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Indigenous Yeasts in Madeira Wines
Impact on the physicochemical and sensorial characterization

DOCTORAL THESIS

Andreia Fátima Santos Miranda
DOCTORATE IN CHEMISTRY

UNIVERSIDADE da MADEIRA
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ORIENTATION
José Carlos Antunes Marques

Dedicado aos meus pais, irmãos, Henrique e Matilde

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RESUMO

O Vinho Madeira é produzido pela fermentação espontânea através de leveduras indígenas presentes em uvas e adegas. A crescente exigência dos consumidores leva à necessidade de uniformizar o processo de vinificação. O presente trabalho visa avaliar a composição química de vinhos Madeira produzidos em diferentes adegas e com uvas colhidas de diferentes localizações (sul e norte). Depois, isolar e identificar as leveduras indígenas de mostos da casta Tinta Negra de diferentes localizações e adegas, e avaliar o impacto do uso de 5 leveduras indígenas não-*Saccharomyces* selecionadas *(Hanseniaspora uvarum, Starmerella bacillaris, Pichia terricola, Pichia fermentans* e *Pichia kluyveri*) na produção de vinho Madeira, como culturas puras iniciadoras, na composição química, perfil volátil e características sensoriais. Os resultados mostraram variabilibidade na composição química nas diferentes adegas, e baixa reprodutibilidade entre lotes da mesma adega. Uvas da região sul apresentaram níveis mais elevados de açúcares (196.32 g/L), polifenóis totais (270.18 mg GAE/L) e potencial antioxidante (128.48 mg Trolox/L). Os estudos da microflora de leveduas indígenas envolvidas na produção de vinho Madeira permitiram identificar 11 espécies nas adegas (*Hanseniaspora uvarum, Starmerella bacillaris, Pichia kluyveri, Pichia fermentans, Saccharomyces cerevisiae, Torulaspora delbrueckii, Candida apicola, Cystobasidium minutum, Pichia terricola, Cystobasidium slooffiae,* e *Wicheramomyces anolalus*) e 6 espécies nas uvas (*Hanseniaspora uvarum, Starmerella bacillaris, Pichia kluyveri, Pichia fermentans, Saccharomyces cerevisiae,* and *Hanseniaspora opuntiae*). Os vinhos produzidos por *Pichia* spp. demonstraram níveis mais elevados de polifenóis totais (367.68 mg GAE/L), enquanto os vinhos produzidos por *Starmerella bacillaris* demonstraram maior acidez (9.23 g/L) e menor conteúdo de voláteis (11.26%). O vinho produzido por *Pichia kluyveri* destacou-se na concentração total de volatéis (24.50%), dos quais 77.35% são estéres, obtendo 37% de preferência dos consumidores, com notas intensas de caramelo e especiarias. Portanto, *Pichia kluyveri* demonstrou potencial para ser utilizada como cultura iniciadora de fermentação na produção de vinho Madeira.

Palavras – chave: leveduras não-*Saccharomyces*; Vinho Madeira, leveduras indígenas; processo de vinification; composição química; análise sensorial.

SUMMARY

Madeira wines are produced by spontaneous fermentation with indigenous yeasts from grapes and wineries. Increasing demands from consumers have led to the need to standardize the winemaking process. This work aims, first, to evaluate the chemical composition of Madeira wines produced by different wineries and with grapes from vineyards located in the south and north regions. Then, to isolate and identify indigenous yeasts in Tinta Negra grape musts from different vineyard locations and wineries and evaluate the impact of using five selected indigenous non-*Saccharomyces* yeasts (*Hanseniaspora uvarum, Starmerella bacillaris*, *Pichia terricola*, *Pichia fermentans,* and *Pichia kluyveri*), as a starter culture, in the chemical composition, volatile profile, and sensory characteristics of Madeira wines. Results showed variability in the chemical composition of samples from different wineries and a lack of reproducibility between batches from the same winery. Grapes from southern vineyard locations presented higher levels of sugars (196.32 g/L), total phenolic content (270.18 mg GAE/L), and antioxidant potential (128.48 mg Trolox/L). Studies about the indigenous yeasts involved in Madeira wine production identified 11 yeast species from wineries (*Hanseniaspora uvarum, Starmerella bacillaris, Pichia kluyveri, Pichia fermentans, Saccharomyces cerevisiae, Torulaspora delbrueckii, Candida apicola, Cystobasidium minutum, Pichia terricola, Cystobasidium slooffiae,* and *Wicheramomyces anolalus*) and 6 yeast species from vineyards (*Hanseniaspora uvarum, Starmerella bacillaris, Pichia kluyveri, Pichia fermentans, Saccharomyces cerevisiae,* and *Hanseniaspora opuntiae*). Wines produced by *Pichia* spp. showed higher total phenolic content (367.68 mg GAE/L), while wines produced by *Starmerella bacillaris* showed higher acidity (9.23g/L) and lower volatile content (11.26%). Wines produced by *Pichia kluyveri* revealed higher total volatile content (24.50%), of which 77.35% were esters, and a score of 37% in a preference test by regular wine consumers, described with intense caramel and spicy notes. Therefore, *Pichia kluyveri* showed potential to be used as a starter culture in the production of Madeira wines.

Keywords: non-*Saccharomyces* yeasts; Madeira wine; indigenous yeasts; vinification process; chemical composition; sensorial analysis.

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List of abbreviations

A_{420} – Absorbance at 420 nm

A_{520} – Absorbance at 520 nm

AAB - Acetic acid bacteria

AOAC - Association of Official Analytical Chemists

AP - Antioxidant Potential

AU - Absorbance units

BI - Browning Index

°C - Degree Celsius

°C/min - Degree Celsius per minute

CI - Color intensity

CO_2 - Carbon dioxide

cfu - colony-forming unit

DBDM - *Dekkera/Brettanomyces* Differential Medium

DRM - Demarcated Region of Madeira

DCM - Dichloromethane

DPPH - 2,2-diphenyl-1-picrylhydrazyl

EtOH - Ethanol

EU - European Union

GAE - Gallic acid equivalent

g/L - Gram per litre

GC-MS - Gas chromatography coupled to mass spectrometry

GYP - Glucose yeast peptone agar

ha - Hectares

HMF - 5-hydroxymethylfurfural

HPLC - High-performance liquid chromatography

HS-SPME - Headspace solid-phase microextraction

IS - Internal standard

IVBAM - Instituto do Vinho, do Bordado e do Artesanato da Madeira

KI - Kovats index

LAB - Lactic acid bacteria

LOD - Limit of detection

LOQ - Limit of quantification

m/z - Mass-to-charge ratio

M0 - Grape juice

MBF - Must before fortification

MeOH - Methanol

MLF - Malolactic fermentation

n.d. - Not detected

n.q. - Not quantified

NaOH - Sodium hydroxide

NIST - National Institute of Standards and Technology

OD - Odor descriptor

OIV - International Organisation of Vine and Wine

OT - Odor threshold

PCR-RFLP - Polymerase chain reaction - restriction fragment length polymorphism

SD - Standard deviation

SPE - Solid-phase extraction

TA - Total acidity

TP - Total Phenolic Content

VA - Volatile acidity

VLQPRD - Vinho Licoroso de Qualidade Produzido em Região Determinada

v/v - Volume to volume

VOCs - Volatile organic compounds

WAE - Wine after estufagem

WAF - Wine after fortification

µg/L - Microgram per litre

ZDM - *Zygosaccharomyces* Differential Medium

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LIST OF PUBLICATIONS

ARTICLES TO BE SUBMITTED:

- **Miranda A**., Pereira V., Jardim H., Marques JC. Evaluation of volatile profile, antioxidant potential and color during the alcoholic fermentation of Madeira wines musts in different wineries.

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- **Miranda A.**, Pereira V., Jardim H., Malfeito-Ferreira M., Marques JC. Volatile profile, chromatic characteristics and sensorial analysis of Madeira wines produced by selected indigenous non-*Saccharomyces* yeasts.

ARTICLES IN REVISION:

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- Leça, J. M., Pereira V., **Miranda A.,** Vilchez J. L., Marques J.C, Malfeito-Ferreira M., Impact of Indigenous Non-Saccharomyces Yeasts Isolated from Madeira Island Vineyards on the Formation of Ethyl Carbamate in the Aging of Fortified Wines, Processes. 2021 (9), 799.

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- **Miranda, A.,** Pereira, V., Marques, J.C. Avaliação do efeito da inoculação de diferentes leveduras indígenas na produção de Vinho Madeira: Atividade antioxidante e polifenóis totais. 6th Infowine Forum: Congresso Internacional de Vitivinicultura, Vila Real, Portugal, 23-24 maio, 2018.

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PART I – GENERAL INTRODUTION

1. MADEIRA WINE

1.1. BRIEF INTRODUCTION

Madeira wine is an internationally recognized fortified wine (17 to 22%, v/v) with a significant impact on the Madeira Island economy. The Island is part of the Madeira archipelago with Porto Santo, Desertas, and Selvagens islands. It is located in the Atlantic Ocean, about 600 km from the North African coast and 970 km southwest of Lisbon (Portuguese capital). The moderate climate and the characteristics of the soil (near the sea, from volcanic origin, rich in organic matter and mineral content, mainly magnesium and iron) greatly impact Madeira wine quality (1, 2). In 1913, Madeira Island obtained the status of Demarcated Region of Madeira (DRM), representing one of the oldest Demarcated Regions of the country (3).

Madeira wine history has been well studied by several authors (2, 4). Briefly, the first mark in the history of Madeira wines exportations was at the end of the 15th century with the discovery of America by Christopher Columbus (2). In the middle of the 18th, during the Age of Exploration, the fortification step was introduced to prevent the wine from spoiling during trips to the New World and East Indies. Soon, the wine producers discovered that the wine exposition to heat during the long sea expedition improved the wine quality. So, in 1794 Pantaleão Fernandes introduced the *estufa* procedure. Currently, the Madeira winemaking process continues to involve the so-called *estufagem* procedure that consists in heating the wine to temperatures of up to 50 ºC for 3 to 4 months (4).

Nowadays, the commercialization of Madeira wines continues to be one of the region's main economic activities. In 2021, Madeira wine commercialization stood at about 31.419 hectoliters (hl), being the European Union (EU) the primary market with 66.3% of sales, especially France (27.8%), Portugal (13.7%), Germany (10.1%) and Belgium (6.1%). In the Portuguese national market (13.7%), the Madeira region assumes considerable importance representing 9.3% of wine sold. Outside the EU, the main markets for Madeira wines are the United Kingdom (10.1%), the United States of America (7.6%), Japan (6.4%), and Switzerland (2.4%) (5).

1.2. VITICULTURE, VINIFICATION AND WINE AGING

Madeira wine has a long tradition and sound reputation in fortified wines dating back to the 18th century, as mentioned previously. Madeira Island´s soil characteristics, climate, grape varieties, and peculiar winemaking and aging processes results in a unique product worldwide known.

1.2.1. Wine growing region

The total area of Madeira Island is about 732 km^2, while the wine-growing region of Madeira wine is about 500 hectares (ha), mostly distributed in three regions: one on the south coast (about 188 ha in Câmara de Lobos) and two in the north coast, namely São Vicente (around 142 ha) and Santana (about 70 ha) (see Fig. 1).

Figure 1 - Main wine growing region of Madeira Island (6).

The traditional conduction system of the vines is commonly known as *latada* or *pergola* system, where the vines are horizontally disposed on wires and suspended off the ground by stakes (at a height between 1 and 2 m) (Fig. 2a). The plantation densities range from 2500 to 4000 plants/ha, and the irrigation is done through canals (called *levadas*) that bring water from the upper points of the island. Since the agricultural land is usually small terraces known as *poios*, sustained by walls of basaltic stones with sharply elevated terrains, mechanization is almost impossible. So, harvesting is still manual, increasing the costs of the entire process. In the second half of the 20th century,

in lands with soft slopes, a new conduction system (*espaldeira*) was introduced, enabling the vines to grow vertically on wired rows (Fig. 2b). The plantation densities in this system range between 4000 and 5000 plants/ha. Currently, about 80% of the vines are planted in *latada*, and the remaining 20% in *espaldeira* (1, 5) .

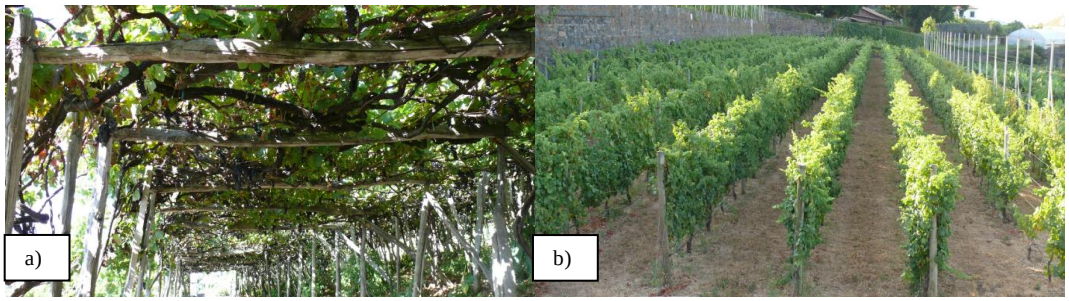


Figure 2 - Traditional conduction systems: *latada* (a) and *espaldeira* (b).

1.2.2. Grape varieties

All grape varieties used in the production of Madeira wines belong to the *Vitis vinifera* L. species. According to the Madeira wine official classification for grape varieties, these can be divided into *Recommended* and *Authorized* (see Table 1). However, the most common varieties (also called traditional varieties) are Sercial, Verdelho, Boal, and Malvasia (white varieties) and Tinta Negra (red variety) (5).

Table 1 - Recommended and Authorized grape varieties for Madeira wine production (5).

	Recommended	Authorized
Red varieties	Tinta Negra Bastardo Tinta da Madeira Malvasia Cândida Roxa Verdelho Tinto	Complexa Tinto Negro Triunfo Deliciosa
White varieties	Sercial Verdelho Boal (Malvasia Fina) Malvasia Cândida Terrantez	Listrão Caracol Carão de Moça Moscatel de Málaga Malvasia de S. Jorge Valveirinho Rio Grande

According to the residual sugar content, Madeira wines can vary from extra-dry to sweet wines (see Table 2), while in terms of color, they can range from very pale (typical for dry wines) to dark brown (sweet wines) passing through golden tones (5).

Table 2 – Madeira wine designations according to the sweetness degree (5).

Type of wine	Total sugar content (minimum limit) g/L	Total sugar content (maximum limit) g/L	Baumé scale
Extra-dry	Do not exist	49.1	< 0.5º
Dry	49.1	64.8	<1.5º
Medium-Dry	64.8	80.4	1.0º – 2.5º
Medium Sweet	80.4	96.1	2.5º - 3.5º
Sweet	96.1	Do not exist	>3.5º

In the next paragraph, a short description is provided according to the main characteristics of the recommended grape variety studied in the present work, namely Tinta Negra.

Tinta Negra is the grape variety most used for Madeira wine production. The introduction of this variety dates back to the 18th century, and it was planted again at the end of the 19th century after phylloxera devastated the vineyards. Tinta Negra is known to be more robust than the white varieties, being, for that reason, quite resistant to some pests. Additionally, it can be easily adapted to the specific conditions of Madeira Island. The grapes are black with a light pulp, elliptic-globose shape, vary from small to medium, and have soft and thin skins (Fig. 3). Tinta Negra musts can achieve 9 to 12% of the alcoholic potential. It is mainly cultivated in the south of the island (Câmara de Lobos and Funchal) and São Vicente, in the north. This variety accounts for 80 to 85 % of the total Madeira wine production. It can produce dry, medium dry, medium sweet, and sweet Madeira wines, according to the different levels of residual sugars (1, 5).

Figure 3 – *Tinta Negra* grape variety.

1.2.3. Winemaking and aging process [1, 5]

Approximately 1600 producers are registered as individual growers of grapes for the Madeira wine vinification. Indeed, considering the high number of individual growers, it is still difficult to establish wine traceability in terms of its grape-growing region even with all the efforts made. However, since measures were introduced in the 1980s to ensure uniformity and protect the traditional winemaking process, currently less variability is introduced during vinification.

Madeira wine results from a unique winemaking process, mainly characterized by spontaneous grape must fermentation by natural indigenous yeasts, present in the grapes and the wineries, and by its distinct aging process. The winemaking process of Madeira wine is schematized in Fig. 4, and the main phases are briefly described below.

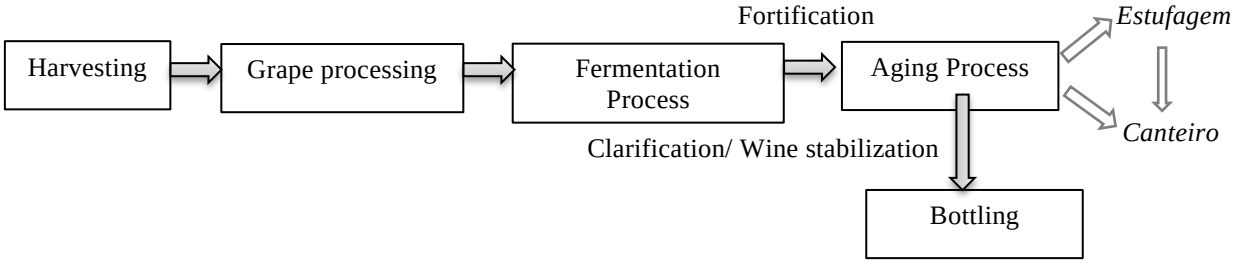


Figure 4 - Scheme of Madeira wine winemaking and aging process.

- Harvesting – The grapes are picked at their maturity peak (at the desirable sugar content and acidity), with the minimum alcohol potential required of 9%. This happens usually between the end of August and the middle of October. Then, the grapes are placed in boxes (25 and 50 kg) and transported to wine cellars, where the phytosanitary conditions are assessed. After this, the grapes are weighed, and the probable degree of alcohol is verified using a refractometer. The result of this analysis, together with the grape variety, set the price of the grapes. Finally, after being accepted, grapes start to be processed according to the type of wine intended.
- Grape processing – Grapes are first de-stemmed to avoid a bitter flavor in the final wine and then crushed to release the must and split berries. At this stage, a 5% aqueous solution of potassium metabisulphite ($K_2S_2O_5$) is usually added to avoid the growth of bacteria. Rectified concentrated must is also added to adjust sugar levels during or/and after fermentation to a maximum allowed amount of 8%. Madeira wine producers can also add pectolytic enzymes that promote a better extraction of volatile and non-volatile compounds, intensifying the color and aroma of the musts.
- Fermentation – The fermentation process of Madeira wines can be performed in two ways: *bica aberta* or *curtimenta*. In the first one, musts are fermented without the grape solids (skin and seeds) and it is usually used to produce dry and medium dry Madeira wines. On the other hand, the *curtimenta* process involves fermentation with grape solids and is commonly used to produce sweet and medium sweet Madeira wines. The fermentation process starts when yeast begins to digest the

fermentable sugars (mostly fructose and glucose) in the grape juice, converting them into ethanol and carbon dioxide. Although inoculated and selected pure yeast cultures are used worldwide in the wine industry, in Madeira winemaking, fermentation occurs spontaneously by yeasts naturally present in grapes and cellar equipment and tanks. Must fermentation occurs in stainless steel tanks in the wine producing-cellars, under controlled temperature (usually below 25 ºC) and density. The fermentation is carried out according to the type of intended wine (sweet, medium-sweet, medium-dry or dry). In this sense, sweet wines undergo a soft fermentation (not longer than 5 days) to maintain higher levels of residual sugar. In contrast, dry wines are submitted to a longer fermentation (up to 8 days) to obtain low sugar levels.

- Fortification step – The International Organisation of Vine and Wine (OIV) defines fortified wines as ones with a total alcohol content above 17.5 % (v/v). Moreover, at least 4% (v/v) of the final wine's alcohol content must come from the fermentation process (7). These specifications apply to the production of Madeira wines. During the fermentation step, the levels of sugars decrease at a ratio of 18 g/L to produce 1º (v/v) of alcohol. Once the desired sweetness levels are obtained (usually below 130 g/L), according to the type of wine intended, the fermentation is completely stopped by the addition of natural grape spirit (containing 95 % (v/v) of ethanol). This step also prevents the metabolism of lactic acid bacteria (LAB, transformation of malic acid into lactic acid), avoiding acetic acid formation. After the fortification procedure, the alcohol content of Madeira wines is usually between 17 and 18 % (v/v).
- Clarification and stabilization – Wine clarification and stabilization are generally performed by fining agents (bentonite clays and gelatins). This procedure takes about 30 days. Unlike filtration, these fining agents remove grape fragments, dead yeast cells, and other soluble substances (such as phenols, tannins, and proteins), improving the final wine's appearance. At this stage, some oenological corrections can be done, for example, to the alcohol content. Finally, the wines are decanted into other stainless steel tanks and submitted to the aging process.
- Aging process – Madeira wine aging can be achieved by two distinct processes: *canteiro* and *estufagem*. In the *canteiro* system, the wines are stored in oak casks for at least 2 years on the top floor of cellars. The wines are naturally warmed by the sun due to the temperatures of Madeira's sub-tropical climate. Also, this

oxidative aging is promoted deliberately since the wines are exposed to certain volume of air at the top of the cask, enabling the development of the wine's intense and complex aromas. Madeira wine's evaporation while in casks averages almost 4 to 5 % per year. The *canteiro* process is usually used for the aging of Madeira wines produced from white grape varieties (Sercial, Verdelho, Boal, and Malvasia) and for some high-quality wines made from the red variety Tinta Negra. *Canteiro* wines can only be commercialized after 3 years of aging. In the *estufagem* process, the wine is heated at 45 to 55 ºC for 3 to 4 months, in stainless steel tanks called *estufas*. Usually, the wines that undergo the *estufagem* aging process are made from the Tinta Negra grape variety. This process aims to accelerate aging. Then, according to Instituto do Vinho, do Bordado e do Artesanato da Madeira (IVBAM), which regulates the production of Madeira Wine, the wines undergo maturation for at least 90 days in oak casks on the wine cellar lofts. After this period, an evaluation is carried out by the winemaker, who decides if the wines are ready to be commercialized or should continue aging in oak casks. In order to ensure the wine's quality, IVBAM stipulated that the resulting wine can only be sold after October 31 of the second year from the harvest date.

- Bottling – Before bottling, the wines are submitted to several procedures, namely clarification and oenological corrections (addition of rectified concentrated must, ethanol, and/or sulfites) followed by filtration. After certification by IVBAM, ensuring that the analytical and organoleptic parameters are within the legal limits required for each wine type, the wine can be bottled, commonly in dark green or black bottles with 5, 37.5, 50, or 75 cL capacity.

