences between the samples in each wine.

	CS78			CS79			CS80		
V. Phenols (µg/L)	C*	Db21	Db4801	C	Db21	Db4801	C	Db21	Db4801
4VG	15,6	nd	nd	nd	nd	35,2	nd	nd	nd
4VP	23,8a	2372,2b	2918,5c	nd	1814,9b	481,5c	23,7a	180,6b	148,2b
4EG	77,6a	101,0b	87,0b	55,9a	60,6a	507,4b	124,5a	115,3a	87,3a
4EP	nd	129,2a	124,7a	nd	91,4a	118,6a	nd	1754,4b	807,1b

*C – control, non-inoculated wines stored under the same conditions as treated wines

nd- not detected.

The composition of the most important higher alcohols in wines was not significantly changed in the wines of both grape varieties (Tables 5.6 and 5.7) or was lower in the inoculated wines, except for 2-Methyl-1-butanol and 3-Methyl-1-butanol in TN77. Methanol decreased in all wines, except for CS80 inoculated with *D. bruxellensis* PYCC4801.

Table 5.6. Higher alcohols and methanol, of three wines of Touriga Nacional 60 days after inoculation with *D. bruxellensis* 21 or *D. bruxellensis* PYCC4801. The values are the averages of two independent assays. Different letters indicate statistically significant differences between the samples in each wine.

	TN75			TN76			TN77		
Compounds (mg/L)	C*	Db21	Db4801	C	Db21	Db4801	C	Db21	Db4801
Methanol	20,70a	13,64b	16,21b	13,31a	10,50b	nd	nd	nd	nd
1-Propanol	16,63a	6,83b**	5,62b**	5,80a**	5,14a**	7,53a**	12,70a	6,44b**	18,50a
1-Butanol	33,34a	22,90b	23,85ab	33,60a	16,30ab	25,20b	10,82a	7,50a**	17,80b
2-Methyl-1-propanol (isobutyl alcohol)	nd	nd	nd	nd	nd	nd	nd	nd	nd
2-Methyl-1-butanol (active amyl alcohol	58,41a	51,40a	50,04a	77,63a	68,73a	80,14a	46,50a	62,51ab	71,00b
3-Methyl-1-butanol (isoamyl alcohol)	149,60a	132,90b	87,55ab	207,40a	185,70b	220,70b	113,20a	137,90b	159,90b

*C - control, non-inoculated wines stored under the same conditions as treated wines.
** < LQ
nd - not detected

Table 5.7. Higher alcohols and methanol of three wines of Cabernet Sauvignon 60 days after inoculation with *D. bruxellensis* 21 or *D. bruxellensis* PYCC4801. The values are the averages of two independent assays. Different letters indicate statistically significant differences between the samples in each wine.

	CS78			CS79			CS80		
Compounds (mg/L)	C*	Db21	Db4801	C	Db21	Db4801	C	Db21	Db4801
Methanol	nd	nd	nd	nd	nd	nd	nd	nd	nd
1-Propanol	11,70a	7,30b	6,13b	6,80a	6,22a	14,00b	4,90a	5,21b	6,00b
1-Butanol	13,80a	15,40a	14,40a	13,60a	9,90b	22,30c	11,50a	11,30a	12,80a
2-Methyl-1-propanol (isobutyl alcohol)	nd	nd	nd	nd	nd	nd	nd	nd	nd
2-Methyl-1-butanol (active amyl alcohol	72,00a	75,41a	69,40a	58,84a	52,60a	57,30a	58,50a	57,30a	50,50a
3-Methyl-1-butanol (isoamyl alcohol)	195,72a	198,60b	182,82b	168,65a	147,10b	157,70b	167,00a	163,20b	160,60b

*C – control, non-inoculated wines stored under the same conditions as treated wines.
**< LQ
Nd – not detected

The sum of the ethyl esters analysed was higher in the inoculated wines of both grape varieties. This effect was even higher in the wines inoculated with *D. bruxellensis* PYCC4801 than with strain 21. Isoamyl acetate did not contribute to the increase of esters in the inoculated wines, in fact it was lower in most of them. This is in agreement with the esterase activity found in *Brettanomyces/Dekkera,* responsible for the formation of ethyl esters, along with the hydrolysis of acetate esters, such as isoamyl acetate, described to occur in beer (Spaepen and Verachtert, 1982) and in wines (Romano et al, 2008). Curtin *et al*., (2013) also got an increase in most of the esters analysed by three strains of *D. bruxellensis*. Ethyl acetate increased in most of the wines mainly in those inoculated with *D. bruxellensis* PYCC4801. The increase of ethyl acetate might be related with the production of acetic acid, which is known in *Brettanomyces/Dekkera* and it is stimulated by the presence of oxygen (Ciani *et al.*, 2003).