1.3. MADEIRA WINE TRADITIONAL CATEGORIES [5]

Madeira wine is a VLQPRD (*Vinho Licoroso de Qualidade Produzido em Região Determinada*) and has the highest category attributed to wines, being designated as DOP (Protected Designation of Origin – Denominação de Origem Protegida). This designation is given to wines strictly produced in a specific geographical region. The natural and human resources involved must also be from the same region where the wine is produced, ensuring its quality and uniqueness. Also, all Madeira wine bottles in the market have the Selo de Garantia, a guarantee seal, from IVBAM, ensuring that its

production and sale were authorized and that the wine is according to the required standards.

The most common Madeira wines have 3, 5, 10, 15, and 20 years old (*blends*). Regarding the traditional categories of Madeira wines established by IVBAM (*Portaria nº 38/2015),* the wines can be classified into 3 categories: *Frasqueira, Colheita* and *Solera. Frasqueira* is produced solely from one of the recommended grape varieties and aged in oak casks for at least 20 years, representing Madeira wine's high quality. *Colheitas* are wines from a specific harvest, aged for at least 5 years in oak casks. *Solera* wines are produced from one recommended grape variety and aged in oak casks for at least 5 years. After this period, a volume of up to 10% can be withdrawn annually and replaced by equal amount of a younger wine from the same variety, up to 10 additions.

2. WINE CHEMICAL COMPOUNDS – A BRIEF INTRODUCTION

The followed section will provide a brief introduction about the wine chemical compounds evaluated in the present work, namely organic acids, phenols and volatile compounds.

2.1. ORGANIC ACIDS

2.1.1. Wine acidity

Acidity is one of the most important characteristics in wines, affecting the final wine quality. Acids contribute to the flavor of wines, conferring a fresh taste and modifying the sweetness perception. Sour-tasting wines originate from high acidity levels grapes, while wines with insufficient acidity are usually flat or insipid (8, 9). The concentration of acids in grapes depends on several factors, such as grape variety, soil fertility and irrigation, pruning, viral infection, and grape maturation (9). Indeed, high temperatures during grape ripening result in musts with high sugar content and low acidity (which leads to high pH). Wines with high pH are more susceptible to microbiological spoilage which, consequently, can cause organoleptic depreciation due to the accumulation of toxic products (8, 9). In this sense, several chemical methods (strictly regulated by the OIV) based on the addition of authorized organic acids (such as tartaric, malic, citric, and lactic) have been extensively used by wine producers in order to increase wine acidity (10). Recently, several authors have reported the increase in wine acidity by using biological methods, namely the addition of bacteria or yeast during the fermentation process, depending on the species and strain used in the inoculation (11-13).

In wineries, wine acidity is usually measured by the pH, representing the quantity and the acid strength (obtained by the dissociation constant, namely the proportion of the hydrogen ions liberated). Depending on the type of wine, the pH can vary between 2.9 and 3.9. In white wines, pH values usually range from 3.1 to 3.4, while for red wines, it ranges from 3.3 to 3.6 (14, 15). Additional to pH, there are two categories of wine acidity: volatile and total acidity. A briefly description of the two is given below.

- Volatile acidity (VA)

VA refers to the free and combined forms of volatile acids that can be easily removed by steam distillation (15, 16). VA determination can be performed by wine acidification or by steam distillation. In the first one, the wine is acidified with tartaric acid (approximately 0.5 g/20 mL), displacing the volatile acids from their salts (17). However, the most commonly used method for VA determination is according to the Association of Official Analytical Chemists (AOAC) procedure which consists of wine distillation followed by titration with a base (usually NaOH). The amount of NaOH used in the neutralization is directly proportional to the levels of volatile acids in the wine samples (16). The main volatile acid in wines is acetic acid, followed by other minor acids rarely present above their threshold levels, such as formic, propionic and butyric. For that reason, the VA is usually expressed in terms of acetic acid (15). Although formic acid concentration in wines is invariably very low, it can be formed as a metabolite in many biochemical reactions, namely in baking processes. Even at low concentrations, this volatile acid contributes positively to the wine's final quality, with both bactericidal and fungicidal effects (18). Another important compound involved in the wine's VA is ethyl acetate, which results from the esterification of acetic acid. In this sense, the monitoring of this oenological parameter must consider primarily acetic acid and ethyl acetate concentrations. The presence of these compounds at low concentrations can enhance the fruitiness flavor of the wine. However, above the perceptible levels, they can add undesirable aromas to wine (such as vinegar and nail varnish odors), evidencing microbiological problems (19, 20). Although there are legal tabulated limits in wines (VA <1.2 g/L expressed as acetic acid), according to the OIV legislation (10), the odor perception threshold is strongly dependent on the wine type and style (8, 19, 21, 22).

- Total acidity (TA)

TA contemplates the fixed and volatile acidities, consisting of all types of acids present in grapes, musts, and wines, mostly organic and inorganic acids and some amino acids (14). The fixed acidity represents the non-volatile organic acids and is usually expressed in terms of tartaric acid (14, 15). The main inorganic acids found in wines are dissolved gases (such as sulfurous and carbonic acids) and/or other acids such as sulfuric, nitric, hydrochloric and phosphoric. However, according to the OIV, once these acids are present in trace amounts (barely affecting the acidity perception) they

are not included in the expression of TA (14, 15, 23). In this sense, TA can be defined as the amount of organic acids in grapes or wines. In wineries, the TA is frequently determined (according to OIV methods) by the wine neutralization with a base (NaOH at 0.1 M) and expressed in terms of tartaric acid (23, 24). Depending on the type of wine, TA desired values can lie between 5.5 and 8.5 g/L (usually higher in white wines) (25). TA, together with the content of alcohol and sugar, contributes to the final balance of wine flavor (17).

2.2.2 Main organic acids in wines

The organic acids initially present in grapes have an important role during fermentation (through the induction of yeast growth and vitality) and in the maturation and color extraction, influencing the wine's sensory complexity and flavor balance. In addition to organic acids being essential to the wine's sensory qualities (fresh and sour attributes), they also contribute to microbiological, tartaric, and proteic stability (16, 25, 26). The main organic acids in wines are tartaric, malic, lactic, acetic, citric, and succinic. Some are initially present in grapes, as is the case of tartaric, malic, and citric acids, while the others occur during alcoholic and malolactic fermentations (acetic, lactic, succinic, and formic acid) (9, 14). As described in Fig. 5, all these organic acids are carboxylic acids whose acidity is associated with the functional carboxyl group (-COOH) and its ionization capacity to release hydrogen ions (H+) into aqueous systems (25).

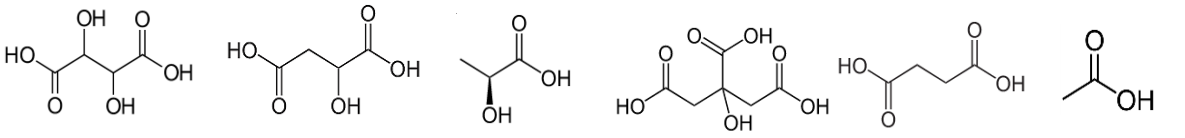


Figure 5 - Structure of main organic acids found in wines.

The evaluation of organic acids during the winemaking and aging processes is an essential tool to detect some eventual wine alterations or defects. Tartaric, malic, and citric acids contribute to about 90% of the wine's total acidity. Moreover, there are organic acids with enough volatility to contribute to the wine aroma, such as the case of acetic acid (8, 14).

A brief description of the main organic acids in wines is presented below.

Tartaric acid

Tartaric acid occurs naturally in many plants, being synthesized and accumulated in significant amounts in the *Vitaceae* genera, which is why it is called the "wine acidity" (14, 25). From the chemical point of view, tartaric is considered the strongest wine acid, with a pK_a of 3.1. It can lower the pH of the musts, inhibit bacterial growth, and acts as a preservative after fermentation (27).

Tartaric acid can be present in unripe grapes at very high levels (up to 15 g/L), mainly in the grape skin and the outer part of the pulp. This acid is not metabolized during the vine's ripening, maintaining its levels constant during this stage. Tartaric acid can be present in grapes in its natural form *L(+)*-tartaric acid (14, 27). In musts, the levels of tartaric acid are influenced by the region's climate. Indeed, in vineyards exposed to high temperatures, the tartaric acid levels are about 2 to 3 g/L, while in cooler regions, the levels increase to around 6 g/L. The levels of this organic acid in wines vary between 2 to 5 g/L, with higher levels found in white wines compared to red wines. Tartaric acid sets the wine's pH between 3.0 and 3.5 and, therefore, can affect the wine's final properties such as flavor, color, and chemical and microbiological stability (8, 14). In order to correct some acidity deficiencies, it is possible to add tartaric acid to musts (up to 1.5 g/L) and wines (up to 2.5 g/L), according to the legislation of the European Community (14).

Tartaric acid can be present in wines as a free acid or as tartrate salts. These tartrate salts tend to precipitate during fermentation and aging due to the ethanol increasing concentration, producing potassium bitartrate crystals. So, to avoid crystal deposition in the final product, a cold stabilization of wines (at temperatures below -7.2 ºC) is usually performed before the wines are bottled. However, the tartrates precipitation can continue to occur due to the conversion of the *L* forms into *D* isomers, which are less soluble. Also, other salts can contribute to the wine's stability, such as calcium salts. In this case, this salt's presence is more difficult to control since its precipitation is not activated by refrigeration. The precipitation of potassium and calcium salts is usually accompanied by an increasing wine pH due to the loss of TA (14, 27, 28).

Malic acid

Malic acid, one of the most important organic acids in grapes and wines, represents about half of the TA. This acid is also abundant in green apples and is

commonly called the "apple acid" (14, 15). In grapes, this acid is usually present in its natural form, *L(-)*-malic acid, which is structurally similar to tartaric acid. Even though the levels of malic acid are low in grape skins before the maturation period, its concentration increases in both skins and pulp after grape maturation (25). Therefore, the amount of malic acid is an important parameter in determining the harvest date since its concentration decreases during grape maturation. Before grapes color changes, the level of malic acid is about 25 g/L. As the color changes, there is an increase in grape size, and consequently, the levels of malic acid decrease by half due to dilution. Also, the amount of this organic acid in grapes strongly depends on the region's climatic conditions. Indeed, when grape maturation occurs in cool climates, the malic acid content is usually high (between 4 to 6.5 g/L) because it is not extensively synthesized or consumed, providing a sour taste to the final wine. On the other hand, when grapes mature in warmer regions, the levels of malic acid are usually around 1 to 2 g/L, producing wines with a flat taste. In order to avoid microbial wine spoilage, malic acid is usually manually added (acidification process) (14, 15, 27).

The acidification process can be performed before fermentation or during wine treatments and usually consists of adding tartaric acid within the legal concentration limits mentioned before (in tartaric section). On the other hand, when the levels of wine acidity are too high, it can be corrected through deacidification processes by adding potassium bicarbonate ($KHCO_3$) or calcium carbonate ($CaCO_3$) (14, 25). Another kind of wine deacidification can be achieved by malolactic fermentation (MLF). During the MLF, malic acid (pK_a 3.46) is decarboxylated enzymatically by the metabolic activity of the LAB, causing the transformation of the dicarboxylic acid (malic acid) into a monocarboxylic acid (lactic acid), increasing the pH. This kind of wine fermentation is more frequent in red wines than in white ones (14, 27, 29).

Lactic acid

The lactic acid found in wines originates during alcoholic and malolactic fermentations. As previously described, the main source of lactic acid is the MLF, through the action of LAB. LAB found in grapes, musts, and wines belong to the Lactobacillaceae (genus *Lactobacillus*) and Streptococcaceae (genus *Oenococcus* and *Pediococcus*) families (14, 17). LAB can also metabolize citric acid, producing other organic acids (lactic and acetic acids) and other compounds such as diacetyl and acetoin.

The concentration of glucose influences the extension of metabolization of each product (30).

During alcoholic fermentation, lactic acid can be produced via pyruvic acid. This acid is also usually produced during alcoholic fermentation through the metabolism of carbohydrates. However, it is quickly transformed into the lactic acid isomers *L(+)* from bacterial activity, and *D(-)* originated through yeasts (14). As such, *L*(+)-lactic acid is usually associated with the MLF (25). Therefore, the lactic acid levels found in wines depend on the type of wine produced and the type of fermentation involved since the wines that undergo MLF show higher levels of lactic acid (900 to 2600 mg/L) (8).

Citric acid

Citric acid is prevalent in citrus fruits but is scarce in ripe grapes, and for that reason, it is present in low levels (0.5 to 1 g/L) in wines (14). Citric acid plays an important role in the Krebs cycle during the grape's development and in the fermentation process by slowing the yeast's growth (27). During the must fermentation, the level of citric acid decreases due to its conversion into acetic acid by yeast metabolism. This oenological effect promotes the formation of acetonic compounds (mainly acetoin, diacetyl, and 2,3-butanediol) that greatly impact wine aromas (21).

Succinic acid

1,4-butanedioic acid, also called succinic acid, is a di-acid that is present in grapes in low concentrations. This non-volatile acid is mainly formed during alcoholic fermentation due to yeast metabolism (17). So, the succinate level in wines strongly depends on the yeast strain involved in the fermentation process. For example, the concentrations of succinic acid in the presence of *Saccharomyces cerevisiae* are variable and generally below 2 g/L, whereas its concentration was shown to be higher in wines fermented with *Saccharomyces bayanus* (21). This acid is important for the wine's microbiological stability, by providing resistance to bacterial attack and for the wine's final flavor, conferring a bitter and salty taste (15, 17, 27).

Acetic acid

Acetic acid is the main volatile acid found in wine and can contribute for the acidity and odor of wines. At low concentrations, acetic acid can contribute to wine

aroma by producing acetate esters that confer fruity notes. Contrarily, high concentrations of this acid can be detrimental to wines since it confers a vinegar odor and a disagreeable mouthfeel. Therefore, it is essential to control this volatile acid's levels to ensure the quality of the wine (8, 21).

Acetic acid formation in wines depends on the presence of yeasts, LAB, and acetic acid bacteria (AAB). Although most yeast species can produce acetic acid during the fermentation process, the amount of acid produced depends strongly on the species of yeast involved in this stage (17, 21,31). In addition, the concentration of this acid is closely related to initial sugar levels in the must, increasing with higher levels of sugars (17, 21). During MLF, the levels of acetic acid can increase due to the presence of LAB that metabolize the unfermented sugars present in musts (20). Moreover, the increase of acetic acid is also associated with the presence of AAB (from the genera *Acetobacter* and *Gluconobacter*) due to their ability to oxidize ethanol, producing acetic acid in an aerobic environment. These bacteria are favored in environments rich in sugars and alcohols. They can influence the wine quality through the contamination of grapes and during alcoholic fermentation and wine storage (15, 16, 20).

2.2. PHENOLIC COMPOUNDS

Phenols are secondary metabolites in plants derived from the acetate and shikimate pathways (32). These non-volatile compounds, widespread in nature, are important compounds found in grapes and wines that contribute to the wine's sensory properties, such as taste (bitterness and astringency), color (especially in red wines), and aroma, and are involved in protein interaction, oxidation reactions and other aging-associated processes (33). Grape phenolics are usually found in juice, pulp, seeds, and skins, having accumulated during grape ripening (32). Most of the phenols in wine originated from the grapes. However, their concentration is strongly influenced by several factors such as vineyard-related factors, the winemaking process, and wine storage conditions (see Table 3) (33-35). The MLF also contributes to the levels of phenols in wine due to deacidification where a decarboxylation of malic to lactic acid occurs, which promotes a pH change and, consequently, the hydrolysis of glucosides (36).

Table 3 – Factors that influence the levels of phenols in grapes, musts and wines (adapted from (35)).

Vineyards factors	Winemaking process	Wine storage
Grape variety Climatic conditions Organic and conventional cultivations Biostimulants	Pre-fermentative maceration Yeast strain and bacteria Additives Fining agents Post-fermentative maceration Filtration	Temperature Barrels

In addition to the phenol's oenological properties, they are known for having significant health benefits ("French paradox") by promoting the reduction of heart disease risks due to their pharmacological properties, such as antioxidant, anti-inflammatory, antiviral, antibacterial, antifungal, anti-allergic, anti-hypertensive, and anti-carcinogenic (37-39). However, phenols health effects depend on their levels and bioavailability. Higher levels of phenols are found in wines when compared to other beverages, which increases the wine's value (35).

The chemical structure of phenols is based on one or more hydroxyl groups directly connected to one or more aromatic rings (33). Additionally, their structure can vary from simple phenolic acid structures to a high molecular mass polymeric form (35). Also, phenols present in grapes are usually found in their conjugated form, either by direct bonds between a sugar and a carbon atom of the aromatic ring (C-glycosides) or by the connection to sugar residues through β-glycosidic bonds (O-glycosylated) (35, 39, 40). Phenols are mainly bonded to glucose (the most abundant sugar in grape skins), but also to other minor sugars (such as xylose, galactose, rhamnose, and arabinose), organic acids, lipids, amines, or even associated with other phenols (27, 35, 40).

Phenols are commonly classified into two groups: non-flavonoids and flavonoids. A brief characterization of each group is described below, according to the compounds evaluated in the present study.

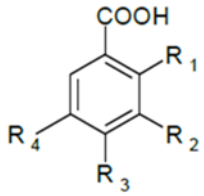
2.2.1. Non-flavonoids

Non-flavonoids are generally structurally simpler than flavonoids and are mainly composed of phenolic acids (hydroxybenzoic and hydroxycinnamic acids) and stilbenes (33, 35, 39). In red wines, these non-flavonoids concentration can range from 60 to 566 mg/L (41). Phenolic acids constitute the main fraction of wine phenolics, and

even though they are colorless in hydroalcoholic solutions, they can be oxidized into yellow compounds. Also, due to some yeast and bacterial action, phenolic acids can be precursors of volatile phenolic acids. Generally, the levels of volatile phenols in wines are low, but they can influence the wine's sensory characteristics, giving rise to unpleasant aromas due to their high odorant activity (21, 42). A brief description of phenolic acids and stilbenes is given below.

- Hydroxybenzoic acids

Hydroxybenzoic acids have a C_6-C_1 structure (an aromatic six-carbon ring with one carbon bonded to a carboxylic group). Several hydroxybenzoates can be found in wines, presenting different substitutions at the benzene ring (R_2, R_3, R_4, and R_5). The most common hydroxybenzoic acids in wines are gallic, *p*-hydroxybenzoic, vanillic, syringic, gentisic, and protocatechuic, as depicted in Fig. 6 (21, 27, 43).



Hydroxybenzoic acids	R_1	R_2	R_3	R_4
Gallic acid	H	OH	OH	OH
Gentisic acid	OH	H	H	OH
p-hydroxybenzoic acid	H	H	OH	H
Protocatechuic acid	H	OH	OH	H
Syringic acid	H	OCH_3	OH	OCH_3
Vanillic acid	H	OCH_3	OH	H

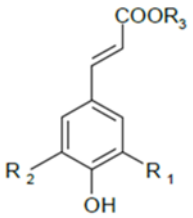
Figure 6 - Chemical structure of the hydroxybenzoic acids found in wines.

The concentration of hydroxybenzoic acids in red wines varies from undetectable to 218 mg/L (41). Frequently, gallic acid is the most abundant phenolic acid found in wines, with concentrations of about 70 and 10 mg/L for red and white wines, respectively (44). On the other hand, salicylic and gentisic acids are usually found in trace amounts (21, 42, 43). Additionally, ellagic acid is commonly found in wines, resulting from the breakdown of ellagitannins (ellagic acid polymers) or gallic or ellagic acids with glucose, since ellagic acid results from the interaction of two molecules of gallic acid (25, 27).

- Hydroxycinnamic acids

Hydroxycinnamic acids have a C_6-C_3 structure. These phenolic acids are the most abundant group of phenols found in musts and wines in free form, but essentially

as esters of tartaric acid esters. The main esterified hydroxycinnamates in wines are caffeic, coumaric, sinapic, and ferulic acids (14, 45) depicted in Fig. 7.



Hydroxycinnamic acids	R_1	R_2	R_3
Caffeic acid	OH	H	H
Caftaric acid	OH	H	Tartaric acid
p-coumaric	H	H	H
p-coutaric acid	H	H	Tartaric acid
Ferulic acid	OCH_3	H	H
Fertaric acid	OCH_3	H	Tartaric acid
Sinapic acid	OCH_3	OCH_3	H

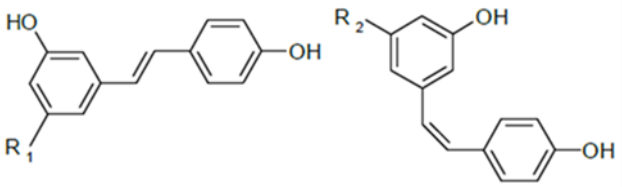
Figure 7 – Chemical structure of the hydroxycinnamic acids found in wines.

The main esterified hydroxycinnamates are the hydroxycinnamoyltartaric acids, namely caffeoyltartaric (caftaric acid), *p*-coumaroyltartaric (coutaric acid) and feruloyltartaric acids (fertaric acid), which after their hydrolysis originate their correspondent free form, respectively caffeic, *p*-coumaric, and ferulic acid. These hydroxycinnamoyltartaric acids are easily oxidizable through browning processes and resulting in formation of *o*-quinones (slightly colored), which in turn promote the oxidation of other compounds from wine, thus forming brown polymers such as flavan-3-ols (25, 46, 47). Trace amounts of sinapic acid are frequently found in wines in its free form (42). Hydroxycinnamic acids can be found in both isomeric forms (*trans* and *cis*), however, the *trans* isomer is the most abundant since it is more stable (21, 43). These phenolic acids are found in about 100 mg/L in red wines and 30 mg/L in white wines, with the *trans*-caftaric acid being usually the most representative (around 50%) of the hydroxycinnamates (27, 35, 39).

Moreover, during alcoholic fermentation, yeasts carry out the enzymatic decarboxylation of *p*-coumaric and ferulic acids, promoting the formation of volatile phenols (vinyl phenols and vinyl guaiacols, respectively) that play an important role in the wine's aroma due to their high odor activity. In addition to the enzymatic route, volatile phenols can migrate to the wine from the cask's wood during wine aging (21, 42, 43).

- Stilbenes

Stilbenes are bioactive compounds formed by two benzene rings bonded by a carbon chain (Fig. 8) (42).



Stilbenes	R_1	R_2
trans-resveratrol	OH	-
trans-piceid	OGlc	-
cis-resveratrol	-	OH
cis-piceid	-	OGlc

Figure 8 - Chemical structure of stilbenes found in wines (27).