The concentration of the two fatty acids acids analysed, isovaleric acid and caprylic acid (octanoic acid) was higher in the inoculated wines. But the differences were more notorious and statistically significant on Cabernet Sauvignon wines. Again, *D. bruxellensis* PYCC4801 produced, in general, higher values than strain 21. The production of isovaleric acid by *Brettanomyces/Dekkera* is well documented (Renouf and Lonvaud-Funel, 2007; Licker *et al*., 2008) but the production of caprylic acid is less known. Both compounds give unpleasant rancid-like smell and taste. The metabolic pathways involved in the production of fatty acids and the conditions influencing their production by *Brettanomyces/Dekkera* are not yet well elucidated, but it was shown that the degradation of the amino acids L-leucine, L-isoleucine and L-valine is involved in the formation of respectively isovaleric acid, 2-methylbutyric and isobutyric acid (Oelofse, 2008).

The concentration of the three monoterpenes analysed didn't change or was lower in the Touriga Nacional wines but it was found to be higher in the inoculated Cabernet Sauvignon wines in comparison to the non-inoculated wines. It has been shown that *Brettanomyces/Dekkera* strains are able to release glycosidically bound terpenes, due to beta-glucosidase activity, from naturally present glycosides, thereby increasing free aroma active compounds (Vilhena *et al*., 2007). It is interesting to note a grape variety effect, which can be related with different compositions on glycosidically bound terpenes between Touriga Nacional and Cabernet Sauvignon.

The results show that apart from the volatile phenols, *D. bruxellensis* is capable of changing the composition of other chemical families of wines. The changes provoked by these organisms were similar in both grape varieties except for terpenic compounds.

Table 5.8. Esters, fatty acids and terpenes of three wines of Touriga Nacional 60 days after inoculation with *D. bruxellensis* 21 or *D. bruxellensis* PYCC4801. Different letters indicate statistically significant differences between the samples in each wine.

	TN75			TN76			TN77		
Compounds (mg/L)	C*	Db21	Db4801	C	Db21	Db4801	C	Db21	Db4801
Ethyl hexanoate	0,45a	0,90a	1,40b	0,60a	1,14b	2,00c	0,52a	0,90a	1,30b
Ethyl octanoate	nd	0,46a	0,50a	nd	1,00a	1,03a	0,28a	0,57a	0,62a
Ethyl decanoate	nd	0,17a	0,15a	0,46a	0,30a	0,24a	nd	0,14a	0,17a
Ethyl dodecanoate	nd	0,81a	0,81a	0,15a	1,11b	1,09b	0,07a	0,60b	0,63b
Sum ethyl esters	0,45	2,34	2,86	1,21	3,55	4,48	0,87	2,21	2,72
Ethyl acetate	173,10a	150,00a	180,30a	78,70a	94,70a	112,70a	132,40a	71,50a	96,90a
Isoamyl acetate	0,80a	0,92a	1,26b	1,35a	1,55a	1,73a	0,79a	0,70a	0,78a
2 Furfural	0,72a	1,24b	1,39b	0,58a	0,45a	0,88a	0,53a	1,02b	1,07b
Isovaleric acid	0,50a	0,64a	0,73a	0,77a	0,77a	1,17b	0,75a	0,80a	1,08b
Octanoic acid	0,31a	0,41a	0,74a	0,89a	1,37ab	1,91b	0,41a	0,60a	0,80a
Compounds (µg/L)									
Linalool	nd	19,26a**	22,71a	155,24a	114,44ab	126,48b	49,00a	nd	nd
α Terpineol	nd	22,19ab	26,26b	26,61a	39,51b	43,25b	19,16a**	22,15a	23,21a
Citronellol	nd	nd	nd	nd	18,1a**	18,33a**	nd	46,40b	nd
Sum terpenes		22,19	48,97	181,85	172,05	188,06	68,16	76,05	24,78

*C – control, non-inoculated wines stored under the same conditions as treated wines.
** < LQ
nd - not detected

Table 5.9. Esters, fatty acids and terpenes of three wines of Cabernet Sauvignon 60 days after inoculation with *D. bruxellensis* 21 or *D. bruxellensis* PYCC4801. Different letters indicate statistically significant differences between the samples in each wine.