They are mostly found in their glycosylated forms in the grape skin. During vinification, these compounds are transferred to the must, especially if a maceration step is included, which promotes the contact between the must with the grape solids (seed and skin) (48).

The most important stilbene found in grapes and wines is resveratrol (3,5,4′-trihydroxystilbene) due to its well-known antioxidant and anticarcinogenic properties (21, 43). Even though both free isomeric forms (*cis* and *trans*) and their *β*-glucoside conjugated forms (*cis*- and *trans*-piceid) can be found in grapes and wines, the *trans* isomer is the most representative (21, 42, 43, 48). *trans*-resveratrol levels in wines can range from 0.1 to 0.8 mg/L in white wines and 0.2 to 14 mg/L in red wines (43, 49). The levels of *trans*-resveratrol in wines are higher when grapes are exposed to biotic and abiotic stresses and when specific yeast strains are involved in fermentation (39, 50). Indeed, yeasts with *β*-glucosidase activity tend to increase the concentration of free resveratrol in wine (50, 51).

2.2.2. Flavonoids polyphenols

Flavonoids are mainly found in grape seeds and skins. The presence of these phenolic compounds in the final product strongly depends on the vinification practices and the yeast strains used during fermentation (25, 52). Even though the flavonoids found in grapes are usually in their glycoside form (hydrolyzed during the fermentation process), in wines, these phenolic compounds can be found in both free form or polymerized with sugars, non-flavonoids, or other flavonoids (25, 42, 52). Commonly, flavonoids are the most abundant phenols is wines, representing about 85% of the phenolic content of red wines (with levels ranging between 1000 and 1800 mg/L) but are less frequent in the white wines (less than 50 mg/L) (25).

Flavonoids are formed by a C_{15} (C_6-C_3-C_6) structure connected by a 3-carbon chain cyclized through oxygen (Fig. 9). The multiple radicals bonded to this carbon skeleton promote the diversity of this phenolic family (33, 35). Moreover, the flavonoids in grapes and wines are formed by two hydroxyl groups in ring A (position 5 and 7) (35). The most common flavonoids found in wines are flavonols, flavan-3-ols, and anthocyanins. Other flavonoids, such as flavanonols, flavones, and condensed tannins can be present in smaller concentrations (53). In the following paragraphs, a brief description of the main flavonoids in wines is presented.

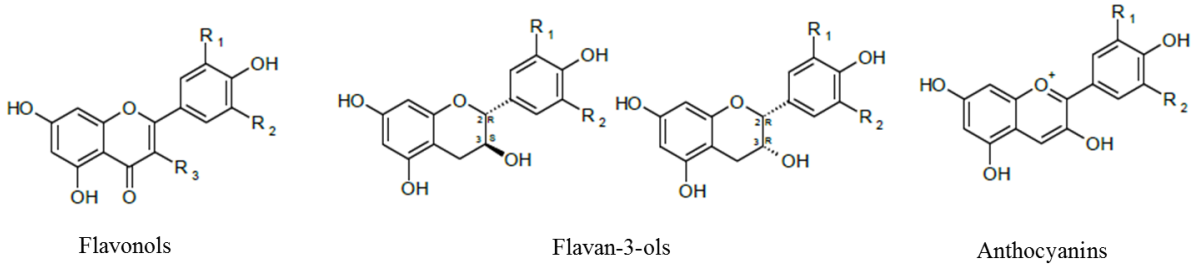


Figure 9 - Chemical structure of flavonoids in wines.

- Flavonols

Flavonol compounds are structurally distinct from other flavonoids due to a double bond between positions 2 and 3 of the ring, a ketone group in position 4 and a hydroxyl group in position 3. These compounds are frequently found as glycosides, linked to a sugar moiety (33, 35, 39).

The main flavonols in grapes and wines are myricetin, quercetin, kaempferol, syringetin, isorhamnetin, and laricitrin (54). Flavonols (represented by yellow pigments) have an essential role, especially in wine color, but also in the wine's sensory perception (astringency and bitterness) (33, 39). Indeed, these flavonoids are involved in the color stabilization of young red wines due to their co-pigmentation interaction with anthocyanidins (35). Red wines present higher flavonols concentrations (the maximum described is 60 mg/L) than white wines due to vinification practices, since the vinification of white wines does not often include a maceration process (which promotes contact between grape juice and grape solids) (27, 35).

- Flavanols (Flavan-3-ols)

Flavan-3-ols are commonly more abundant in grape seeds but are also present in its skins and stems. Flavan-3-ols can be found in their monomeric, oligomeric, or

polymeric forms. In grapes and wines, they are mostly found as monomers, being *(+)*-catechin, *(-)*-epicatechin, *(+)*-gallocatechin, and *(-)*-epigallocatechin the most commonly detected (27, 35, 53, 55).

Oligomeric and polymeric flavanols are also known as proanthocyanidins, once they form anthocyanidins (delphinidin or cyanidin) when heated under acidic conditions. Proanthocyanidins can be classified as procyanidins (composed of *(+)*-catechin and *(-)*-epicatechin monomers) and prodelphinidins (composed of *(+)*-gallocatechin and *(-)*-epigallocatechin monomers). Moreover, the proanthocyanidins can also be classified as condensed tannins since they can react with proteins, promoting their precipitation (27, 56).

The levels of flavan-3-ols can range from 4 to 120 mg/L in red wines and from 15 to 25 mg/L in white wines (35, 39, 57). Like flavonols, flavan-3-ols play an important role in wine's color and sensory characteristics (58). Indeed, flavan-3-ols are the main phenolic compounds responsible for the oxidative browning of white wines. Under oxidative conditions, oxygen is reduced to hydrogen peroxide, which can form a hydroxyl radical in the presence of some metal ions (such as copper (II) or iron (II)). Then, these radicals can oxidize the tartaric acid present in wines and originate aldehydes that consequently react with flavanols (mainly *(+)*-catechin). As a result, yellow-colored compounds (xanthylium cations) are formed (27, 59, 60).

- Anthocyanins

Anthocyanins are natural water-soluble pigments, present mainly in grape skins, responsible for the red color of grapes and wines (42, 61). These phenolic compounds accumulate during grape ripening and are extracted from the must during fermentation, enhancing with maceration due to contact of grape skins with the must (25). The color of anthocyanins depends on factors such as pH (red in low pH and purple to blue in high pH), sulfur dioxide levels, and wine co-pigments (35). These phenols are composed mainly of glycoside derivatives of anthocyanidins in which the sugar moiety contributes to the stability of the anthocyanidins and enhances water solubility (25).

The main anthocyanins in grapes and wines are the 3-*O*-monoglycosylated and the 3-*O*-acylated forms of cyanidin, peonidin, malvidin, delphinidin, and petunidin (61). The acylated anthocyanins are relatively more stable and can include glucose esterification with lactic, acetic, caffeic, and *p*-coumaric acids (62). In fact, this interaction with other phenolic acids (known as co-pigmentation) is an important

phenomenon for the stabilization of the anthocyanidins and, therefore, their color (63). The concentration of anthocyanins in wines depends on factors such as grape variety, grape maturation, and climatic conditions (61). Additionally, the strain of yeast used during fermentation also influences the concentrations of anthocyanins in the final product (51, 52, 64). These compounds are abundant in red wines (90 to 400 mg/L) but absent in white wines (35, 39, 44).

2.3. VOLATILE COMPOUNDS

Volatile compounds play an essential role in the wine's characteristics and are directly associated with its quality. Several volatile organic compounds (VOCs) have been identified in wines, at concentrations ranging from a few nanograms to several milligrams per liter (65, 66). However, not all VOCs are impacting odorants. Odorants are volatile compounds found at concentrations above their odor threshold (OT). Indeed, wine aroma comprises a mixture of several hundred odorants (65). Various factors can influence the wine's volatile composition and concentrations, namely the environmental conditions during plant growth (soil characteristics, climate), grape variety and ripeness, the fermentation variables (temperature, juice nutrients, pH, and the strain and type of yeast and bacteria present), post-fermentation treatments (clarification with finning agents), storage and aging conditions (27).

Wine aromas are frequently categorized according to the stage of the production process during which they originate. They can be primary aromas (compounds found in grapes and that persist during vinification), secondary aromas (result from the fermentation processes), and tertiary aromas (formed during storage and/or aging processes) (67).

A brief description of the wine's main classes of aromas is given below.

2.3.1. Primary aromas

Primary aromas, also known as varietal aromas, correspond to the secondary products of the plant metabolism and are usually abundant in grape skins. The compound and its concentration strongly depend on the climatic conditions, variety and cultivation practices, and increases during grape ripening (27).

Even though the varietal aromas in grapes and grape juice can be present in their free forms (VOCs, odorants), the majority of these compounds are found in their glycosylated forms (odorless), bonded to sugars by *O*-glycosidic linkage (68). The varietal aromas are mainly monoterpenes, C_{13}-norisoprenoids, methoxypyrazines, and volatile thiols (formed by sulfur compounds with thiol functions) (54, 69). Brief descriptions of each family are provided bellow:

Monoterpenes

Monoterpenes are the main responsible for wine varietal volatile aromas. In grapes, these compounds frequently occur as non-volatile glycosides; however, during the fermentation and aging processes, they can be hydrolyzed (enzymatically or/and chemically) to their free form, enhancing the wine aroma (70). Fig. 10 depicts the most prominent monoterpene odorants found in wines – linalool, α-terpineol, geraniol, nerol and citronellol (71, 72).

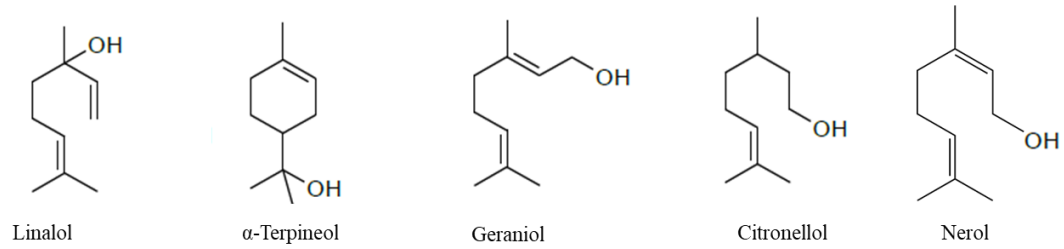


Figure 10 - Chemical structure of the monoterpenes found in wines.

C_{13}-norisoprenoids

C_{13}-norisoprenoids are important contributors to the wine aroma, especially with floral and fruity notes. These compounds result from the chemical, enzymatic, and/or photochemical oxidation of carotenoids. The most important C_{13}-norisoprenoids that contribute remarkably to several wine aromas are β-damascenone and β-ionone. Other compounds can also be found, namely vitispirane (camphor or eucalyptol aromas) and 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN, described as petrol-kerosene-like aromas) (27).

Methoxypyrazines

Methoxypyrazines are produced in grapes through the amino acid metabolism and are associated with green, herbaceous, and vegetal aromas (3, 73). The main methoxypyrazines found in wines are 2-methoxy-3-isobutylpyrazine (described as

green gooseberries or bell peppers), 3-sec-butyl-3-methoxypyrazine (described as bell pepper or pea-like), and 3-isopropyl-2-methoxypyrazine (described as green bean and asparagus) (3, 74, 75). Since the OTs of these compounds are very low (only a few ng/L), they usually contribute to the wine aroma (73).

Volatile thiols

Volatile thiols (mercaptans) possess low OTs and, for that reason, can influence wine quality. The most common compounds found in wines are 3-sufanylhexyl acetate (passion fruit aromas, with an OT of 4 ng/L), 3-sulfanylhexan-1-ol (grapefruit notes and an OT of 60 ng/L), and 4-methyl-4-sulfanylpentan-2-one (aroma descriptor of boxwood, with OT levels of 0.8 ng/L) (76, 77).

2.3.2. Secondary aromas

Secondary aromas, also known as fermentative aromas, result from the chemical and biochemical reactions that occur during alcoholic and malolactic fermentations. The fermentation process is catalyzed by the action of different yeast strains that belong to the grape microbiota: *Saccharomyces* and non-*Saccharomyces* species (78, 79). Fermentative VOCs represent the largest amount of volatiles found in wines (79). Although the total level of secondary aromas in wines varies between 0.3 and 1.5 g/L (80), the concentrations of most individual volatile compounds are below their OTs and, for that reason, do not contribute to the typical wine bouquet (79). Besides alcohol, glycerol, and diols, other important secondary aromas, such as alcohols, esters, fatty acids, carbonyl compounds, and lactones, are formed by yeasts during the fermentation process (27).

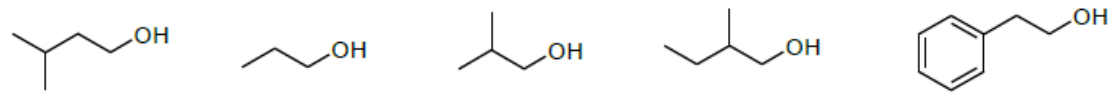
Alcohols

Alcohols are organic compounds that may contain one (ethanol) or more hydroxyl groups (-OH). Ethanol is the most important alcohol in wine being produced as a result of the yeast fermentation. This compound also play several roles in the wine aging (15).

Alcohols with more than two carbon atoms are commonly called higher alcohols (or fusel alcohols) (15). These compounds are abundant VOCs produced by the deamination and decarboxylation of amino acids (65%) and from sugars catabolism

(35%) (3, 25, 70). The formation of higher alcohols is affected by several factors, such as grape variety, the initial must sugar content, the maceration process, pH, assimilable nitrogen, aeration, temperature, and the yeast strains involved in the fermentation process (81). The formation of fusel alcohols is favored when the fermentation is conducted under high temperatures, in contact with grape skins, and in the presence of oxygen (25).

Higher alcohol levels in wines can vary between 140 and 420 mg/L, usually with 3-methyl-1-butanol representing about half the total alcohols content (70, 80). Other fusel alcohols can also be present in wines, namely 1-propanol, 2-methyl-1-butanol, 2-methyl-1-propanol, and 2-phenylethanol (27, 70). The chemical structure of the main higher alcohols found in wines is depicted in Fig. 11. Alcohols, such as 2-phenyletanol, can contribute positively to the wine's aroma, with flowery and fruity notes, when in concentrations below 300 mg/L. On the other hand, if the concentrations of these VOCs are higher than 400 mg/L, they can negatively impact in the final wine aroma with unpleasant and pungent notes (70, 81).



3-methyl-1-butanol | 1-propanol | 2-methyl-1-propanol | 2-methyl-1-butanol | 2-phenylethanol

Figure 11 - Chemical structure of the higher alcohols found in wines.

In addition to the alcohols already mentioned, there are longer-chain higher alchols that may contributes for the wine final aroma, such as 1-hexanol, *trans*-2-hexen-1-ol, 1-heptanol, 1-octanol and 1-decanol (15). Hexanol is often present in wines together with *cis*- and *trans*-2-hexen-1-ol and *trans*-3-hexen-1-ol, revealing a grassy flavor to the final wines. It is mainly found in grape must, as a result of the grape crushing techniques, due to the action of the enzymes on linoleic acid (8).

Esters

Esters are some of the most important fermentative compounds that greatly influence the wine aroma, especially with fruity and floral notes. These secondary compounds are usually formed by yeast during fermentation due to the reaction of alcohols with acids through enzymatic esterification, releasing water (79, 82). More than 160 esters have been reported in wines. Frequently, these are ethyl esters of organic

acids (short or medium chain, C_2 to C_{12}) and acetate esters (short chain C_4 to C_6), most of them in concentrations above their OTs (few µg/L) (25, 83, 84). Fig. 12 depicts the structure of the most significant esters found in wines (27, 84).

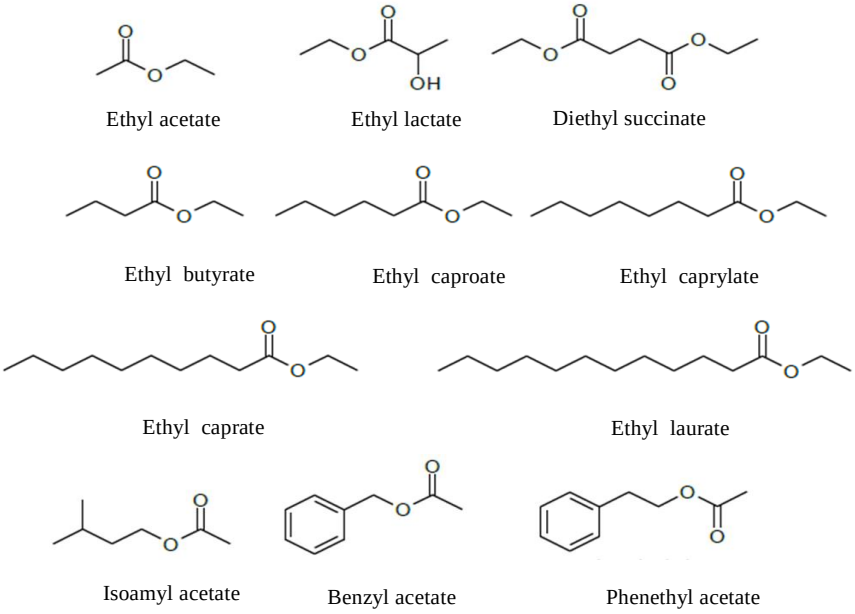


Figure 12 - Chemical structure of the esters in wines (27).

The chemical nature and the amount of esters formed during fermentation are greatly influenced by the temperature and the yeasts involved. Indeed, when fermentation occurs at low temperatures (about 10 ºC), the synthesis of acetate esters is favored, promoting the formation of isoamyl, isobutyl, and hexyl acetate compounds. On the other hand, higher fermentation temperatures (15 to 20 ºC) stimulate the formation of higher esters, namely ethyl caprate, ethyl caprylate, and phenethyl acetate (25). As mentioned before, yeasts influence the formation of esters. *Saccharomyces cerevisiae* has been reported to produce significant levels of isoamyl acetate and hexyl acetate. The highest synthesis rates of ethyl octanoate were observed in fermentations with *Saccharomyces cerevisiae* and with *Pichia fermentans*, while *Saccharomyces bayanus* produced the highest rates of 2-phenylethyl acetate and ethyl decanoate (85, 86).

Fatty Acids

During alcoholic fermentation, yeasts can synthesize short and medium- (propionic, butyric, caproic, and caprylic and capric acids) or long-chain fatty acids

(oleic and linoleic acids), usually C_2 to C_{12}. The chemical structures of these fatty acids are represented in Fig. 13 (82).

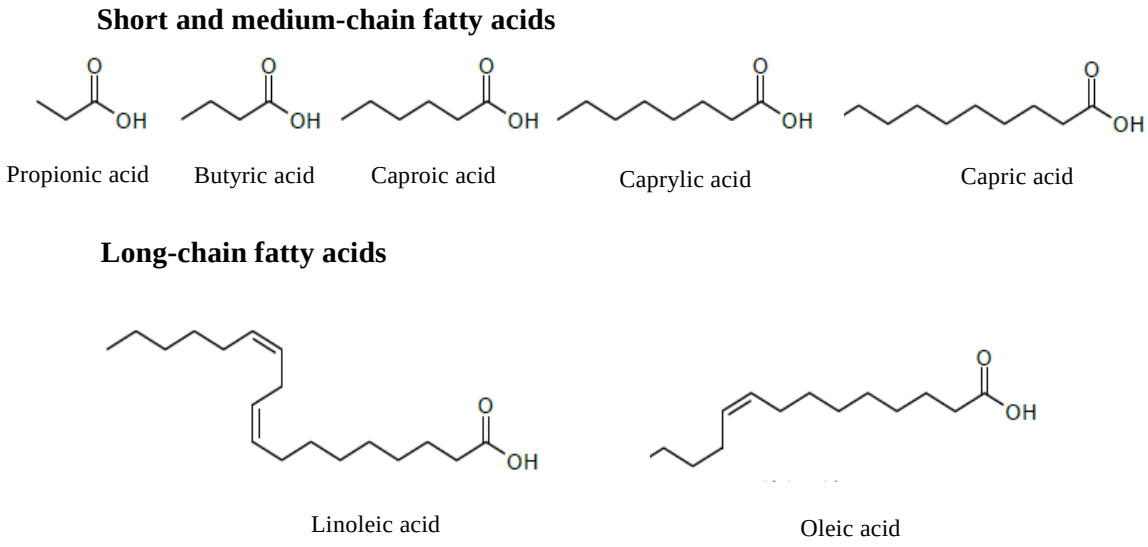


Figure 13 – Chemical structure of the fatty acids found in wines (adapted from (27)).

The concentrations of fatty acids in wines usually vary between 500 and 1000 mg/L. Even though these acids can contribute negatively to the wine's aroma (cheesy, rancid, and vinegar notes), the levels detected are usually below their OTs (87).

Carbonyl compounds

Carbonyl compounds in wines can be divided in two groups of volatiles: aldehydes and ketones. Aldehydes can be formed through several reactions, such as amino acid deamination or transamination, microbial activity during fermentation, Strecker degradation, and fatty acid oxidation (88). Acetaldehyde (ethanal) is the most predominant aldehyde, representing about 90% of the total aldehyde content in wines. This volatile compound can be formed during fermentation, by the decarboxylation of pyruvate or during aging, through the chemical or biological oxidation of ethanol (82, 84). Other minor straight-chain aldehydes (C_3-C_7) derived from the fermentative process can also be present in wines. Others can be formed during aging in barrels (such as vanillin, cinnamaldehyde, and benzaldehyde) or by carbohydrate degradation (furfural and 5-hydroxymethylfurfural) (8, 25). Some of the main aldehydes found in wines are illustrated in Fig. 14.

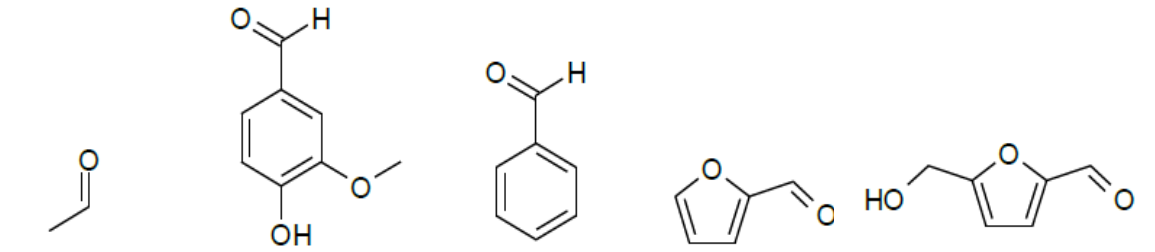


Figure 14 – Chemical structure of the aldehydes found in wines.

Frequently, ketones have a low influence on wine flavor since their OTs is high. However, the main exceptions are diacetyl (2,3-butanedione), acetoin (3-hydroxybutan-2-one), and 2,3-pentanedione. The chemical structure of these compounds is shown in Fig. 15. Diacetyl is produced through the oxidative decarboxylation of acetolactate (a by-product of the condensation of pyruvate with acetaldehyde), while acetoin is formed in the absence of oxidative decarboxylation (27, 84). Acetoin can also be produced by the direct reduction of diacetyl. At low concentrations (1 to 4 mg/L), diacetyl is characterized by its pleasant buttery aroma (hazelnut–like), whereas at high concentrations, it turns into a negative buttery lactic odor. Acetoin is characterized as a sugary butter-like aroma, and 2,3-pentanedione can change from buttery to plastic notes depending on the diols involved (25, 82, 89) .

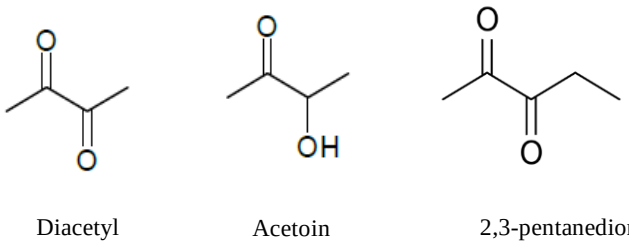


Figure 15 - Chemical structure of the main ketones in wines.

Lactones

Lactones are a subclass of esters usually formed by yeasts during alcoholic fermentation (25). Lactone formation is associated with the internal esterification between hydroxyl and carboxyl moieties of the same molecule (82). Lactones can be divided into γ-lactones (or furanones), structurally represented by 5-membered rings,

and δ-lactones (or pyranones), structurally constituted by 6-membered rings (8). Fermentative lactones (γ-butyrolactone is the most abundant) do not have a significant impact on the wine's aroma since they are usually present below their OT (82).

2.3.3. Tertiary aromas

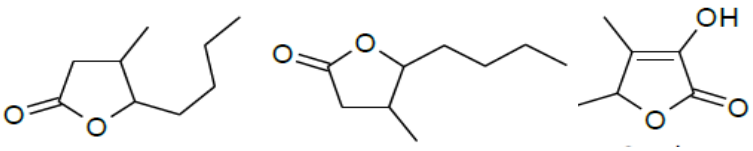
Tertiary aromas (also known as aging aromas) are developed during wine aging, either in wood barrels or bottles. During this stage, several chemical reactions take place, promoting the development of the wine's final bouquet (90).

The composition and quality of the wine's aging bouquet depend on several factors such as grape origin (e.g., grape variety, climacteric conditions), the fermentation process (yeast strain and temperature), storage conditions (temperature and duration), and the oak characteristics (geographical origin of the casks, oak species, toasting, cask age) which influences the diffusion of molecules from the oak to the wine (90-92).

During aging, the levels of some varietal and fermentative aromas decreases (namely terpenes and acetate esters such as isoamyl acetate, hexyl acetate, and isobutyl acetate), resulting in the loss of freshness and fruity notes characteristic of the young wines. On the other hand, the levels of ethyl esters of diprotic acids, such as diethyl and monoethyl succinate, increase due to chemical esterification. The concentration of lactones, furanic compounds, volatile phenols, and acetals, responsible for the typical aging aromas (caramel, spicy, dried fruits, toasty, and woody notes), also increases (90, 93-96).

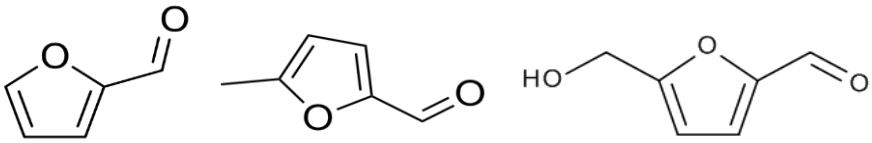
Some of the the main wine aging markers are depicted in Fig. 16.

Lactones



(E)-oak lactone (Z)-oak lactone Sotolon

Furan compounds



2-furfural 5-methyl-2-furfural 5-hydroxymethyl-2-furfural

Figure 16 – Chemical structure of the lactones and furan compounds found wines.

Three pathways have been described regarding the formation of furan compounds: caramelization, sugar dehydration by Maillard reaction, and carbohydrate pyrolysis. 5-hydroxymethyl-2-furfural (HMF) is frequently the most prominent furan compound in aged wines and is associated with spices, caramel, and dried fruit notes. Its formation results from the breakdown of pentoses and hexoses by heating and Maillard reactions (96, 98, 99). Additionally, lactones that accumulate during the aging process greatly influence the wine aroma. Sotolon (3-hydroxy-4,5-dimethyl-2(5H)-furanone), is one of the most important lactones formed during wine aging in oak casks and is associated with potent nutty, burnt, sweet, and curry notes (with a low OT of 5 μg/L) (100). Effectively, sotolon contributes to the typical aroma of some oxidatively aged fortified wines, such as Madeira wines (93, 98, 101, 102).

3. NON-*SACCHAROMYCES* YEASTS IN WINES

3.1. Wine yeasts

Wine is the product of complex biological and biochemical interactions between grape juice and several microorganisms such as yeasts and bacteria. In 1866, Louis Pasteur elucidated for the first time the bio-conversion of grape juice into wine, and since then, this process has been extensively studied (103). For the most part, yeasts are responsible for alcoholic fermentation by converting grape sugars into ethanol, carbon dioxide (CO_2), and other secondary products (103-105).

Yeasts are the most simple of the eucaryotes, and they are taxonomically defined as unicellular fungi that can reproduce by budding or binary fission. Current taxonomies recognize about 100 genera with more than 700 species, of which about 20 are the most relevant for winemaking (103, 105). The first level of yeast classification is based on the sexual phase during the life cycle: aspects of sexual phase (Ascomycotina and Basidiomycotina) or the lack of sexual phase (Deuteromycotina). The yeasts in grape skins usually belong to the Ascomycetes class (7, 103, 105). The haploid spores of Asmocyetes are contained in the ascus, whereas, in Basidiomycetes, the spores are in an external structure called basidium (7). Also, yeasts can have two valid names: teleomorphic name (sexual state producing ascospores) and the anamorphic name (asexual state that do not produce ascospores). Yeast classification can be difficult since not only do some yeasts not sporulate easily, but their ability to produce ascospores can be lost during long-term storage (106). Furthermore, their morphological, biochemical, physiological, and genetic properties allow taxonomic subdivisions into orders, families, genera, and species (105).

Yeast populations found during fermentation can be derived from vineyards, grapes, wine cellars (equipment and surfaces), and external sources, when selected cultures are added to promote and improve wine fermentation (103, 105). The yeast species commonly found in grapes and wines are similar worldwide; however, several factors can significantly affect its microflora (103-105, 107):

- Vineyards: altitude and climatic conditions (such as maritime influence, temperature, rainfall, and humidity).
- Grape variety: the thickness of grape skin and cultivar.
- Viticultural practices: irrigation, fertilization, and the use of fungicides.

- Harvesting: grape temperature, method of harvest (mechanical *Vs*. manual), type and time of transport to the wine cellar and the time before crushing.
- In wineries: transfer of the winery's indigenous yeasts from equipment surfaces, crushing methods, sulfite addition, enzyme addition, temperature control, cellar hygiene, and clarification process.

The yeast species found in grapes that are involved in wine production can be arbitrarily divided into *Saccharomyces* and non-*Saccharomyces*.

3.2. *Saccharomyces* yeasts

Regarding the *Saccharomyces* yeasts, *Saccharomyces cerevisiae* is one of the most important yeast species responsible for wine production. Even though *S. cerevisiae* is present in grape skins at low concentration, they are in great number in winery equipment and during must fermentation. Indeed, this yeast plays an essential role in the metabolism that converts grape sugars into alcohols and CO_2, in the release of aroma precursors, and in the formation of secondary aromas (103, 105, 108). For that reason, despite the wine's highly complex microbial ecology, *S. cerevisiae* became the wine yeast *par excellence* due to its fermentation performance (103, 108).

Currently, in the wine industry, fermentation is usually conducted by a selected starter culture of a *S. cerevisiae* strain. The first use of *S. cerevisiae* starter culture in wine production was reported by Muller-Thurgau in 1980, who adapted the technology used by Christian Hansen for the Carlsberg Brewery (104, 108). Nowadays, one of the winemaker's most common practices is the addition of active dry yeast to standardize wine production and maintain its quality throughout the years, avoiding the risk of spoilage. The wine market offers a wide range of dehydrate cultures of yeast strains that can promote good implantation, specific skills for the different types of wines, and also improve some wine characteristics such as the formation of varietal and fermentative aromas, tolerance to ethanol, glycerol production, and specific enzymatic activities (108).

Despite the advantages of using a pure culture of *S. cerevisiae*, wines produced with this pure yeast monoculture demonstrated lack of complex flavor and vintage variability due to the difficult implantation/persistence of the individual yeast strain within the indigenous yeast population. Indeed, it is reported that the world's highest quality wines are produced in the presence of non-*Saccharomyces* yeasts. This improves

the wine flavor and proprieties and, therefore, its final quality (103, 104, 108). Additionally, several reports suggest including non-*Saccharomyces* yeast species as part of the mixed starter culture, together with *S. cerevisiae*, to improve the wine quality by enhancing fermentation performance and minimizing the risk of spoilage (108-110).

3.3. Non-*Saccharomyces* yeasts

At first, non-*Saccharomyces* yeasts were considered as secondary mechanisms during alcoholic fermentation and were even thought to negatively affect the wine's final quality, being considered spoilage yeast since several species may produced high levels of undesirable compounds (namely acetic acid, acetoin, ethyl acetate and acetaldehyde) (111-112). However, in the past three decades, the role of non-*Saccharomyces* yeasts during wine fermentation has received increasing attention due to the production of some metabolites that greatly contribute to wine flavor diversity, and consequently, regional microbial populations have been explored, once they confer special signatures to wines and enhance its regionality (112, 113).

Non-*Saccharomyces* yeasts are either ascomycetous or basidiomycetous and currently, taxonomies recognize 149 genera, comprising nearly 1500 species. Until now, more than 40 species have been identified in grape musts (see Table 4) (106).

Table 4 - Teleomorphic and anamorphic forms and respective synonyms of some non-*Saccharomyces* yeasts found in the Ascomycetous genera reported in grape and must fermentation (106, 114).

Teleomorphic form	Anamorphic form	Synonyms[a*]
Citeromyces matritensis	*Candida globosa*	
Debaryomyces hansenii	*Candida famata*	*Pichia hansenii*
Dekkera bruxellensis	*Brettanomyces bruxellensis*	
Hanseniaspora guilliermondii	*Kloeckera apis*	
Hanseniaspora occidentalis	*Kloeckera javanica*	
Hanseniaspora osmophila	*Kloeckera corticis*	
Hanseniaspora uvarum	*Kloeckera apiculata*	
Hanseniaspora vineae	*Kloeckera africana*	
Lachancea kluyveri	-**	*Saccharomyces kluyveri*
Lachancea thermotolerans	-**	*Kluyveromyces thermotolerans; Candida dattlia*
Metschnikowia pulcherrima	*Candida pulcherrima*	*Torulopsis pulcherrima*
Meyerozyma guilliermondii	*Candida guilliermondii*	*Pichia guilliermondii*
Milleronzyma farinosa	-**	*Pichia farinosa*

Pichia fermentans	*Candida lambica*	
Pichia kluyveri	-**	*Hansenula kluyveri*
Pichia membranifaciens	*Candida valida*	
Pichia occidentalis	*Candida sorbosa*	*Issatchenkia occidentalis*
Pichia terricola	-**	*Issatchenkia terricola*
Saccharomycodes ludwigii	-**	
Starmerella bombicola	*Candida bombicola*	*Torulopsis bombicola*
Torulaspora delbrueckii	*Candida colliculosa*	*Saccharomyces rosei*
Wickerhamomyces anomalus	*Candida pelliculosa*	*Pichia anomala; Hansenula anomala*
Zygoascus meyerae	*Candida hellenica*	
Zygosaccharomyces bailii	-**	*Saccharomyces bailii*
--*	*Candida zemplinina*	*Starmerella bacillaris*

[a]* synonyms found literature; --* no teleomorphic form; -** no anamorphic form.

3.3.1. Origin of non-*Saccharomyces* yeasts in wine production

The origin of non-*Saccharomyces* yeasts and how they reach the grape juice has been the subject of several studies. Currently, it is known that the non-*Saccharomyces* yeasts in the fermentation process are essentially from two sources: grapes/vineyards and/or the winery environment.

Grape and vineyard

Non-*Saccharomyces* yeasts are found in grapes and, in lesser number, on wine cellar equipment. In unripe grapes, the number of non-*Saccharomyces* yeasts is low (10-10^3 cfu/g), but it increases during grape maturation due to sugar diffusion from the inner tissue to the surface of the grape skin or due to contact with damaged berries, reaching numbers of 10^4 to 10^6 cfu/g in ripe grapes. Studies evidence that these microorganisms tend to colonize around the grape stomata, where small amounts of exudate are secreted (105). The most common non-*Saccharomyces* yeasts found in vineyards, representing about half of the grape yeast flora, are *Hanseniaspora* and its anamorph counterpart *Kloeckera*. Other yeast genera present in grapes are *Metschnikowia*, *Pichia*, *Candida*, *Starmerella*, *Cryptococcus*, *Zygosaccharomyces*, *Wickerhamomyces*, *Torulaspora*, *Sporidiobolus*, *Kluyveromyces*, and *Hansenula* (106, 113). On the other hand, *Saccharomyces* species are relatively scarce in grapes.

Winery environment

Little attention has been given to the diversity of non-*Saccharomyces* yeasts in wine cellars since the main sources of these yeasts are grapes and vineyards. However,

during grape crushing, the non-*Saccharomyces* yeasts in grapes and wine cellars are carried to the must and, therefore, contribute to the wine's quality (103, 115). Because winery equipment (such as valves, crush/press equipment, and barrels) is difficult to clean, this facilitates microbial adhesion. In this sense, one of the most important features that characterize the winery microbiota is the yeast's ability to survive and colonize future vintages (116). However, the cellar's resident flora can be minimized by employing hygiene procedures (105).

Even though several studies have shown that *S. cerevisiae* is the most abundant specie in winery environments, due to its resistance to high levels of ethanol and SO_2, accounting for about 30 to 40% of the total yeast populations, other non-*Saccharomyces* species may also colonize winery surfaces (116). Indeed, more recent studies have revealed that many isolates belonging to non-*Saccharomyces* species (namely *S. bacillaris*, *H. uvarum* and *H. guilliermondii*) demonstrated high persistence from year to year in wine cellars (115, 116). Compared to *Saccharomyces* species, the proportion of non-*Saccharomyces* yeasts in wineries may vary according to the winery, different parts of the cellar, and different time of the year (115, 117, 118). Furthermore, the main genera reported in winery environments are *Hanseniaspora*, *Pichia, Candida*, *Torulaspora*, *Aureobasidium*, *Metschnikowia*, *Bullera*, *Cryptococcus, Debaryomyces*, *Dekkera*, *Rhodotorula*, *Kluyveromyces*, *Sporobolomyces*, *Sporidiobolus*, and *Williopsis* (115, 117, 119-123).

3.3.2. Importance of non-*Saccharomyces* species in wine production

The most important contribution of non-*Saccharomyces* species is in terms of wine's flavor and depends on the concentration of the metabolites that are formed. The yeast's survival and growth depend on specific environmental conditions of the must, such as glucose and fructose concentrations, high osmotic pressure, SO_2 levels, temperature, an increase in ethanol concentrations, and anaerobic conditions (105, 106).

Non-*Saccharomyces* yeasts are mostly present in the initial stage of fermentation and apparently do not survive the entire process, due to the increase in the concentrations of toxic metabolites, such as ethanol and SO_2. However, recent studies have shown that these yeasts can appear both during and in the final stage of

fermentation, probably as a result of the improvement of cellar technology (reduction in SO_2 concentrations used) and hygiene (103, 106, 124-126).

Non-*Saccharomyces* yeasts in grape musts are usually divided into three groups (105, 106, 124):

a) Highly aerobic yeasts, such as *Pichia* spp., *Candida* spp., *Rhodotorula* spp., *Debaryomyces* spp., and *Cryptococcus albidus*.
b) Apiculate yeasts with low fermentative activity, such as *Hanseniaspora uvarum* (*Kloeckera apiculata*), *Hanseniaspora occidentalis* (*Kloeckera javanica*), and *Hanseniaspora guilliermondii* (*Kloeckera apis*).
c) Yeasts with fermentative activity for example, *Zygosaccharomyces bailii*, *Torulaspora delbrueckii*, *Kluyveromyces marxianus* (*Candida kefyr*), *Candida colliculosa*, and *Metschnikowia pulcherrima* (*Candida pulcherrima*).

During spontaneous fermentation, there is a sequence of yeast dominance, first by non-*Saccharomyces* species and then by *S. cerevisiae*, which completes the fermentation. *H. uvarum* and *Candida* spp. are usually present in higher numbers at the start of fermentation, while other non-*Saccharomyces* species, such as *Pichia*, *Rhodotorula*, *Metschnikowia*, and *Cryptococcus*, are commonly found in low levels (103, 105, 106, 126). However, during the early stages of vigorous fermentation, most of these non-*Saccharomyces* yeasts tend to disappear due to their slow growth and due to inhibition by the combination of several factors, such as low pH, high ethanol and SO_2 levels, and oxygen deficiency (106, 124, 127, 128). Also, nutrient competition and the dominance of *S. cerevisiae* can contribute to the suppression of non-*Saccharomyces* species (106, 129). Some non-*Saccharomyces* spp. are reported to survive fermentation, namely *Z. bailii* and *Pichia* spp. (106, 130, 131). The characteristics of individual species contribute to their extent in fermentation as well as their growth and survival. Also, different strains within the same species can have different growth kinetics (126).

Non-*Saccharomyces* yeasts in wines are usually associated with post-fermentation spoilage or/and barrel aging. The main species in bottled wines are ethanol tolerant, such as *Brettanomyces* spp. and *Zygosaccharomyces* spp. Their presence is associated with the filtration procedure and the cellar's hygiene conditions during bottling (105, 123).

3.3.3. Enzymatic activity of non-*Saccharomyces* wine yeasts

Several non-*Saccharomyces* species contribute to the enzymatic reactions that occur during must fermentation, as described in Table 5.

Table 5 – Main enzymatic activity of non-*Saccharomyces* wine yeasts (132).

Enzymatic activity	Genera
β-glucosidase	*Candida, Debaryomyces, Hanseniaspora, Hansenula, Kloeckera, Kluyveromyces, Metschnikowia, Pichia, Saccharomycodes, Schizosaccharomyces, Zygosaccharomyces*
Protease	*Candida, Kloeckera, Pichia*
Esterase	*Brettanomyces, Debaryomyces, Rhodotorula*
Pectinase	*Candida, Cryptococcus Kluyveromyces, Rhodotorula*
Lipase	*Candida*

β-glucosidases are frequently found in plants, namely grapevines. In addition, some microorganisms, including yeasts, can produce this enzyme. *β*-glucosidases in grapes, bacteria and filamentous fungi have low stability under winemaking conditions and can be practically inactive at pH 3.0 to 4.0 and in the presence of glucose (133). Also, the activity of this enzyme can be inhibited by alcohol and temperature (106).

Glycosidically bound volatiles are highly complex and diverse, namely the aglycone moiety. The sugar parts consist of different di-glycosides and *β*-D-glucopyranosides (6-O-α-L-arabinofuranosyl-β-D-glucopyranosides, 6-O-α-L-rhamnopyranosyl-β-D-glucopyranoside, 6-O-α-L-arabinopyranosyl-β-D-glucopyranoside, 6-O-β-D-apiofuranosyl-β-D-glucopyranosides, 6-O-β-D-xylopyranosyl-β-D-glucopyranosides, and 6-O-β-D-glucopyranosyl-β-D-glucopyranosides), while the aglycon part is usually formed by terpenols or other flavor precursors, such as C_{13}-norisoprenoids, phenolic acids, volatile phenols, or cyclic alcohols (113, 134).

The liberation of terpenols and other flavor precursors during fermentation can be explained by the *β*–glucosidase activity of several non-*Saccharomyces* genera (*Hanseniaspora Kloeckera*, *Candida*, *Debaryomyces Metschnikowia*, *Pichia Kluyveromyces*, *Saccharomycodes*, *Schizosaccharomyces*, and *Zygosaccharomyces*) (132). Fig. 17 depicts the enzymatic hydrolysis of terpene glycosides. Briefly, the enzymatic hydrolysis of glycosides is carried out in two steps: first, α-L-rhamnosidase, α-L-arabinosidase, or β-D-apiosidase act to release rhamnose, arabinose, or apiose and

the corresponding *β*-D-glucosides; then, *β*-D-glucosidase acts and promotes the terpenol liberation (135).

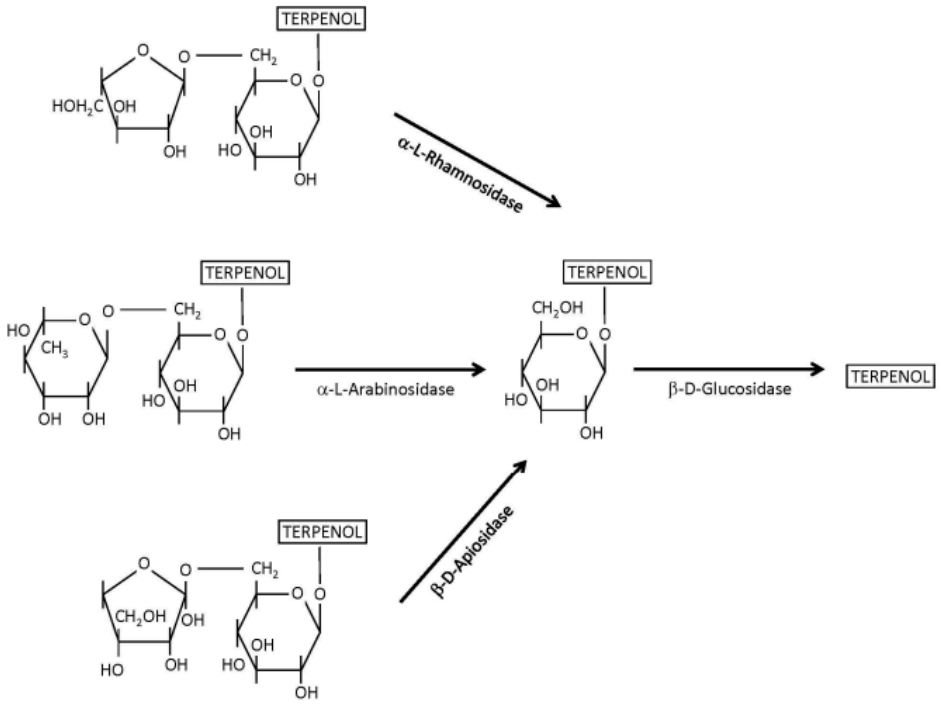


Figure 17 - Sequential enzymatic hydrolysis of flavor precursors (135).