	CS78			CS79			CS80		
Compounds (mg/L)	C*	Db21	Db4801	C	Db21	Db4801	C	Db21	Db4801
Ethyl hexanoate	0,70a	1,19b	1,76b	0,63a	1,00b	1,52c	0,50a	0,84ab	1,30b
Ethyl octanoate	0,21a	1,04b	1,03b	0,22a	0,58ab	1,01b	0,28a	0,63b	0,73b
Ethyl decanoate	nd	0,23b	0,21b	nd	0,20b	0,36b	0,09a	0,27b	0,21b
Ethyl dodecanoate	nd	nd	nd	nd	nd	0,04b	nd	0,07b	0,05b
Sum ethyl esters	0,91	2,46	3,00	0,90	1,78	2,93	0,87	1,81	2,29
Ethyl acetate	80,48a	113,49b	115,48b	126,25a	76,99b	116,24ab	113,74a	107,08a	127,43b
Isoamyl acetate	0,78a	1,65b	1,09ab	0,95a	0,66b	0,98a	0,81a	2,82c	2,08b
2 Furfural	0,43a	0,84b	0,89b	0,73a	0,80a	0,66a	0,60a	0,15b	0,40a
Isovaleric acid	0,86a	0,86a	1,22b	0,76a	0,93a	1,12b	1,01a	1,50a	1,39a
Octanoic acid	0,76a	1,18b	1,74c	0,91a	1,46b	1,76b	0,78a	0,97a	1,08a
Compounds (µg/L)									
Linalool	nd	25,26a	29,02a	56,60a	59,60a	62,54a	nd	33,15a	29,55a
α Terpineol	nd	44,03a	48,14a	23,20a	26,98a	43,53b	nd	nd	nd
Citronellol	nd	32,56a	38,84a	nd	nd	23,36c	nd	nd	nd
Sum terpenes		101,85	116,00	79,80	83,58	129,43		33,15	29,55

*C – control, non-inoculated wines stored under the same conditions as treated wines.
nd - not detected

5.4. References

Ciani, M., Maccarelli F., Fatichenti, F. 2003. Growth and fermentation behaviour of *Brettanomyces*/*Dekkera* yeasts under different conditions of aerobiosis. *World Journal of Microbiology & Biotechnology* **19**: 419–422.

Conterno, L., Aprea, E., Franceschi, P., R., Vrhovsek, U., 2013: Overview of *Dekkera bruxellensis* behavior in an ethanol-rich environment using untargeted and targeted metabolomic approaches. *Food Res. Int.* **51**: 670-678.

Curtin, C. D., Geoffrey Langhans, Paul A., Henschke, Paul R. Grbin. 2013. Impact of Australian *Dekkera bruxellensis* strains grown under oxygen-limited conditions on model wine composition and aroma. *Food Microbiology,* **36**, 241-247

Ginjom, Irine, D'Arcy, Bruce, Caffin, Nola, & Gidley, Michael. (2011). Phenolic compound profiles in selected Queensland red wines at all stages of the wine-making process. *Food chemistry, 125*(**3**), 823-834.

Hernández, Teresa, Estrella, Isabel, Pérez-Gordo, M, Alegría, E. G, Tenorio, Carmen, Ruiz-Larrrea, Fernanda, & Moreno-Arribas, M. V. (2007). Contribution of malolactic fermentation by *Oenococcus oeni* and Lactobacillus plantarum to the changes in the nonanthocyanin polyphenolic composition of red wine. *Journal of agricultural and food chemistry*, 55(**13**), 5260-5266.

Joseph, C. M. L., Gorton, L. W., Ebeler, S. E. and Bisson, L. F. 2013. Production of volatile compounds by wine strains of *Brettanomyces bruxellensis* grown in the presence of different precursor substrates. *American Journal Enology Viticulture*. **64**:231-240.