Although *Pichia* and *Hanseniaspora* yeasts can produce *β*-glucosidase, its activity depends on the yeast strain. For instance, *β*-glucosidase from *Pichia anomala* can be stimulated in the presence of ethanol (110 to 130%), whereas *β*-glucosidase from *Hanseniapora* spp. was reported to be substantially inhibited by this substance (133, 136).

The proteins in grapes impact the clarification and stabilization of must and wine. The yeast's proteases hydrolyze the peptide linkage between the amino acid units of the protein and, therefore, improve the clarification process. Nitrogen sources can influence protease activity, as has been observed in several strains of non-*Saccharomyces* species, such as *Candida pulcherrima*, *K. apiculata* and *Pichia anomala* (132).

Esterases found in non-*Saccharomyces* yeasts from the *Brettanomyces*, *Rhodotorula*, and *Debaryomyces* genera can be involved in the formation of wine aroma compounds (132)

Pectinases have significant application in winemaking, namely in the clarification of grape juice, wine filtration, and color extraction, and may also promote

the increase of terpenol levels in musts. Although pectin esterase activity increases during grape ripening and this enzyme can be produced by non-*Saccharomyces* yeasts in the must (such as *Candida*, *Cryptococcus*, *Kluyveromyces*, and *Rhodotorula*), the addition of commercial pectinase preparations is a common industrial practice (132).

Lipases are enzymes that can degrade lipids from the grape or the yeast's autolytic reactions, releasing fatty acids into the must or wine and, therefore, can influence the wine's quality (132). Even though the properties of peroxidase and lipoxygenase enzymes found in grapes have been well established, only a few publications describe lipases produced from non-*Saccharomyces* species, especially from the genus *Candida* (132, 137).

3.3.4. Contribution of non-*Saccharomyces* species in wine production

Current consumer and market trends demand wines with lower ethanol content (the main product from fermentation). In this sense, several studies have reported using non-*Saccharomyces* species to produce wines with low levels of ethanol (106, 138-140). For example, *Williopsis saturnus* and *Pichia subpelliculosa* can produce wines with 3% (v/v) after fermentation under intensive aerobic conditions (141).

Another metabolite produced during must fermentation is glycerol, which contributes to the regulation of the redox potential in the yeast cell. Glycerol influences the wine's smoothness, sweetness, and complexity, and its desirable concentration depends on the type of wine (106, 142). Several non-*Saccharomyces* yeasts are described to increase glycerol levels during fermentation, such as *L. thermotolerans* and *C. zemplinina* (143-145). However, the increase of glycerol is usually related to the increase in acetic acid levels, which can negatively influence wine quality (142). So, to avoid the increase of acetic acid (and consequently the VA), some non-*Saccharomyces* yeasts are being used in mixed fermentations with *S. cerevisiae* (145-147).

Non-*Saccharomyces* yeasts can also produce organic acids, especially succinic acid. The levels produced, which can influence wine quality, correlate with high ethanol production and ethanol tolerance (136, 148, 149).

The yeast metabolism significantly impacts the phenolic content and composition of the final wine, especially due to the adsorptive effect of yeast cell walls and the interactions between phenols and yeast secondary metabolites, such as pyruvic

acid and acetaldehyde (136, 150). Also, *β*-glucosidase produced by yeasts can hydrolyze the phenolic glycosidic bonds to release phenolic aglycones (136). Non-*Saccharomyces* yeasts can enhance the levels of phenolic contents in wines. For instance, the levels of anthocyanins and stilbenes increase in fermentations with *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Candida zeylanoides*, and *Zygosaccharomyces bailii* (51).

The production of flavor compounds by different non-*Saccharomyces* yeasts is well documented (106, 136, 151-153). Most of these compounds are found in grapes as glycosylated flavorless precursors, which can be hydrolyzed by *β*-glucosidase to form free volatile organic compounds that can enhance the wine's final aroma (106). Nevertheless, the degree of *β*–glucosidase activity depends on the yeast species and strain involved in the fermentation (106, 154-156). Moreover, other extracellular enzymes in non-*Saccharomyces* yeasts, such as proteases and lipases, can potentially degrade proteins and lipids and then promote the release of esters, alcohols, acids, and other compounds (132).

Recently, *β*-glucosidase preparations have been added to grape juice to improve the release of bound volatile compounds. In this sense, intracellular *β*-glucosidase from *Debaryomyces hansenii* and *Issatchenkia terricola* was shown to increase the levels of monoterpenes and norisoprenoids in Muscatel wines (157, 158). Fermentations with *Hanseniaspora* sp. and *Pichia anomala* increased the levels of volatile compounds in Traminette grape juice and wines (133), and *Sporidiobolus pararoseus* yeasts promoted the release of volatile terpenols in white and red wines (159).

Several non-*Saccharomyces* yeasts are described to increase the concentration of terpenols in wines due to co-fermentation with *S. cerevisiae*, namely *Debaryomyces pseudopolymorphus, Debaryomyces vanriji, C. zemplinina,* and *T. delbrueckii* (140, 154, 160). Non-*Saccharomyces* yeasts can be divided into two groups based on their flavor contribution to the wine aroma: neutral yeasts (non-*Saccharomyces* yeasts that produce little or no flavor compounds) and flavor-producing yeasts (yeasts that produce flavor compounds) (106). Flavor-producing yeasts, which include several non-*Saccharomyces* species such as *P. anomala* (*Hansenula anomala*), *K. apiculata, C. pulcherrima, H. guilliermondii, H. osmophila*, and *P. membranifaciens*, are known to be extensive producers of esters (106, 161, 162).

The production of higher alcohols depends on the species and strains involved during the fermentation process. This is important during wine production since low levels of higher alcohols can add complexity to the wine, while high concentrations may

negatively contribute to the wine quality (106). Indeed, *H. guilliermondii* and *H. uvarum* mixed starter cultures with *S. cerevisiae* enhancing the production of desirable compounds (163).

Non-*Saccharomyces* yeasts can also affect wine color due to the presence of anthocyanins during the fermentation process. In fact, *P. guilliermondii* and *S. cerevisiae* promote the formation of vinylphenolic pyranoanthocyanin molecules, which significantly influence wine's color stability (106, 164).

3.3.5. Use of non-*Saccharomyces* species as starter cultures in wine production

The role of non-*Saccharomyces* species through the fermentation stage is greatly significant since, as previously mentioned, they strongly contribute to the wine quality. Indigenous yeasts provide distinctive regional features to wines, so their use as starter cultures is advised (165). Indeed, some non-*Saccharomyces* yeasts are already commercialized as starter cultures combined with *S. cerevisiae* (namely, *Pichia kluyveri*, *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, and *Lachancea thermotolerans*). Other non-*Saccharomyces* species, such as *Hanseniaspora uvarum* and *Starmerella bacillaris*, are still the subject of study for industrial applications (166).

The impact of the non-*Saccharomyces* species used in the present study on the oenological parameters is briefly described below.

Hanseniaspora uvarum

The apiculate yeast *Hanseniaspora uvarum* (anamorph *Kloeckera apiculata*) is frequently found in the highest amount in grapes and musts and remains active in the first stages of spontaneous alcoholic fermentation. Therefore, it contributes significantly to wine quality (106, 165). Even though *H. uvarum* plays an important role on the wine's chemical composition, the physiological properties of oenological interest, such as ethanol production, volatile acidity, and production of primary (e.g., glycerol and acetaldehyde) and secondary metabolites (e.g., ethyl acetate and hydrogen sulfite) are influenced by the strain involved in the fermentation (165).

Mixed fermentations of *H. uvarum* with *S. cerevisiae* have increased the production of primary metabolites (glycerol and acetaldehyde) and secondary

metabolites such as terpenes, C_{13}-norisoprenoids, ethyl esters, acetate esters, fatty acids, and higher alcohols (167-169).

Starmerella bacillaris

The non-*Saccharomyces Starmerella bacillaris* yeast (synonym *Candida zemplinina*) was isolated for the first time in Napa Valley (California, USA) in 2002 (114). Interestingly, in Chardonnay musts, this yeast was able to ferment fructose exclusively without affecting the levels of glucose (170). *S. bacillaris* has been reported in several spontaneous alcoholic fermentations in different countries (121, 171-173), and plays an important role in the winemaking industry due to its fructophilic character and low production of ethanol during fermentation (174).

S. bacillaris has very interesting oenological characteristics, such as its ability to grow at low temperatures and high levels of sugars and produces low levels of acetic acid and acetaldehyde and significant levels of glycerol. *S. bacillaris* can also survive until the end of fermentation due to its ability to tolerate high levels of ethanol (174-176). Additionally, mixed fermentations of *S. bacillaris* with *S. cerevisiae* have shown great potential for the chemical and aromatic profile due to the production of high levels of esters and thiols in Sauvignon blanc wines (177), as well as reduced levels of metabolites that can be negative for the quality of red wines, such as volatile fatty acids (178).

***Pichia* species**

Several *Pichia* species yeasts have been found in must fermentation, namely *P. kluyveri, P. fermentans, P. terricola, P. membranifaciens, P. occidentalis, P. manshurica,* and *P. kudriavzevii*. However, *Pichia* species are not isolated from grapes as often as *H. uvarum* (about 44%) and *S. cerevisiae* (around 28%). Of all *Pichia* species, *P. kluyveri* is the most studied and is the only commercially available specie in the market since it can improve the composition of volatile compounds such as fruity esters, terpenes, and thiols. Currently, there are two commercial starter cultures based on *P. kluyveri*: FROOTZEN® (from Hansen®, Denmark), which contribute to an increase in the concentrations of thiols and varietal aromas, and WLP605 (from Vintner's Harvest®, USA). Consequently, it promotes an increase in floral and rose petal aromas. The oenological effect of both of these commercial yeasts is favored by a sequential fermentation with a *S. cerevisiae* strain after 48 h (179-181).

In 2005, *Pichia fermentans (Candida lambica)* was reported in microvinifications of Macabeo wines (106, 182). The starter culture of *P. fermentans* with *S. cerevisiae* promoted an increase in glycerol and some volatile compounds, such as ethyl acetate, acetaldehyde, 1-propanol, *n*-butanol, 1-hexanol, 2,3-butanediol, and ethyl octanoate. Additionally, this mixed culture was also able to enhance the concentrations of polysaccharides, improving the wine's taste and body (106, 183).

Pichia terricola (synonym of *Issatchenkia terricola*) is part of the grape's native flora and significantly impacts the oenological parameters since it has low fermentative performance and can increase ethyl acetate concentrations (161, 184). On the other hand, the mixed starter culture of *P. terricola* with *S. cerevisiae* improves the wine quality and flavor due to the production of wines with low volatile acidity and increased the amount of volatile compounds (185).

4. SCIENTIFC OVERVIEW IN MADEIRA WINES

In the last decades, several scientific studies have been published about Madeira wines' composition and winemaking/aging processes to garner more information on how to maintain the characteristic high quality of these fortified wines. In this sense, research about Madeira wines resulted in 5 doctoral thesis. First, Câmara (2004) focused on the volatile profile of Madeira wines produced by the white grapes varieties (186). Then, Pereira's (2011) work was based on the impact of the *estufagem* process on the Madeira wine composition (namely on organic acids, biogenic amines, volatile compounds, furans, amino acids, and polyphenols). In 2012, Pereira (2012) developed reliable tools based on Madeira wine's intrinsic features in order to predict its age (6) and in the same year, Perestrelo (2012) studied the phenolic compounds and volatile profile of *Vitis vinifera L*. grapes used for the production of Madeira wines (187). Recently, Leça (2021) focused on the mitigation and control of ethyl carbamate in the production of Madeira wines (188).

Madeira wine is a peculiar and unique fortified wine, so high standards are required for the merchants and consumers. Many scientific publications about Madeira wine have been found, mainly on the volatile profile of Madeira wine aging (*canteiro* and *estufagem*) (3, 93, 95-98, 101, 102, 189-194). Perestrelo *et al.* (2019) established a platform based on Madeira wine descriptors with a total of 82 VOCs belonging to different chemical families (esters, higher alcohols, terpenic compounds, fatty acids, furanic compounds, norisoprenoids, lactones, acetals, volatile phenols, and sulfur compounds) (96). Similar to Pereira *et al.* (2014) studies (192), the authors showed that the Madeira wine aging process promoted a decrease in some varietal and fermentative aromas with freshness and fruitiness notes and increased the tertiary aromas (such as caramel, spicy, toasty, woody, and dried fruits notes) due to the Maillard reaction and also the diffusion of some compounds from the oak to the wine. Additionally, in order to ensure Madeira wine authenticity, several studies have determined some VOCs that can be potential aging markers, such as sotolon, 2-furfural, ethyl 2-furoate, HMF, 5-methylfurfural, diethoxymethane, 1,1-diethoxyethane, 1-(1-ethoxyethoxy)-pentane, 1,1-diethoxy-2-methylpropane, *cis*-oak-lactone, *trans*-oak-lactone, *trans*-dioxane, *cis*-dioxane, and 2-propyl-1,3-dioxolane (101, 102, 191, 195).

Regarding the non-volatile composition of Madeira wines, numerous reports have been published regarding the determination of the levels of phenols and the

antioxidant capacity. For example, Gonçalves *et al.* (2013) quantified hydroxybenzoic and hydroxycinnamic acids in red and white wines. They showed that gallic acid was the most abundant phenol, ranging from 6 to 29 mg/L in red wines and from 1 to 17 g/L in white wines (196), while Paixão *et al*. (2008) studies revealed phenol levels of 429 mg/L in red wines, with caffeic acid the most abundant compound in white wines (14.62 mg/L) (197). Pereira *et al.* (2013) evaluated the phenolic levels in different styles of Madeira wine during the *estufagem* process and identified 6 hydroxybenzoic acids, 3 hydroxycinnamic acids, 3 flavonols, 1 stilbene and 3 flavan-3-ols (198).

Several other studies have also been reported based on Madeira wine's chemical composition and its impact on the wine's final complexity. For instance, Miranda *et al*. (2017) studied the evolution of volatile acidity main contributors (acetic acid and ethyl acetate) and their impact on the olfactory perception, revealing that the consumer's odor rejection threshold increases with the wine age and sweetness degree (22). Madeira wine organic acids composition has also been reported by Pereira *et al.* (2010) and Rudnitskaya *et al.* (2010), showing that tartaric acid was the most predominant acid (199, 200). Additionally, Madeira wine's color has been assessed during wine aging (198, 201).

Current concerns in food safety and quality have led to the need to identify and mitigate the occurrence of some possible contaminants in wines. In this sense, some studies have been performed on the determination of copper in Madeira wines (202, 203). Furthermore, due to the *estufagem* aging process, characteristic of Madeira wines, special attention has been given to potentially carcinogenic compounds, such as 5-hydroxymethylfurfural (HMF) and ethyl carbamate. In this sense, several works have been done regarding the methodologies, evolution, and mitigation of these compounds in Madeira wines (93, 204-208).

Although Madeira wine's chemical composition has been extensively studied, the papers based on the yeast microbiota involved in the production of these wines are scarce. As far as we know, only two scientific studies have been published about this matter. The first preliminary study was presented by Pereira (2012) in her doctoral thesis (6). The results revealed that the predominant indigenous yeast involved in Madeira wine fermentation belong to the non-*Saccharomyces* species. Furthermore, the wines produced by the *Malvasia* and *Boal* grape varieties showed a predominance of *Hanseniaspora uvarum*, while the predominant species were *Pichia terricola* in *Verdelho* and *Torulaspora delbrueckii* and *Pichia guilliermondii* in *Sercial*. In addition,

at the end of fermentation, the most predominant yeast species was *Saccharomyces cerevisiae* in all wines, as well as the notable presence of *Saccharomyces bayanus* in wines produced by *Malvasia* grapes. Most recently, Castillo *et al.* (2020) reported the characterization of *S. cerevisiae* strain isolated from grape musts used for the production of Madeira wines and its impact on the volatile organic metabolites profile (209).

Additionally, in collaboration with the present work, Leça *et al.* (2021) studied the impact of non-*Saccharomyces* yeasts on the formation of ethyl carbamate in the production of Madeira wines, revealing that *P. terricola* and *S. bacillaris* showed lower potential for the formation of this compound (< 100 µg/L) (206).

5. ANALYTICAL TECHNIQUES APPLIED FOR MADEIRA WINE CHARACTERIZATION

Several analytical techniques have contributed to the chemical characterization of Madeira wines. The following paragraphs briefly describe the methodologies used in the current work, according to the resources available when this investigation was carried out. A detailed description of the procedures followed for each methodology is presented in the corresponding chapters.

The characterization of the aroma profile of Madeira wines has been commonly performed using two extraction techniques: solid phase extraction (SPE) and headspace solid phase microextraction (HS-SPME), followed by capillary gas chromatography coupled to mass spectrometry detection (GC-MS) (22, 194, 210). SPE has been extensively used for the analysis of volatile aroma compounds. This analytical technique involves a liquid-solid partitioning in which the analytes are bound to active sites on the surface of a solid sorbent (211). The HS-SPME technique first involves exposing the fiber indirectly to the sample through the vial headspace and then the desorption of the trapped compounds into the GC injector. This analytical technique is highly sensitive and reproducible, can be easily automated, and does not require the use of a solvent or previous sample preparation (210).

Global spectrophotometric determinations using ultraviolet-visible spectroscopy (UV-vis) enable the evaluation of the total phenolic content (TP), antioxidant potential (AP), and color. The determination of the TP is usually carried out according to the OIV procedure (OIV-MA-AS2-10) by following the Folin-Ciocalteu's method (212). Previously, studies about the AP of Madeira wines identified a good correlation between the 2,2-diphenyl-1-picrylhydrazyl (DPPH) methodology (which measures the ability of wines to scavenge free radicals) and the TP (Folin-Ciocalteu's method), and for that reason (198), both analytical procedures were taken into account in the current work. In the present study, the evaluation of Madeira wine's color considers two methodologies: the Glories method and the CIELab coordinates system. The Glories method (OIV-MA-AS2-07B) measures the absorbance at three wavelengths: 420, 520, and 620 nm (212). This method allows us to calculate the intensity of the wine color (CI) and study the browning development (absorbance at 420 nm) typical of oxidatively aged wines, such as Madeira's. However, since this routine

technique does not reflect the overall visual perception of the consumer, the CIELab coordinates system (OIV-MA-AS2-11-R2006), which takes into account all visible spectrum information (transmittance from 380 to 770 nm at 5 nm intervals) (212) is often used for the detailed evaluation of Madeira wine color (194, 198, 201).

Similar to other works (95, 190, 197-199), the analysis of Madeira wine's chemical composition (phenols, organic acids, sugars, glycerol and ethanol) was performed by high performance liquid chromatography (HPLC) coupled with a diode array detector and a refraction index detector. Regarding the molecular techniques used in this work, a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) followed by DNA sequencing (159, 213) allowed the identification of the indigenous yeast microbiota involved in the production of Madeira wines.

6. OVERVIEW AND OBJECTIVES

Madeira wine is produced by spontaneous alcoholic fermentation. Yeast microbiota significantly impacts must fermentation and, consequently, the wine's sensorial profile and chemical composition. Also, grape vine's molecular and chemical composition and winery conditions influence the wine's quality.

In order to ensure the requirements of increasingly demanding markets, it is essential to standardize the winemaking process while simultaneously maintaining the characteristic high-quality of Madeira wines. To our knowledge, studies about the yeast microbiota involved in the production of Madeira wines are scarce. Generally, the present work aims to evaluate Madeira wine's chemical composition and isolate, identify, and characterize the yeast microbiota of musts produced with the Tinta Negra grape variety from the main vineyard locations, as well as from 3 of the main regional wineries. Then, we intend to study, for the first time, the impact of the inoculation of 5 indigenous non-*Saccharomyces* yeasts on the physicochemical and sensorial parameters of Madeira wine.

The present work is divided into 4 chapters:

Chapters 1 and 2 focus on evaluate the chemical composition of wines produced by different wineries and with grapes from different vineyard locations (south and north regions of Madeira Island) and to determine if there is variability in the wines and how it can compromise the vinification standardization.

The work presented in Chapter 3 aims to isolate, identify, and characterize the microbiota of indigenous yeasts from Tinta Negra grape musts, from different vineyard locations and wineries. The impact of the inoculation of selected 5 non-*Saccharomyces* yeasts, as starter pure culture, on the sugar content, phenolic composition, and antioxidant activity was also studied.

Finally, in Chapter 4, the volatile, chromatic and sensorial profile of Tinta Negra Madeira wines produced by the selected non-*Saccharomyces* yeasts, as starter culture, was evaluated.

PART II - EXPERIMENTAL

Chapter 1

Evaluation of volatile profile, antioxidant potential and color during the alcoholic fermentation of Madeira wines musts in different wineries.

This chapter is based on:

Evaluation of volatile profile, antioxidant potential and color during the alcoholic fermentation of Madeira wines musts in different wineries.
Miranda A., Pereira V., Jardim H., Marques JC.
(Prepared to be submitted)

Abstract

Madeira wines are produced by the spontaneous fermentation of grape juice by indigenous yeasts microbiota. The present study intends to characterize the volatile profile, phenolic content, antioxidant potential, and color of musts from the Tinta Negra grape variety, produced in 3 different wineries (W1, W2 and W3), during the vinification process. Three samples were collected from 2 fermentation stages, the must at the start of fermentation (M0) and the must before fortification (MBF), and then evaluated their chemical characterization. The results showed that all chemical parameters vary between samples from different wineries. The main volatile compounds found in the musts from all wineries are alcohols and esters, which increase in concentration between M0 and MBF. The musts from W1 stood out due to higher levels of total volatile compounds (95.72 mg/L). Regarding the total phenolic content (TP), the levels increased in samples from W1 and W3. TP levels from W3 reached 858.82 mg GAE/L, while on W2 the amount of TP decreased about 29.88% during the fermentation process. Significant differences were found between the batches in all wineries. The antioxidant potential (AP) increased during the fermentation process and a high reproducibility between batches from W1 and W3 were observed, reaching levels up to 531.58 mg Trolox/L. Concerning the must's color, red predominated over yellow must before fortification, with samples from W1 standing out due to the higher reproducibility between all batches. Results indicate that the variability observed in samples from different wineries shows that the winemaking treatments may influence the wine chemical composition. Moreover, the differences between batches from the same winery suggest that it is necessary to identify the parameters that contribute to this variability. Therefore, it is important to standardize the vinification process to produce high-quality and uniform Madeira wines.

1. Brief introduction

Wines are produced by the fermentation of grape musts, and their classification is based on the alcoholic content, chemical composition, color, and taste (27, 214). Madeira is a fortified wine (17-22%, v/v) with complex flavor resulting from its peculiar winemaking and aging processes. Tinta Negra *Vitis vinifera* L. is a versatile grape variety that can produce wines with different sweetness levels, from dry to sweet, and is responsible for 80-85% of Madeira wines total production.

Even though the wines chemical composition mainly depends on grapes, wineries also significantly impact the wines final proprieties and quality. Indeed, in wineries, several factors can influence the wine composition, namely the presence of indigenous yeasts on equipment surfaces, cellar hygiene, winemaking (grape crushing, sulfite and/or enzyme addition, and clarification processes), and aging processes (27, 103, 105, 115). Although each winery has its own characteristic wine, it is important to maintain reproducibility by standardizing the winemaking and aging processes.

The wine quality is mainly influenced by its aroma, resulting from a combination of several VOCs that can be divided into varietal aromas (originate from grapes), fermentative aromas (formed during fermentation), and post-fermentative aromas (result from aging). During alcoholic fermentation, many VOCs, such as higher alcohols, volatile acids, ethyl esters, aldehydes, acetate esters, and others, are produced by yeast metabolism (8).

Phenolic compounds also play an important role in wine quality by contributing to the wine's color and taste (astringency and bitterness). These compounds are associated to several bioactive effects, such as preventing cardiovascular diseases and decreasing the risk of certain cancers. For that reason, the interest of studying the wines phenolic content and antioxidant capacity has been growing (215). Also, several studies point out that wine browning occurs mainly due to the oxidation of phenolic compounds associated with sugars, lipids, phenols, and amino acids present in wines (201).

With these aspects in mind, the present work aims to study the reproducibility of must batches from different wineries at the beginning of fermentation and before fortification. For that purpose, musts from Tinta Negra grapes were collected during the fermentation process of Madeira wines from different wineries and then study the volatile profile, total phenolic content, antioxidant activity, and color.

2. Material and Methods

2.1. Vinification process and sampling

The objects of this study were musts fermented at 3 Madeira wine wineries (W1, W2, and W3). Musts from Tinta Negra *V. vinifera* grapes were collected from all 3 wineries on the harvest of 2017 and the vinification was carried out by indigenous yeasts, without the addition of commercial yeasts. Although each winery has its own practices, fermentation was performed in an industrial scale through *bica aberta* procedure (fermentation without grape solids) under a controlled temperature (at around 20 ºC), and with the addition of potassium metabisulfite to the grape juice, usually up to 60 mg/L. The fermentation was stopped when the total sugar content reached about 50 g/L by adding grape spirit (containing 95% (v/v) of ethanol), raising the alcohol content to about 17% (v/v). Three samples of 3 different fermentation tanks were collected from each winery at 2 stages of fermentation: grape juice (M0), with a density from 1.070 to 1.075 g/mL, and the must before fortification (MBF), with a final density of from 0.998 to 1.004 g/mL.

2.2. Volatile organic compounds

The volatile profile of musts samples from all wineries was evaluated by SPME followed by GC-MS analysis, based on a method developed by López *et al.* (2002) (216). The activation of LiChrolut EN resin, from Merck (Darmstadt, Germany), prepacked in 30 mg cartridges, was performed using 1 mL of dichloromethane (DCM) (Fisher Scientific, Massachusetts, EUA), 1 mL of methanol (MeOH) (Chem-Lab, Zedelgem, Belgium), and 1 mL of an 18% ethanol (EtOH) solution (Sigma- Aldrich, Missouri, EUA). A solution of synthetic wine was prepared with 6 g/L in tartaric acid and 18% of ethanol, then adjusted the pH to 3.50 with 1M of NaOH. For the extraction, 12.5 mL of must spiked with 10 µL of internal standard (500 mg/L of 3-octanol in synthetic wine) (IS) from Acros Organics (Massachusetts, EUA) was used, followed by elution with 350 µL of DCM, and then drying with sodium sulfate anhydrous. Finally, 1 µL of the extract was injected into the GC-MS. Two injections of each extract were analyzed in duplicate. The volatile compounds were separated on a TRACE GC Ultra coupled to an ISQ single quadrupole mass spectrometry detector, on electronic impact

ionization mode, from Thermo Scientific (Hudson, NH, USA). The compounds were injected, by inserting the syringe into the GC injection port, for 5 min at 240 ºC. The column used was a DB-WAXetr (60 m × 0.250 mm with 0.50 µm film thickness) from Agilent J&W (Folsom, CA, USA) and the carrier gas was helium at a flow rate of 1 mL/min. The transfer line and ion source temperatures were both kept at 230 ºC. The oven temperature program started at 50 °C for 3 min, then increased to 240 °C at 4 °C/min, and finally kept at 240 °C for 17.5 min. The total analysis time was 68 min. Data were acquired using the Xcalibur software. The mass range 30-300 m/z was recorded and the compounds were identified by comparing their mass spectra obtained with those of pure standards and/or those in the Wiley 6.0 and NIST08 databases. Furthermore, the Kovats indexes (KI) were calculated and compared with those found on the Pherobase or NIST online databases. The amount of each volatile compound was expressed in terms of IS as relative concentration.

2.3. Total phenolic content and antioxidant potential

Samples were assayed for total phenolic content (TP) by following Folin-Ciocalteu's method adopted from OIV (OIV-MA-AS2-10) (212). Gallic acid was used as a standard (Fluka Biochemika AG, Buchs, Switzerland). Briefly, 100 µL of sample/standard solution were added to the following reagents: 5 mL of distilled water, 0.5 mL of Folin-Ciocalteu reagent (Fluka Biochemika AG, Buchs, Switzerland), and 2 mL of a 20% (w/v) aqueous solution of Na_2CO_3 (Panreac Química S.A., Barcelona, Spain). Then, the volume was adjusted to 10 mL, and the solutions were mixed. The absorbance was measured at 750 nm after a 30 min reaction period using a UV-vis spectrophotometer, model UV-2600 from Shimadzu (Kyoto, Japan), equipped with the UVProbe 2.42 software, also from Shimadzu (Kyoto, Japan). TP was determined based on the following standard curve of gallic acid (Fluka Biochemika AG, Buchs, Switzerland) in the range 25 - 750 mg/L: A_{750}= 0.0011 GAE (mg/L) + 0.0213 (R^2= 0.998). Samples were analyzed in triplicate.

The antioxidant potential (AP) of musts was evaluated by the ability of samples to scavenge DPPH free radicals, and the procedure was adapted from Pereira *et al.* (2013) (217). First, the calibration curve was defined using Trolox solutions in the range of 25 to 1250 mg/L. A 60 µM 2,2-diphenyl-1-picrylhydrazyl solution (DPPH) in methanol (MeOH), both from Sigma Aldrich (EUA), was prepared daily. A volume of

22 µL of standard solution/sample was added to 3 mL of DPPH solution, and the absorbance was determined at 515 nm after 20 min, every 30 seconds. MeOH was used as a blank, and samples were analyzed in triplicate.

2.4. Color

Samples were analyzed on a UV-vis 2600 dual beam spectrophotometer from Shimadzu (Kyoto, Japan), using quartz cells with a 1 cm optical path and ultrapure water as blank. Before the analysis, samples were filtered through 0.20 µm PP Chromafil Xtra syringe filters (from Macherey-Nagel, Düren, Germany). The browning index (BI) was evaluated by measuring the absorbance at 420 nm (A_{420}). Glories parameters were based on OIV methodology (OIV-MA-AS2-07B), where the color intensity (CI) is conventionally given by the sum of the absorbance at 420, 520 and 620 nm. CIELab coordinates were determined by measuring the transmittance from 380 to 770 nm at 5 nm intervals, according to OIV methodology (OIV-MA-AS2-11-R2006) (212). The coordinate L^* represents lightness (in which $L^*=0$ indicates black/dark and $L^*=100$ indicates white/light), a^* is the green/red color component (in which $a^*>0$ indicates the color red and $a^*<0$ indicates the color green), and b^* is the blue/yellow component (in which $b^*>0$ indicates the color yellow and $b^*<0$ indicates the color blue) (218, 219). Data were acquired using the Shimadzu UVProbe 2.42 software, and the CIELab coordinates were determined, considering the illuminant D65 (daylight source) and a 10° incidence angle, using the Color Analysis 3.10 software, both from Shimadzu (Kyoto, Japan).

2.5. Data analysis

All results are presented as mean ± standard deviation (SD), and significant differences were evaluated by the analysis of variance (One-way ANOVA, Holm-Sidak method) for 95% of probability, using the statistical software SigmaPlot, version 12.0.

3. Results/ Discussion

The purpose of the current work was to study the reproducibility between different batches of musts and evaluate the diversity between wineries. To achieve that,

the essays performed focused on the characterization of the volatile composition, total phenolic content, antioxidant potential, and color of Madeira wine musts collected at the beginning and end of alcoholic fermentation from different wineries.

3.1. Volatile Composition

Madeira wine is characterized by a complex aroma due partly to its peculiar winemaking process. To evaluate the impact of the vinification process on the volatile profile of musts, VOCs from musts collected at 2 stages of fermentation (M0 and MBF) were analyzed. The compounds identified and their respective relative concentrations are compiled in Table 6.

Table 6 - Individual VOCs (as µg 3-octanol/L) found in different wineries (W1, W2 and W3) during the fermentation process (M0 and MBF stages).

	M0 (µg/L)			MBF (µg/L)		
	W1	**W2**	**W3**	**W1**	**W2**	**W3**
Carbonyl compounds (5)						
acetoin**	149.24 ±19.14*a*	167.16±35.71*a*	163.90±19.18*a*	n.d.	71.05 ± 3.42*b*	n.d.
ɣ-butyrolactone**	77.60 ± 4.99*a*	37.14 ± 6.73*c*	46.48 ± 6.02*c*	66.35 ± 6.10*b*	43.13 ± 8.21*c*	46.33 ± 3.37*c*
4-*tert*-butylcyclohexanone**	41.33 ± 3.94*a*	15.74 ± 2.02*c*	14.68 ± 1.87*c*	37.82 ± 3.71*a*,*b*	13.04 ± 3.01*c*	33.92 ± 6.01*b*
benzaldehyde**	n.d.	n.d.	33.20 ± 0.75*a*	n.d.	n.d.	n.d.
decanal**	n.d.	39.53 ± 4.49*a*	n.d.	36.82± 5.84*a*	n.d.	27.60 ± 1.69*b*
Total	***268.17***	***259.57***	***258.26***	***140.99***	***127.22***	***107.85***
Esters (21)						
isoamyl acetate*	779.43± 78.63*a*	n.d.	17.96 ± 3.35*d*	375.95 ± 32.27*c*	607.83± 86.30*b*	393.14 ± 68.01*c*
2-phenethyl acetate**	8.39 ± 0.79*a*	n.d.	n.d.	n.d.	n.d.	n.d.
ethyl hexanoate*	n.d.	27.19 ± 1.86*c*	n.d.	112.42 ± 11.78*b*	126.14± 11.29*b*	171.61 ± 16.81*a*
hexyl acetate*	n.d.	n.d.	n.d.	n.d.	64.52 ± 4.05*b*	106.98 ± 10.85*a*
ethyl lactate**	n.d.	9.28 ± 1.25*d*	12.01 ± 2.46*d*	494.83 ± 36.73*b*	322.85± 26.71*c*	567.31 ± 58.64*a*
ethyl octanoate**	n.d.	n.d.	n.d.	107.28 ± 15.46*a*	82.19 ± 18.56*b*	109.19 ± 4.02*a*
ethyl-3-hydroxybutyrate**	n.d.	n.d.	n.d.	29.26 ± 3.66*b*	30.27 ± 2.71*b*	41.31 ± 2.37*a*
ethyl DL-leucate***	n.d.	n.d.	n.d.	76.98 ± 4.89*b*	98.81 ± 9.89*a*	n.d.
ethyl decanoate**	n.d.	n.d.	n.d.	85.52 ± 5.84*a*	62.35 ± 0.07*b*	50.34 ± 3.80*c*
diethyl succinate**	n.d.	n.d.	n.d.	248.36 ± 5.83*b*	285.34± 31.91*a*	n.d.
ethyl dec-9-enoate**	n.d.	n.d.	n.d.	n.d.	10.75 ± 2.10*b*	20.84 ± 2.66*a*
trimethylene acetate***	n.d.	n.d.	n.d.	162.03 ± 9.60*b*	191.05± 29.06*a*	148.30 ± 13.06*b*
ethyl phenylacetate**	n.d.	n.d.	n.d.	n.d.	19.00 ± 0.85*a*	n.d.
ethyl-4-hydroxybutanoate**	16.37 ± 1.09*a*	n.d.	6.07 ± 0.83*b*	1211.61±94.52*a*	653.28 ± 6.06*b*	928.47 ± 33.34*c*
2-phenethyl acetate**	n.d.	7.47 ± 1.17*c*	10.96 ± 2.22*c*	125.34 ± 9.76*b*	123.59 ± 6.67*b*	153.08 ± 25.74*a*
ethyl laurate**	n.d.	n.d.	n.d.	n.d.	5.53 ± 0.47*a*	n.d.
(E)-geranylacetone**	n.d.	10.56 ± 0.36*a*	n.d.	n.d.	n.d.	n.d.
propyl acetate**	n.d.	8.04 ± 1.16*a*	n.d.	n.d.	n.d.	n.d.
diethyl malate**	n.d.	n.d.	n.d.	1251.25±106.48*a*	266.47± 52.81*c*	966.43 ± 37.11*b*
ethyl palmitate**	n.d.	n.d.	n.d.	31.01 ± 5.90*a*	n.d.	n.d.
monoethyl succinate**	n.d.	n.d.	n.d.	1981.22± 71.10*a*	1887.53±173.84*a*	1705.75±274.22*a*
Total	***804.18***	***62.54***	***47.00***	***6293.05***	***4837.50***	***5362.74***
Fatty acids (10)						
acetic acid*	85.87 ± 1.40*c*	20.88 ± 1.51*d*	7.18 ± 1.13*d*	174.47 ± 28.83*a*	151.30 ± 7.03*b*	134.70 ± 18.51*b*
isobutyric acid**	40.84 ± 1.57*c*	40.67 ± 8.51*c*	68.49± 13.48*b*	n.d.	270.81 ± 25.73*a*	n.d.
isovaleric acid**	24.70 ± 1.70*d*	20.77 ± 3.59*d*	40.41± 11.08*d*	197.99 ± 14.32*b*	387.95 ± 67.61*a*	122.09 ± 17.06*c*
hexanoic acid*	91.11 ± 7.70*c*	35.62 ± 5.71*d*	35.94± 17.67*d*	548.30 ± 41.15*b*	656.64 ± 13.49*a*	527.15 ± 18.82*b*

(E)-2-hexenoic acid**	41.20 ± 0.89*a*	n.d.	n.d.	n.d.	n.d.	n.d.
octanoic acid*	28.49 ± 3.29*b*	n.d.	n.d.	n.d.	890.16 ± 80.88*a*	n.d.
nonanoic acid*	48.58 ± 5.70*b*	n.d.	n.d.	n.d.	n.d.	329.88 ± 42.62*a*
decanoic acid**	45.43 ± 5.74*b*	n.d.	n.d.	580.66 ± 66.40*a*	n.d.	n.d.
9-decenoic acid**	n.d.	n.d.	n.d.	n.d.	n.d.	157.81 ± 9.60*a*
phenylacetic acid**	n.d.	n.d.	n.d.	n.d.	148.31 ± 24.67*a*	n.d.
Total	***406.22***	***117.94***	***152.02***	***1501.42***	***2505.30***	***1271.63***
Alcohols (21)						
isobutyl alcohol*	340.92± 44.17*c*	229.61±10.29*d*	142.57±10.80*d*	2771.52±354.55*b*	2844.79± 85.18*ab*	3083.99±135.66*a*
1-butanol**	21.96 ± 3.04*d*	19.13 ± 3.68*d*	n.d.	59.75 ± 5.38*b*	31.21 ± 3.96*c*	41.92 ± 1.35*a*
3-methyl-1butanol**	n.d.	565.67±15.79*d*	345.38± 3.77*d*	52627.34±2214.78*a*	29248.89±2588.86*c*	48147.42±3343.64*b*
1-pentanol**	n.d.	n.d.	n.d.	n.d.	15.82 ± 0.88*a*	n.d.
4-methyl-1-pentanol**	n.d.	n.d.	n.d.	33.58 ± 2.64*a*	n.d.	n.d.
3-methyl-1-pentanol**	n.d.	n.d.	n.d.	42.43 ± 2.07*a*	20.00 ± 0.13*c*	35.93 ± 3.10*b*
1-hexanol*	1314.09±69.04*b*	958.12±13.75*c*	265.12±27.05*d*	1566.02 ± 76.17*a*	891.04 ± 114.30*c*	1392.20 ± 61.17*b*
3-pentanol**	n.d.	n.d.	20.95 ± 2.41*b*	24.88 ± 3.02*a*	13.47 ± 2.17*c*	18.89 ± 2.81*b*
3-ethoxy-1-propanol**	n.d.	14.41 ± 2.38*b*	8.70 ± 0.22*c*	n.d.	62.30 ± 11.63*a*	n.d.
(E)-3-hexen-1-ol**	n.d.	n.d.	n.d.	28.98 ± 1.53*a*	n.d.	31.70 ± 3.25*a*
(Z)-3-hexen-1-ol**	117.52± 10.55*b*	103.37±16.48*b*	57.88 ± 1.54*c*	139.76 ± 4.29*a*	143.40 ± 12.30*a*	124.78 ± 15.53*a*
(E)-2-hexen-1-ol**	516.42± 40.76*a*	43.04 ± 4.05*b*	n.d.	n.d.	n.d.	14.30 ± 2.29*c*
(Z)-2-hexen-1-ol**	22.3 9± 4.30*a*	n.d.	n.d.	n.d.	n.d.	n.d.
1-heptanol**	n.d.	n.d.	n.d.	n.d.	64.49 ± 6.10*a*	15.68 ± 0.78*b*
2,3-butanediol**	31.14 ± 5.93*b*	24.36 ± 3.99*b*	31.57 ± 4.57*b*	354.60 ± 61.80*a*	405.75 ± 35.13*a*	389.77 ± 53.50*a*
1-octanol**	n.d.	n.d.	n.d.	n.d.	47.56 ± 6.04*a*	n.d.
2-pentanol**	n.d.	n.d.	n.d.	n.d.	6.33 ± 1.18*b*	231.58 ± 35.82*a*
2,7-dimethyl-4,5-octanediol***	n.d.	n.d.	n.d.	31.99 ± 4.30*a*	n.d.	n.d.
benzyl alcohol**	137.99 ± 9.63*c*	41.06 ± 5.35*de*	48.54 ± 2.33*d*	167.15 ± 8.93*b*	36.63 ± 4.55*e*	204.84 ± 10.61*a*
2-phenylethanol*	358.01± 23.53*d*	217.25± 4.36*d*	239.69±14.98*d*	27023.79± 393.40*a*	14039.15±1683.07*c*	19397.68±3163.80*b*
dodecanol**	n.d.	n.d.	25.44 ± 4.24*a*	24.13 ± 4.62*a*	n.d.	n.d.
Total	***2860.44***	***2216.02***	***1185.84***	***84895.91***	***47870.84***	***73130.69***
Miscellaneous Compounds (4)						
methionol**	n.d.	n.d.	n.d.	326.17 ± 16.66*a*	89.80 ± 5.12*c*	282.57 ± 27.42*b*
α-citronellol**	n.d.	n.d.	n.d.	9.22 ± 0.58*a*	n.d.	n.d.
N-(3-methylbutyl)acetamide**	n.d.	n.d.	19.47 ± 1.84*a*	345.04 ± 5.62*b*	181.76 ± 27.82*c*	326.65 ± 20.75*b*
tyrosol**	n.d.	62.10± 10.38*a*	87.68 ± 0.86*a*	2213.08 ± 236.70*b*	638.78± 74.71*c*	1859.27 ± 58.94*d*
Total	***0***	***62.10***	***107.15***	***2893.51***	***910.34***	***2468.49***
Total VOCs amount	**4339.01**	**2718.17**	**1750.27**	**95724.88**	**56251.19**	**82341.4**

Different letters in the same row denote statistically significant differences at (p<0.05); n.d. (not deteted);* MS data and Kovats index in agreement with those of authentic compound; ** MS data and Kovats index in agreement with those in literature;*** MS data in agreement with those in NIST08 and Pherobase libraries. Stages: M0 – grape juice; MBF – must before fortification.

According to the data in Table 6, a total of 61 volatile compounds were identified, including 21 alcohols, 21 esters, 10 fatty acids, 5 carbonyl compounds, and 4 miscellaneous compounds. As expected, the fermentation process promoted a concentration increase in all VOCs families (from M0 to MBF), mainly on esters, alcohols, and fatty acids (3). The exceptions were the carbonyl compounds that showed a decrease from 41.76 to 52.57 % (from M0 to MBF) in total concentration, since these volatile compounds are essentially present in grapes and are usually oxidized into the corresponding alcohols during fermentation (220). The total VOC concentration varied between wineries and the samples collected from W1 stood out for having the highest total concentration of VOCs, up to 95.72 mg/L.

The most abundant VOC family (in average 88.79 % of the total VOCs) found in samples from all wineries was alcohols and the compounds with the highest concentrations were 3-methyl-1-butanol and 2-phenylethanol, followed by isobutyl alcohol and 1-hexanol. 3-methyl-1-butanol and 2-phenylethanol are formed during fermentation by deamination and decarboxylation reactions of leucine and phenylalanine, respectively. 2-phenylethanol can contribute positively to Madeira wine's aroma with fruity, flowery, and honey notes (3, 96). The concentration of alcohols strongly depends on several factors such as the yeast strains involved in the fermentation process, the pH of the must, and the maturation level of the grapes (221). Significant differences (p<0.05) between the levels of 3-methyl-1-butanol in the MBF samples from all wineries were observed. This was the most abundant alcohol in must samples, representing about 62.00, 61.10 and 65.84% of the total alcohol concentration in W1, W2 and W3, respectively. Similar amounts of 3-methyl-1-butanol were found in the literature for Madeira wines (3, 96). Regarding, 2-phenylethanol significant differences amounts (p<0.05) were found between the wineries in MBF stage, ranging from 14.04 mg/L in W2 to 27.02 mg/L in W1.