Lamuela-Raventós, Rosa Ma, & Waterhouse, Andrew L. 1994. A direct HPLC separation of wine phenolics. *American Journal of Enology and Viticulture*, **45(1**), 1-5.

Oelofse, A., Pretorius, I., Toit, M. Du, 2008. Significance of *Brettanomyces* and *Dekkera* during winemaking: a synoptic review. South African *Journal of Enology and Viticulture* **29**, 128-144.

Oliveira, Carla Maria, Barros, António S, Ferreira, António César Silva, & Silva, Artur MS. (2015). Influence of the temperature and oxygen exposure in red Port wine: A kinetic approach. *Food Research International,* **75**, 337-347. doi: 10.1016/j.foodres.2015.06.024

Oro L, Canonico L, Marinelli V, Ciani M and Comitini F (2019). Occurrence of *Brettanomyces bruxellensis* on Grape Berries and in Related Winemaking Cellar. *Frontriers in Microbiology* **10,** 1-10.

Renouf, V.; Lonvaud-Funel, A. 2007 Development of an enrichment medium to detect *Dekkera/Brettanomyces bruxellensis*, a spoilage yeast, on the surface of grape berries. *Microbiological Research* 162, (**2**), 154-167.

Romano, A., Perello, M. C., Lonvaud-Funel, A., Sicard, G., & de Revel, G. (2009). Sensory and analytical re-evaluation of ‘‘Brett character’’. *Food Chemistry*, **114**, 15–19.

Spaepen, M., Verachtert, H., 1982. Esterase activity in the genus *Brettanomyces*. *J. Inst. Brew.* **88**, 11–17.

Villena, M.A., Iranzo, J.F.Ú., Pérez, A.I.B., 2007. β-Glucosidase activity in wine yeasts: application in enology. *Enzyme Microb*. *Technol*. **40,** 420–425.

CHAPTER 6

General conclusions

Chapter 6 - General discussion

6.1. General discussion and conclusions

Brettanomyces/Dekkera represent a significant wine spoilage concern due to the capacity to impart undesirable aromas in wine, predominantly through the decarboxylation of hydroxycinnamic acids to vinylphenols and the subsequent reduction to ethylphenols. Isolates have been obtained from wine regions all over the world, but there hasn't been much effort on collecting strains from Portuguese wines.

In first place, this work corroborates that among the *Brettanomyces/Dekkera* genera, *D. bruxellens*is is the main, or most probably the only yeast species responsible for the production of volatile phenols in wines. The involvement of *D. anomala* is still a point of discussion, but recent studies and also this work strongly suggest that *D. bruxellensis* is the key organism. The cultural methodology followed seemed to be suitable for the detection of *Brettanomyces/Dekkera* in wine. The identification of the strains by a DNA based technique confirmed the ability of the media used to selectively isolate *Brettanomyces/Dekkera.* Molecular methods have not been often used to confirm the presumptive detection of *Dekkera/Brettanomyces* achieved by cultural based procedures. This is important for the validation of the robustness of more conventional detection/identification methods. The relatively high percentage of contaminated wines found (37%) demonstrates that the efforts put by wine producers to eradicate the contamination of wines by these spoilage yeasts are still not sufficient.

From the many factors that influence the growth and activity of *D. bruxellens*is, the tolerance to ethanol was characterised, especially focusing on the determination of the limiting ethanol concentration for growth. Phenotypic variation was found among the strains studied, the most tolerant being able to grow in the presence of 14% v/v ethanol. Several studies have highlighted the genetic diversity of *D. bruxellensis* inferring the possibility that some strains might present a greater risk of wine spoilage than others. Furthermore, the effect of growth at different concentrations of ethanol on the survival of *D. bruxellens*is in wine stressful conditions was evaluated. A valuable collection of wine isolates of *D. bruxellensis* was created. Survival and metabolic studies in monovarietal wines were carried out using strains selected from the most tolerant group.