Regarding esters, the second most abundant family (in average 7.28 % of the total VOCs), the total concentrations ranged from 4.84 mg/L (W2) to 6.29 mg/L (W1) in MBF samples. Ethyl 4-hydroxybutanoate, diethyl malate, and monoethyl succinate were the most abundant esters found in the samples from all wineries. Concentrations of ethyl 4-hydroxybutanoate, which is characterized by herbaceous or "green" notes, ranged from 0.65 mg/L to 1.21 mg/L (p<0.05) in MBF samples in W2 and W1, respectively. Also, increasing concentrations of diethyl malate and monoethyl succinate have been previously described during Madeira wine aging (90, 94).

Moreover, isoamyl acetate and ethyl hexanoate can contribute to wine aromas with fruity notes. Isoamyl acetate levels were higher in W2 samples (607.83 µg/L), and no significant differences (p<0.05) were observed between samples from W1 (375.95 µg/L) and W3 (393.14 µg/L). This compound contributes to Madeira wine aroma with banana and sweet notes. Ethyl hexanoate, which aroma resembles apple, was found in higher concentrations in samples from W3 (171.61 µg/L) and no significant differences (p<0.05) were observed between samples from W1 (112.42 µg/L) and W2 (126.14 µg/L). These esters were previously reported as significant contributors to the Madeira wine aroma (3, 90, 94, 96). The variation in ester concentrations between the samples from different wineries may result from several factors, such as the yeast strains

involved in the fermentation process, must acidity, and additional treatments such as clarifications (3, 161, 162).

During fermentation (from M0 to MBF), the fatty acids (average relative abundance of 3.06 % of the total VOCs) levels greatly increased, reaching values of 2.51 mg/L in W2 samples and 1.50 mg/L and 1.27 mg/L in W1 and W3 samples, respectively. The most abundant fatty acids found were the same as previously reported for Madeira wines (3, 192), namely octanoic (only found in W2 samples), hexanoic, and decanoic acids (only found in W1 samples). These VOCs are usually formed by anabolic pathways of yeast metabolism or by β-oxidation of higher fatty acids. At high levels, these acids may contribute negatively to wine aroma with cheesy, rancid, and vinegar odors (3).

3.2. Total Phenolic Content and Antioxidant Potential

Phenols play an important role on the wine organoleptic and sensorial characteristics of wines. Even though, grapes are the main source of phenolic composition and content, the winemaking practices and also aging processes can influence the final amount (223). In this sense, the TP was evaluated for the samples collected at M0 and MBF in all wineries and the results are presented in Fig. 18.

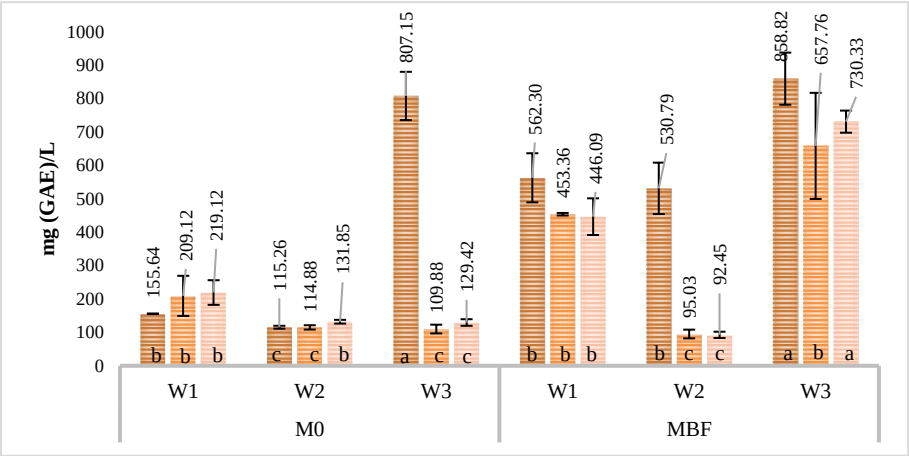


Figure 18 - Total phenolic content during different fermentation stages (M0- grape juice and MBF - must before fortification) in Madeira wine wineries (W1, W2, W3). Different letters in the same stage denote statistically significant differences at (p<0.05).

According to Fig.18, differences between the TP levels of the M0 samples collected from all wineries were found. The samples collected from W1 revealed

reproducibility between the different batches (levels ranged from 155.64 to 219.12 mg GAE/L, with no statistically significant differences (p>0.05)). The samples from W3 showed no reproducibility between batches since the levels of TP significantly varied (p<0.05) from 109.88 to 807.15 mg GAE/L. As expected, at the end of fermentation (MBF stage), the levels of TP increased in the samples from all wineries, much likely due to the action of the yeast strains involved in this stage, which can hydrolyze the glycosidic bonds of phenols and release phenolic aglycones (136). Pereira *et al.* (2013) (198) reported that the TP content of dry Tinta Negra Madeira wines was about 609.98 mg GAE/L, which is in agreement with the results obtained for the samples of 2 of the wineries (W1 and W3) studied in the present work. W3 was the one with higher levels of TP in MBF, ranging from 657.76 to 858.82 mg GAE/L. On the other hand, TP levels decreased in the samples from W2 batches (17.28 and 29.88%), which may be related to the winemaking practices of that winery. Taking into account the amount of TP in wineries before the fortification, significant differences with p<0.05 were found between the batches in each all wineries, revealing lack of reproducibility.

The AP of musts from different wineries was also evaluated through the DPPH assay. The results corresponding to M0 and MBF samples are presented below in Fig. 19.

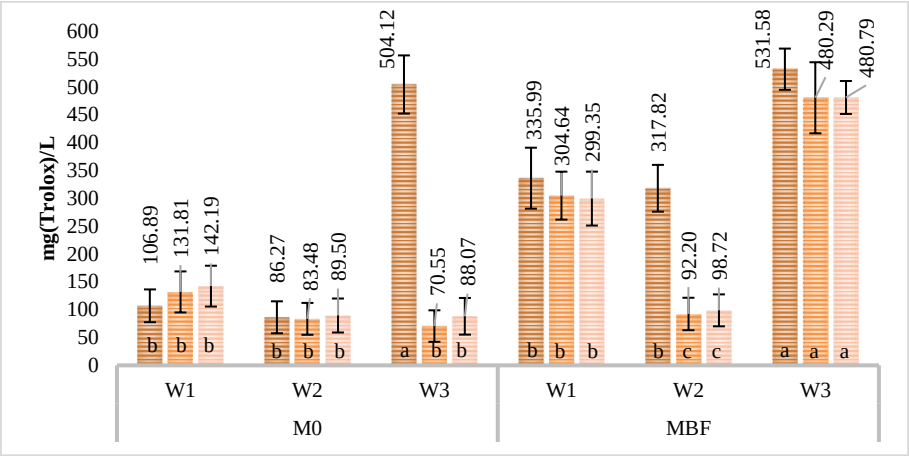


Figure 19 - Antioxidant potential evaluation during different fermentation stages (M0- grape juice and MBF - must before fortification) in Madeira wine wineries (W1, W2, W3). Different letters in the same stage denote statistically significant differences at (p<0.05).

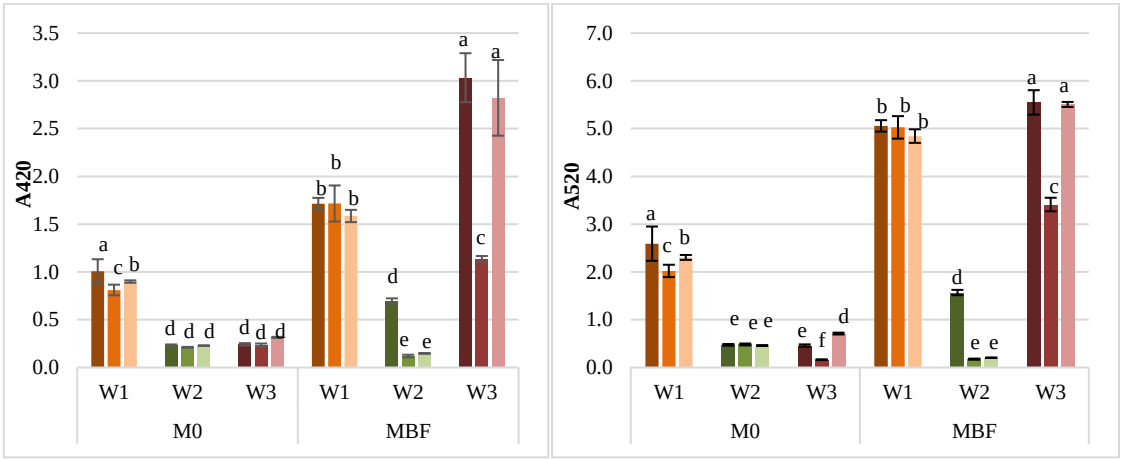
In the M0 stage, the AP levels revealed no significant differences (p>0.05) between the samples from all wineries, except in one batch from W3, which had an AP of 504.12 mg Trolox/L. During fermentation (MBF stage), the AP increased and the highest levels, ranging from 480.79 to 531.58 mg Trolox/L (p<0.05), were observed in

batches samples from W3. The samples from W1 also showed reproducibility between batches (p>0.05), with levels ranging between 299.35 and 335.99 mg Trolox/L. Regarding the samples from W2, AP levels only increased in one batch, but no significant differences were found between the other batches and those from the M0 stage (p>0.05). The AP levels from W3 samples are in agreement with those described previously by Pereira *et al.* (2013) for dry Tinta Negra Madeira wines (about 502.93 mg Trolox/L) (198).

3.3. Color

Color is one of the main characteristics of wines, affecting the wine quality and directly influences the consumer choice. Madeira wines are characterized by its typical color resulting from the oxidative aging process where browning have a significant role. The colors of commercialized Madeira wines can range from pale yellow to dark brown, regardless the grape variety (198, 201). Even though Madeira wines final color results essentially from the aging process, studies about the evolution of color can be helpful for understanding the influence of vinification procedures (198). Thus, wineries samples were evaluated through the Glories method and the CIELab coordinates system.

Glories parameters were determined according to OIV methodology. The measured absorbance at A_{420}, A_{520} and CI are presented in Fig. 20.



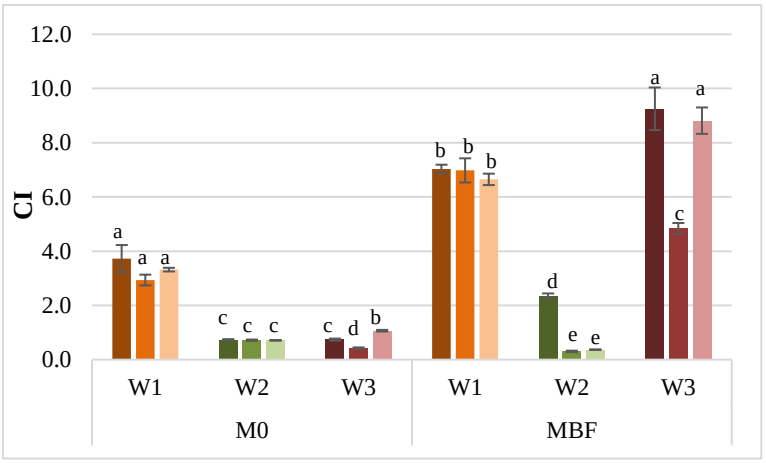


Figure 20 - A_{420}, A_{520}, and color intensity (CI) in M0 and MBF samples (M0- grape juice and MBF - must before fortification) in Madeira wine wineries (W1, W2, W3). Different letters in the same stage denote statistically significant differences at (p<0.05).

Considering the results presented in Fig. 20, even though all fermentations were carried out with musts from the same grape variety (Tinta Negra), there are differences between the mean CI values of different wineries, suggesting that there is variability in the raw materials. Moreover, the CI of MBF samples from different wineries, when comparing to M0 samples, showed that this parameter depends on the winemaking practices of each winery. The samples from W1 were the ones that revealed high color reproducibility, with no significant differences between batches (p>0.05). In general, CI results revealed a similar trend to what was observed for TP content, reinforcing the role of the oxidation of phenols in color (201). The Madeira wine color determination performed by Pereira *et al.* (2013) revealed an A_{420} of 1.58 and CI of 4.22 in young wines before the aging process. These values were lower than the ones determined in the current work, especially in W1 samples, where A_{420} ranged from 1.59 to 1.72 and CI from 6.65 to 7.03, and in W3 samples, where A_{420} varied from 1.14 to 3.03 and CI from 4.84 to 8.81. However, the present work analyzed the must samples and, therefore, the final wine may showed a decrease of color parameters due to the fortification and clarifications practices commonly performed in Madeira winemaking.

Although the Glories method indicates the maximum absorbance of brown pigments and is generally the preferred method by local wineries, CIELab also provides important spectrophotometric data for evaluating wine color. The results obtained for the CIELab chromatic coordinates a^*, b^*, and L^* are presented in Fig 21.

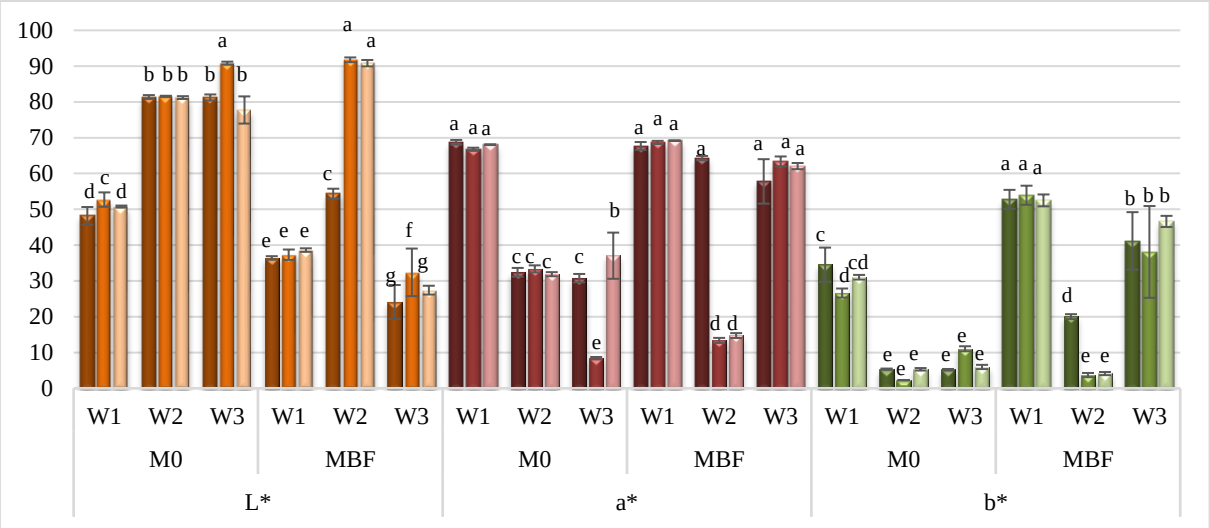


Figure 21 - CIELab chromatic coordinates *a, *b** and *L** in M0 and MBF (M0- grape juice and MBF - must before fortification) in Madeira wine wineries (W1, W2, W3). Different letters in the same chromatic coordinates denote statistically significant differences at (p<0.05).**

Regarding the *L** coordinate, there is a tendency in the samples from two wineries (W1 and W3) to decrease from M0 to MBF. On the other hand, in samples from W2, the *L** increased during fermentation, indicating that the color of the must became lighter. This result, associated with the lower TP levels found in W2 samples, might be explained by the characteristics of the winemaking practices of this winery. Regarding *a** and *b** coordinates, the results obtained in this study were similar to those described by Carvalho *et al.* (2015), where *a** values (positive, red) were higher than *b** (positive, yellow) for Tinta Negra grape musts (201). Additionally, MBF samples from W1 and W3 revealed high reproducibility in *a** and *b** coordinates, between all batches, with no significant differences between them (p>0.05). Once again, there seems to be no clear trend regarding the overall evolution of CIELab coordinates during fermentation, which supports the conclusions previously stated for the Glories parameters.

4. Conclusion

Madeira is a fortified wine with singular characteristics that result from its unique winemaking and aging processes. The great international recognition of theses fortified wines drives each winemaker to produce high-quality Madeira wines with distinguishing characteristics. In this sense, the present study revealed that, during the fermentation process, the volatile profile, phenolic content, antioxidant potential, and

color vary between wineries. Indeed, this variability started from the grape juice and continues through the fermentation process, affecting the reproducibility between different must batches from the same winery. Several factors may be involved, most likely related to the grape maturation, yeast strains involved in the spontaneous fermentation and/or winemaking practices, such as clarifications. Alcohols stands out as the chemical family most abundant in all wineries samples, reaching 88.79% (in average) of total VOCs proportion followed by esters with 7.28%. Winery 3 showed higher levels of TP (858.82 mg GAE/L) and AP (531.58 mg Trolox/L). In order to standardize the vinification process and increase batches reproducibility, guaranteeing the production of high-quality Madeira wines, more studies are needed regarding the indigenous yeast microbiota involved in the fermentation process, as well as establishing a compromise between wine treatments and the intended oenological characteristics.

Chapter 2

Chemical characterization of Madeira wines produced with grapes from different vineyard locations

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This chapter is based on:

Chemical characterization of Madeira wines produced with grapes from different vineyard locations
Miranda A., Pereira V., Jardim H., Marques JC.
(Prepared to be submitted)

Abstract

Tinta Negra *Vitis vinifera* L. grapes, used to produce 80 to 85% of Madeira wines, are mainly cultivated in two vineyard locations on the island: on the south (Câmara de Lobos) and north coast (São Vicente). The present study aims to evaluate the impact of different vineyard locations in the Madeira wine chemical composition in terms of sugars, organic acids, total phenolic content (TP), antioxidant potential (AP), and color. Must and wine samples, produced from grapes collected from 6 locations in 2 distinct winegrowing regions (3 locations in the south and 3 locations in the north of Madeira Island), were evaluated throughout the winemaking and aging processes. The results showed that the initial levels of sugars found in grape juice were higher in samples from the south region (ranging from 174.07 to 196.32 g/L). In addition, glycerol contents increased throughout fermentation, ranging from 4.36 to 6.38 g/L in samples from the south region and from 4.90 to 6.00 g/L in samples from the north. The results revealed that the location where the grapes originated did not influence the organic acid composition of wines. Tartaric acid was found to be the most abundant acid in aged wines, ranging from 2.46 to 3.90 g/L. On the other hand, the TP and AP levels in grape juice were higher in samples from the south region, influencing wine composition, with TP and AP levels up to 270.18 mg GAE/L and 128.48 mg Trolox/L, respectively. In addition, even though the initial grape juice revealed higher Bowning Index (BI) and Color Intensity (CI) in south region, the color showed a similar trend along the vinification and aging processes, with increasing yellow tones typical of these wines. In this sense, understanding the relationship between the vineyard geographic locations and its effect on the variability of the wine chemical composition can be a helpful tool for winemakers to improve Madeira wines quality.

1. Brief introduction

Wine acidity plays an important role in flavor, contributing to a fresh taste. Wines with high acidity have a sour taste, while low acidity can produce insipid or flat flavors. Thus, it is crucial to find a balance between acidity and sweetness. Even though several factors can influence the concentration of acids in grapes, the climatic conditions and the geographic location where the vines are planted have a preponderant impact on their concentrations in wines. Indeed, when grape ripening occurs under high temperatures, the resulting musts show high levels of sugars but low acidity (high pH), increasing the susceptibility to microbiological spoilage (8, 9). In addition, the phenolic content of grapes tends to decrease during ripening; however, different phenolic classes may present distinctive evolution patterns, which are greatly affected by intrinsic (grape variety) and extrinsic factors (geographic location, climate, and soil) (215, 224, 225). *Vitis vinifera* L. grapes are considered a good source of phenolic compounds, which contribute to wine quality (astringency, bitterness, and color) and, due to their bioactive proprieties, confer potential health benefits as well (215).

Yeasts found at the beginning of must fermentation originate from vineyards and grapes. Although the yeasts found in grapes are similar worldwide, several factors can strongly affected wine microbiota and, consequently, its characteristics and quality (103, 105). These factors include altitude and climatic conditions of the vineyards, such as maritime influence, temperature, humidity, and rainfall, as well as grape variety and viticulture practices (such as fertilization, irrigation, and the use of fungicides) (103-105, 107).

Tinta Negra *Vitis vinifera* L. grape vines, used to produce 80 to 85% of Madeira wines, are known for being a robust variety, and for that reason, are easily adapted to different locations in Madeira Island. This grape variety is essentially cultivated in the south region, mainly in Câmara de Lobos, and in the north of the island, in São Vicente (1, 5). The present study evaluate the impact of grapes from different vineyard locations on the chemical composition of Madeira wines. The parameters analyzed include sugars, organic acids, total phenolic content, antioxidant potential, and color.

2. Experimental

2.1. Samples and microvinification

Grapes from the Tinta Negra variety (*Vitis vinifera* L.) were collected in 2017 from two different geographic regions of Madeira Island: from three different locations in the south region – Câmara de Lobos (L1, L2, and L3) – and three different locations from the north region – São Vicente (L4, L5, and L6). Microvinifications were carried out on a laboratory scale (in duplicate) in 3L amber glass flasks, under controlled temperature (20±3 ºC) by spontaneous fermentation and with the addition of potassium metabisulfite up to 60 mg/L. The alcoholic fermentation was stopped when the total sugar content reached about 50 g/L by adding grape spirit (containing 95% (v/v) of ethanol) until an alcohol content of about 17% (v/v) was achieved. Then, to simulate the *estufagem* process, about 100 mL of each wine (in duplicate) were placed into 100 mL borosilicate bottles with a small head space volume and stored at 45±0.5 ºC for 120 days in a Memmert UFE 400 oven (Schwabach, Germany). For each vineyard location, two samples were collected from different production stages: grape juice (M0), must before fortification (MBF), wine after fortification (WAF), and wine after *estufagem* (WAE).