Several grape varieties were characterised and compared focusing on the volatile phenols and on the respective precursor compounds. It was found that these exist mostly as esters of tartaric acid in all grape varieties studied. As for the precursors of volatile phenols in the free form (hydroxycinnamic acids: p-coumaric, ferulic and caffeic), the predominant hydroxycinnamic acid was p-coumaric acid, which is in contradiction with several studies that pointed out caffeic acid as the predominant phenolic acid. Thus, the work of this thesis shows that the production of volatile phenols in wines would mostly depend on the presence of p-coumaric acid. The calculation of the bound/free molar ratios of the precursors of volatile phenols shows big differences between grape varieties, with Touriga Nacional exhibiting the highest difference between bound and free forms. A p-coumaroylated anthocyanin (malvidin-3-O-(6-p-coumaroyl)-glucoside) was detected and was, for the first time, deduced as a potential source of free p-coumaric acid, hence potentially increasing the concentration of an important volatile phenol precursor.

Twenty-two % of the wines analysed had volatile phenols in concentrations above the perception threshold in red wines. The high concentration found in Vinhão may be correlated with the high concentration of precursors in the free form (p-coumaric acid). Following this reasoning, we could also expect high levels of volatile phenols in Syrah wines, which was not the case. Touriga Nacional displayed relatively low amounts of free precursor compounds and, from empirical evidence, wines from this grape variety pose a high risk to develop volatile phenols. Thus the availability of precursors on the free form may not be the only factor explaining the potential of wines to develop volatile phenols.

The ratio 4-ethylphenol/4-ethylguaicol was found to vary greatly, from 1.5:1 to 47:1, showing that this ratio can be very different from what is normally reported (10:1). Differences were found between grape varieties, for example 5:1 for Cabernet Sauvignon and 10:1 for Syrah, while Castelão and Trincadeira presented very high ratios, 15:1 and 37:1 respectively. This work shows relevant differences on the phenolic composition of wines from different grape varieties, being important here to highlight the significant differences found on the precursors of volatile phenols, both on the quantity and the relative proportions of these molecules in each grape variety.

The survival and metabolism of hydroxycinnamic acids by *D. bruxellensis* in monovarietal wines was studied using one the strains isolated in Chapter 2. Wines from Touriga Nacional, Cabernet Sauvignon and Syrah grape varieties were contaminated with

this strain and yeast growth and survival were monitored by viable counting in solid medium and by flow cytometry using the fluorescent dyes. *D. bruxellensis* acquired the VBNC state on all wines of each grape variety studied soon after the inoculation. Yeasts were able to recover the culturable state in most wines, but the time required for the recovery was highly dependent on the wine, being longer on the Syrah wines. Although the entrance of *Brettanomyces*/*Dekkera* on the VBNC state has been commonly described as being induced by the exposure of cells to SO_2, our results strongly suggest that other stress factors may be involved as potential inducers of the VBNC state. Populations on this physiological state were able to produce volatile phenols, meaning that are metabolically active, thus causing the spoilage of the wine. The metabolism of hydroxycinnamic acids by VBNC cells is, however, different from what it would be expected from the activity of *Brettanomyces*/*Dekkera*, leading to the accumulation of vinylphenols at relatively high levels. The survival capacity of *D. bruxellensis,* as determined by flow cytometry, was higher on Touriga Nacional wines than in Cabernet Sauvignon and Syrah wines, which is in accordance with the empirical evidence that wines from this grape variety show a consistent high susceptibility to develop volatile phenols. The different susceptibility of wines to *D. bruxellensis* spoilage may be correlated with the phenolic composition of wines, specifically the bound/free molar ratio and the total concentration of phenolic acids on the free form. The work of Chapter 3 revealed that the concentration of free hydroxycinnamic acids in Touriga Nacional wines is relatively low in comparison with other seven grape varieties. In fact, the precursors of this grape variety were found mainly in bound forms (coutaric and caftaric acids and malvidin-3-O-(6-p-coumaroyl)-glucoside). This specific phenolic composition may be related with the observed higher survival capacity of *D. bruxellensis* on Touriga Nacional wines. Some works demonstrated the antimicrobial capacity of hydroxycinnamic acids over *Brettanomyces*/*Dekkera* and other wine microorganisms. The bound/free molar ratio and the total concentration of phenolic acids on the free form may be to be relevant factors to explain the differences on the susceptibility of wines to *Brettanomyces*/*Dekkera* spoilage. Thus, control strategies based on the manipulation of specific phenolic compounds should be studied and implemented in future work.

Besides the production of volatile phenols, *D. bruxellensis* was able to modify the composition of wines through the production of other metabolites. The two strains used showed esterase activity leading to the increase on the concentration of ethyl esters,

including ethyl acetate. The fatty acids isovaleric acid and caprylic acid also increased in the inoculated wines. The concentration of monoterpenes analysed increased in the Cabernet Sauvignon wines but not in Touriga Nacional wines. The grape variety effect observed can be related with different compositions of glycosidically bound terpenes subjected to the ß-glycosidase activity of *D. bruxellensis.*

The data gathered in this dissertation made an original contribution to the current knowledge on the phenolic composition associated with the production of volatile phenols in wines from different grape varieties and on the behaviour and activity of *D. bruxellensis* in these wines. To summarize the conclusions, the highlights of this thesis work are:

- Corroboration that *Dekkera bruxellensis* is the main microorganism associated with the production of volatile phenols in wines.
- Wine strains exhibit phenotypic variation regarding the tolerance to ethanol.
- The precursors of volatile phenols in wines exist mostly as esters of tartaric acid (caftaric, coutaric and fertaric acids).
- The predominant hydroxycinnamic acid is p-coumaric acid but relevant differences exist among grape varieties.
- Grape varieties have different proportions of bound/free forms of hydroxycinnamic acids.
- *D. bruxellensis* acquired the VBNC state in wines due to stress factors other than SO_2
- VBNC cells were metabolically active, favouring the accumulation of vinylphenols rather than ethylphenols.
- The survival capacity of *D. bruxellensis* was higher on Touriga Nacional wines than on Cabernet Sauvignon and Syrah wines.
- Wine susceptibility to *D. bruxellensis* may be correlated with phenolic acids composition.
- *D. bruxellensis* modified the composition of wines through the production of ethyl esters and fatty acids in Touriga Nacional and Cabernet Sauvignon wines. An increase in free monoterpenes was observed only on Cabernet Sauvignon wines.

6.2 Suggestions of future work

To proceed with the characterisation of grape varieties and also evaluate to what extent viticultural practices, the degree of ripeness, the sunlight exposure, nitrogen availability and the disease status of the grapes influence the composition of precursors of volatile phenols - the concentration of free hydroxycinnamic acids and the proportion of bound and free compounds. Likewise, the effect of some winemaking procedures, such as maceration, the use of enzymes, the activity of yeasts and lactic acid bacteria during the alcoholic and malolactic fermentations, respectively and the ageing on lees should also be better studied.

Further studies should be carried out to better characterise the levels of total hydroxycinnamic acids that exert a significant inhibitory action against *Brettanomyces*/*Dekkera* in wine conditions and whether this inhibition could be exploited as a control strategy. This effect has been previously reported, especially for ferulic acid. Ethanol, acidity and SO_2 may have a synergistic effect with ferulic acid, which deserves more in-depth studies. Future research should focus on identifying how precursors of volatile phenols on the bound form can be converted into free hydroxycinnamic acids, thus making them available to be metabolized by *Brettanomyces*/*Dekkera.*

Also, as a strategy to solve the problem of volatile phenols in wines, an interesting line of research would be the investigation of methods to remove or to decrease the amount of hydroxycinnamic acids by a biological process that converts them into harmless compounds, thus lowering the potential of the wine to form volatile phenols. It is known that hydroxycinnamic (*p*-coumaric and ferulic) acids can be metabolised by microorganisms other than *Brettanomyces/Dekkera* through different pathways, so the potential roles of these organisms in the modulation of the abundance of the substrates/precursors of volatile phenols could be explored.

Another approach would be to decrease the risk of microbial spoilage by the inactivation of the microbial populations of *Dekkera/Brettanomyces* by heat treatment. This would involve the characterisation of the heat resistance (time/ temperature) and the effect of factors such a pH, ethanol concentration, phenolic compounds and SO_2. Sensory evaluation of the effect of the heat treatment on the organoleptic properties of wines should

be carried out. The aim would be to generate data useful for the conception of regimes for the inactivation of *Dekkera/Brettanomyces* in wines and contaminated equipment.