2.2. Ethanol, reducing sugars and organic acids

The concentrations of ethanol, fructose, glucose, glycerol, and organic acids (citric, tartaric, malic, succinic, lactic, formic and acetic) were determined based on the procedure proposed by Miranda *et al.* (2017) (22). The analyses were carried out in a Waters Alliance high-performance liquid chromatograph (HPLC-DAD-RID) equipped with an auto-injector (Waters 2695 separation module), a photodiode array detector (Waters 2996), a refractive index detector, and the Empower Pro software for data handling, all from Waters Corporation (Santa Clara, CA, USA). The chromatographic separation was performed using a Hi-Plex H column (300 x 7.7 mm, and 8 µm particle size) from Agilent (Santa Clara, CA, USA) with an isocratic elution, using an aqueous solution of sulphuric acid (0.0025 M), at a flow rate of 0.6 mL/min. The column temperature was set to 65 ºC and the injection volume was 10 µL. Samples were previously filtered through 0.20 µm PP Chromafil Xtra syringe filters (from Macherey-

Nagel, Düren, Germany). Calibration curves ranged between 100 and 5000 mg/L for tartaric, malic, and acetic acids; from 50 to 5000 mg/L for lactic acid; from 20 to 1000 mg/L for citric and succinic acids; and from 100 to 1000 mg/L for formic acid. The concentration of each compound was calculated using an external standard calibration, and samples were analyzed in triplicate.

2.3. Total phenolic content and antioxidant potential

The TP was evaluated in all different production stages following Folin-Ciocalteu's method, while the AP determination was based on the DHHP assay. Both procedures were performed according to the methodologies described in Chapter 1 (section 2.3).

2.4. Color

The color determination was performed by Glories and CIELab methodology according to procedures previously described in Chapter 1 (section 2.4). The BI was evaluated by measuring the absorbance at 420 nm while the Glories was based on OIV methodology (OIV-MA-AS2-07B) by the determination of CI from the sum of the absorbance at 420, 520 and 620 nm. CIELab coordinates were determined according to OIV methodology (OIV-MA-AS2-11-R2006). The coordinate L^* represents lightness (in which $L^*=0$ indicates black/dark and $L^*=100$ indicates white/light), a^* is the green/red color component (in which $a^*>0$ indicates the color red and $a^*<0$ indicates the color green), and b^* is the blue/yellow component (in which $b^*>0$ indicates the color yellow and $b^*<0$ indicates the color blue).

2.5. Data

All results are presented as mean ± SD, and significant differences were evaluated by the analysis of variance (One-way ANOVA, Holm-Sidak method) for 95% of probability, using the statistical software SigmaPlot, version 12.0.

3. Results/ Discussion

To study the influence of Tinta Negra grape origin on the characteristics and quality of Madeira wines, grape samples from two main vineyard locations, São Vicente and Câmara de Lobos, were collected. Then, the evolution of the wine chemical parameters (sugars, ethanol, organic acids, total phenolic content, antioxidant potential, and color) was assessed throughout the winemaking and aging processes.

3.1. Sugars, glycerol and ethanol contents

Glucose and fructose are the most abundant sugars found in grapes. Other sugars can occur but usually in minor concentrations (14, 25). The levels of glucose, fructose and glycerol were evaluated during the vinification and *estufagem* processes, as reported in Table 7.

Table 7 - Glucose, fructose, and glycerol concentrations during the winemaking and aging of Madeira wines produced with grapes from different vineyard locations.

	South Locations			North Locations		
	L1	**L2**	**L3**	**L4**	**L5**	**L6**
Glucose (g/L)						
M0	85.44±0.19*a*	56.49±0.41*e*	61.88±0.14*d*	79.25±0.27*b*	69.08±0.41*c*	56.51±2.22*e*
MBF	22.60±0.45*a*	20.73±0.24*b*	11.26±0.63*e*	12.37±0.63*d*	19.48±0.24*c*	20.51±0.05*b*
WAF	18.32±1.16*b*	19.54±1.14*a*	9.43±0.57*d*	11.99±0.94*c*	6.96±1.14*e*	n.q.
WAE	18.25±0.25*b*	19.05±0.25*a*	9.32±0.36*d*	11.65±0.65*c*	6.75±0.68*e*	n.q.
Fructose (g/L)						
M0	110.88±0.69*c*	117.58±0.76*b*	128.98±7.83*a*	102.49±0.04*d*	95.58±1.01*e*	119.60±6.20*b*
MBF	45.14±1.71*a*	36.55±2.09*b*	43.32±3.87*a*	30.24±2.51*c*	21.84±0.22*d*	23.70±1.49*d*
WAF	30.43±0.36*b*	19.29±1.63*d*	32.60±1.12*a*	27.17±1.77*c*	5.90±0.35*f*	9.63±0.58*e*
WAE	30.25±0.25*b*	19.23±1.23*d*	32.36±1.23*a*	26.95±0.95*c*	5.35±0.25*f*	9.53±0.36*e*
Glycerol (g/L)						
M0	2.27±0.01*a*	n.d.	n.d.	n.d.	2.23±0.01*b*	n.d.
MBF	10.72±0.72*a*	8.11±0.40*b*	3.72±0.08*e*	6.95±0.29*c*	5.74±0.32*d*	8.11±0.40*b*
WAF	6.42±0.27*a*	4.95±0.22*d*	4.46±0.06*e*	6.05±0.12*b*	5.94±0.47*c*	4.95±0.22*d*
WAE	6.38±0.12*a*	4.89±0.03*d*	4.36±0.02*e*	6.00±0.06*b*	5.78±0.12*c*	4.90±0.18*d*

Different letters in the same row denote statistically significant differences at (p<0.050), according to Holm-Sidak test: n.d. (not detected); n.q. (not quantified). Locations: South Locations (Câmara de Lobos); North Locations (São Vicente). Stages from grape collection: grape juice (M0), must before fortification (MBF), wine after fortification (WAF), and wine after estufagem (WAE).

The results showed that, in M0, the total levels of sugars (given by the sum of glucose and fructose concentrations) were higher in musts produced with grapes from south locations (ranging from 174.07 to 196.32 g/L) than the ones from north locations (varying from 164.66 to 181.74 g/L), demonstrating the influence of geographic

locations on the grape's sugar levels (8, 9). Indeed, the main influence in this sugar locations levels was from fructose representing, in average, more 11% in south regions than in the north coast since glucose is more susceptible to cellular plant respiration at higher temperatures (226). Additionally, musts from the M0 stage revealed that even in the same vineyard location, independently of the region, significant differences (p<0.05) in sugar levels were found between grape juices.

As expected, the results showed, in samples from all vineyard locations, that both sugars (glucose and fructose) followed the same trends, decreasing during the winemaking process, since yeasts readily consume these fermentable sugars as nutrients to produce ethanol and other by-products (25). During fermentation (M0-MBF) the consumed of glucose was similar in both vineyard locations (about 73 %) while the fructose consumption was more evident in the north coast (in average 76%) when compared to the south region vineyards (in average 65%). This fact may be associated with the yeast specie present in this geographic location since some non-*Saccharomyces* evidence a fructophilic character more intense and, therefore, it is more likely to consume fructose (226). Also, the decrease in sugar levels (M0 to WAE) was higher in samples from vineyards in the north, in about 94% in both glucose and fructose. In addition, the glycerol content increased during the fermentation process ranging from 4.36 to 6.38 g/L in samples from the south region and from 4.90 to 6.00 g/L in samples from the north. Although the concentration of glycerol may depend on the grape region origin, similar concentrations (6.82 g/L) were previously reported in South African dry table wines by Nieuwoudt *et al.* (2002) (227).

The ethanol formation during fermentation depends on the grapes initial sugar content and the fermentation performance of yeasts involved during this process (228). Ethanol levels during fermentation and after wine fortification, by the addition of natural grape spirit, are described in Table 8.

Table 8 - Ethanol content during the winemaking and aging processes of musts and wines produced with grapes from different vineyard locations.

	South Locations			North Locations		
	L1	**L2**	**L3**	**L4**	**L5**	**L6**
Ethanol (% v/v)						
M0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MIF	n.d.	1.53±0.09*a*	n.d.	0.19±0.03*c*	0.27±0.01*b*	n.d.
MBF	8.35±0.19*b*	6.18±0.04*d*	6.52±0.24*c*	8.75±0.71*b*	9.30±0.19*a*	8.28±0.09*b*
WAF	17.62±0.45*a*	17.67±0.33*a*	17.32±0.02*ab*	17.34±0.15*a*	16.98±0.09*b*	17.50±0.39*a*
WE	17.58±0.12*a*	17.59±0.05*a*	17.25±0.03*b*	17.18±0.00*b*	16.89±0.05*c*	17.35±0.12*b*

Different letters in the same row denote statistically significant differences at (p<0.050), according to Holm-Sidak test; n.d. (not detected). Locations: South Locations (Câmara de Lobos); North Locations (São Vicente). Stages

from grape collection: grape juice (M0), must before fortification (MBF), wine after fortification (WAF), and wine after estufagem (WAE).

After fermentation, the levels of ethanol formed were higher in wines produced with grapes from north locations (8.28 to 9.30 %) than those from south locations (6.18 to 8.35 %). This was reflected on the consumption of total sugars between M0 and MBF, with a higher decrease in samples from north locations, in about 74.90 to 79.55 %, than in samples from south locations (65.50 to 71.40 %).

3.2. Individual organic acids

Acidity has a significant impact on the Madeira wine quality. Therefore, it was essential to the objectives of the present study to evaluate the evolution of the main organic acids during the winemaking and aging processes and investigate the impact of the grapes origin on the concentrations of these compounds. The amounts of citric, tartaric, malic, lactic, formic, and acetic acid determined in musts and wines produced with grapes from different locations, and collected at various stages of the vinification and aging processes, are presented in Fig. 22. Succinic acid was not detected in any sample.

In general, the results showed that organic acids had similar behavior in the samples produced with grapes from different vineyard locations. Citric, tartaric, and malic acids are mainly present in grapes, while lactic, formic, and acetic acids are mostly formed during the fermentation process (14). Indeed, in the current work, the highest levels of citric, tartaric, and malic acids were found in M0 samples and decreased during fermentation, while the levels of lactic, formic, and acetic acids tended to increase during vinification process. Regarding citric acid, the highest concentrations, ranging from 0.037 to 0.079 g/L, were found in the M0 samples produced with grapes from vineyards in the north. The levels of citric acid decreased during the fermentation in all sample locations, becoming unquantifiable in WAE. These values were lower than those previously described by Pereira *et al.* (2010) for fortified wines (199). Tartaric acid concentrations decreased from values of 4.14 to 8.32 g/L in M0 samples to values of 2.46 to 3.90 g/L in WAE samples. Similarly tendency were observed in malic acid levels, ranging between 0.98 to 2.09 g/L in M0 samples and varied from 0.65 to 1.65 g/L in WAE samples. No influence of the vineyards location in the concentrations of these acids was observed. Similar levels of these both

organic acids were found in the literature in commercialized Madeira wines (199). Lactic, formic, and acetic acid concentrations significantly increased during fermentation and aging, reaching values as high as 0.72 g/L, 0.35 g/L, and 0.50 g/L, respectively. These concentrations were lower than those reported by Pereira *et al.* (2010) for fortified wines (199). Once more, the grapes geographic origin did not seem to significantly influence the concentrations of these organic acids in the final wines samples.

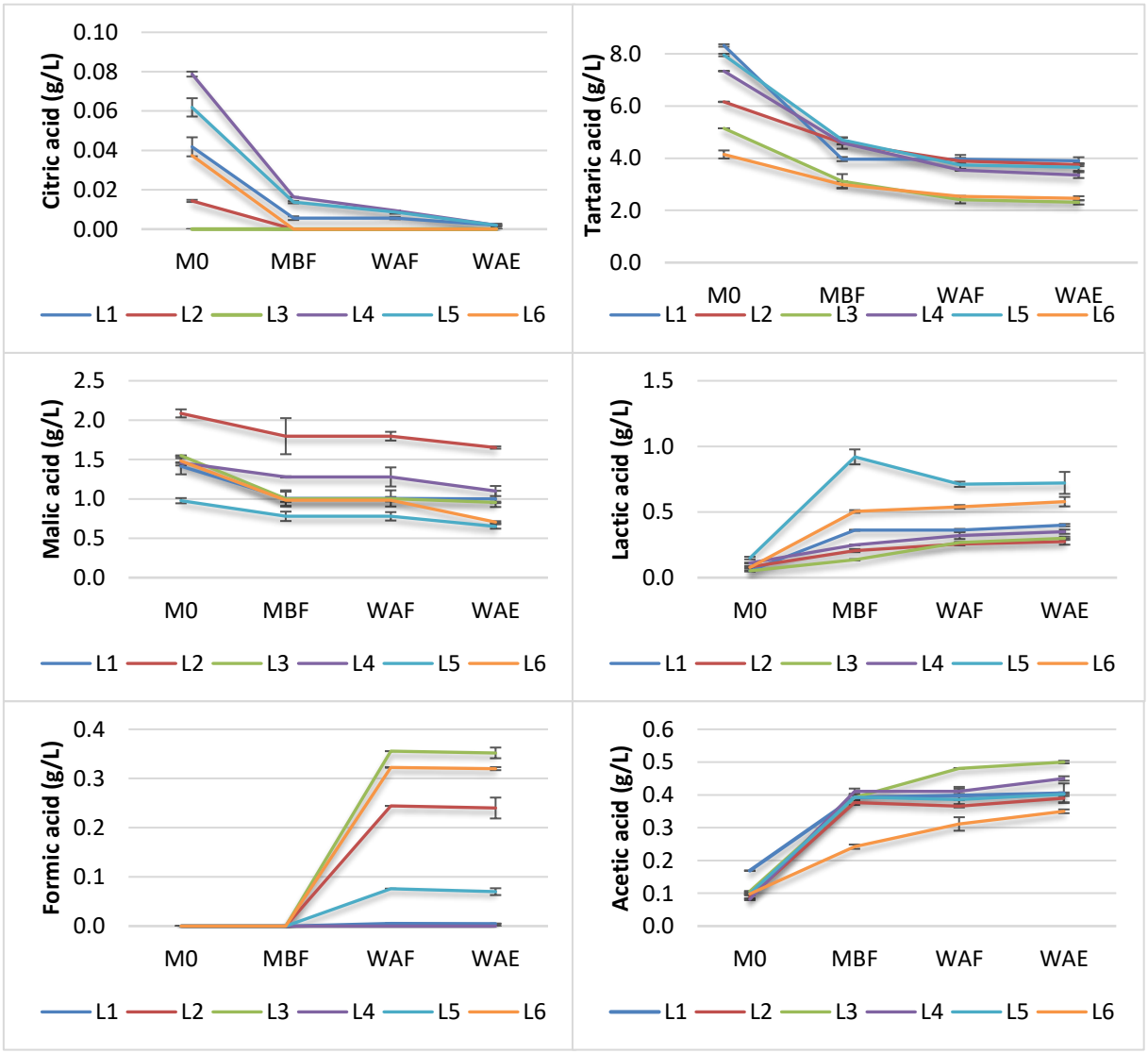

Figure 22 - Individual organic acids levels during the vinification and aging processes of musts and Madeira wines produced with Tinta Negra grapes from different vineyards locations: south (L1, L2, and L3) and north (L4, L5, and L6).

3.3. Total phenolic content and antioxidant potential

The formation of antioxidant compounds in grape vines is affected by the biotic and abiotic stresses that the plant is subjected to. In turn, the location of the vineyards, which is associated with different climate and soil conditions, for example, should influence the levels of these molecules in grapes and, subsequently, in wines (225). The effect of different vineyard locations on the total phenolic composition of Madeira wines along the entire production process was evaluated by spectrophotometric measurements. Fig. 23 illustrates the obtained results.

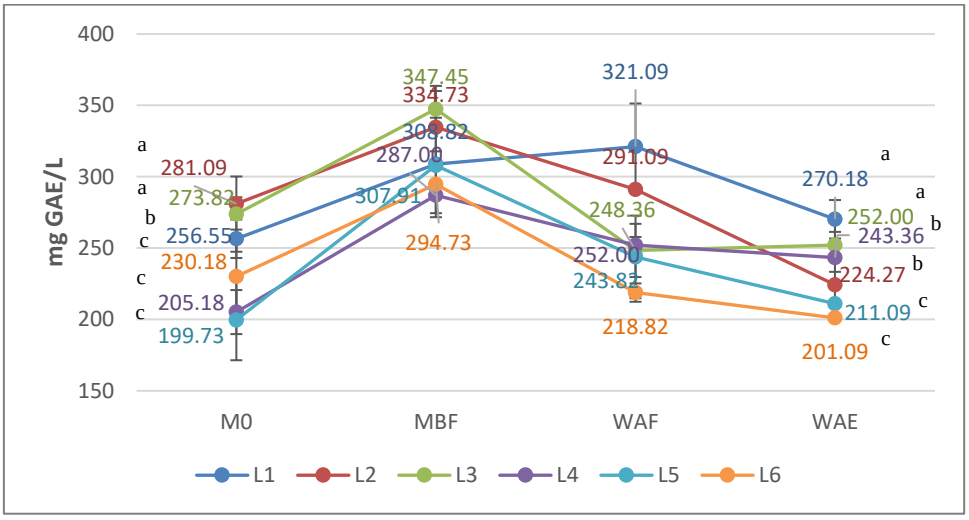

Figure 23 - Total phenolic content of must and wine samples, produced with Tinta Negra grapes from different vineyard locations (South Location: L1, L2 and L3; North Location: L4, L5 and L6), collected during Madeira wine vinification and estufagem processes. Different letters denote statistically significant differences (p<0.05) in M0 (grape juice) and WAE (wine after *estufagem*) stages.

The results demonstrated that the vineyard location influenced the TP content in the grape juice (M0), with higher levels, ranging from 256.55 to 281.09 mg GAE/L, found in samples produced with grapes from the south region. No significant differences (p>0.05) were found between must samples produced with grapes from the different locations within this region. Although samples produced with grapes obtained from vineyards in the north region showed lower TP levels, between 199.73 and 230.18 mg GAE/L, no significant differences were observed in the TP content of these samples. Indeed, significant differences (p<0.05) were found between both regions, being in according to Locatelli *et al.* (2016) (225) studies that reported the influence of the geographic location of the vineyard in the phenolic content of the grapes.

Additionally, the fermentation process, M0 to MBF, promoted an increase in the TP levels of all samples. On the other hand, the fortification and *estufagem* processes caused a decrease in the TP levels (excepted in L1 and L5). In fact, this phenomenon is more notorious in *estufagem* since it promoted a decrease of up to 23% (in the case of samples from L2), in agreement with the studies performed by Pereira *et al.* (2013) (198). Regarding the WAE, TP levels varied from 201.09 to 270.18 mg GAE/L, lower than those described in the literature for Tinta Negra dry Madeira wines after *estufagem* process (198). This difference may result from the fact that fermentation was carried out according to *bica aberta* practices, without grape skins and solids (affecting the phenols content since the main source of these compounds are from grape solids), contrary to the study by Pereira *et al.* (2013). Although, in general, WAE samples produced with grapes from southern locations showed a small tendency for higher TP levels, the results for L2 samples were significantly different (p<0.05) compared to the other samples from southern vineyard locations. So, it is possible to conclude that the TP contents of Madeira wines may be influenced by the vineyard location, as well as the vinification and aging processes.

The antioxidant potential (AP) was determined by the DPPH, and the results are shown in Figure 24.

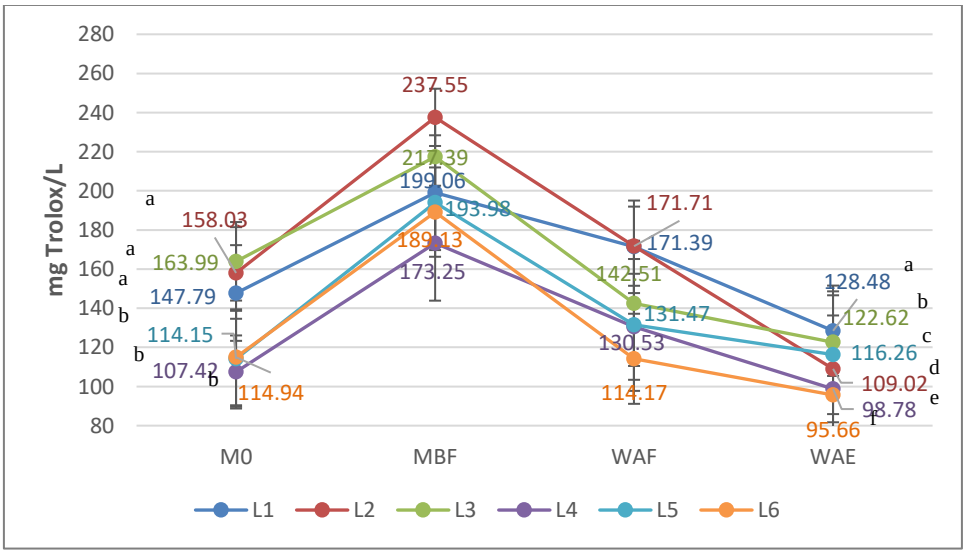

Figure 24 – Antioxidant potential content of must and wine samples, produced with Tinta Negra grapes from different vineyard locations (South Location: L1, L2 and L3; North Location: L4, L5 and L6), collected during Madeira wine vinification and estufagem processes. Different letters denote statistically significant differences (p<0.05) in M0 (grape juice) and WAE (wine after *estufagem*) stages.

The results for AP presented in Fig. 24 showed similar trends as those observed for the TP content. During fermentation, the AP increased about 50.32 % and 69.94 % in the musts produced with grapes from the south and north vineyard locations, respectively. The fortification process promoted a decrease in AP up to 39.64% (in L6 samples), followed by a decrease up to 36.51% during the *estufagem* process (in L2 samples). Although to a lesser extent, the AP levels decreased during *estufagem* has been previously reported during the aging of dry Tinta Negra Madeira wines (198). In addition, grape juices (M0) produced with grapes from vineyards in the south showed higher AP levels than those produced with grapes from vineyards in the north, with no significant differences (p>0.05) between samples from each region. On the other hand, even though the corresponding WAE samples continued to present the highest AP levels, there were significant differences (p<0.05) between samples. The AP levels of all WAE samples ranged from 95.66 to 128.48 mg Trolox/L. As mentioned before, the fact that higher AP levels were reported in another study about Madeira wines (198) may be associated with the characteristics of the fermentation process used.

3.4. Color

Madeira wines are characterized by a brownish color that results from the oxidative aging process and can range from pale yellow to dark brown. Two methodologies were employed to study the color evolution of the musts and wines in this study: the Glories method and the CIELab coordinates system. The Glories parameters (A_{420}, A_{520}, and CI) are presented in Fig. 25.

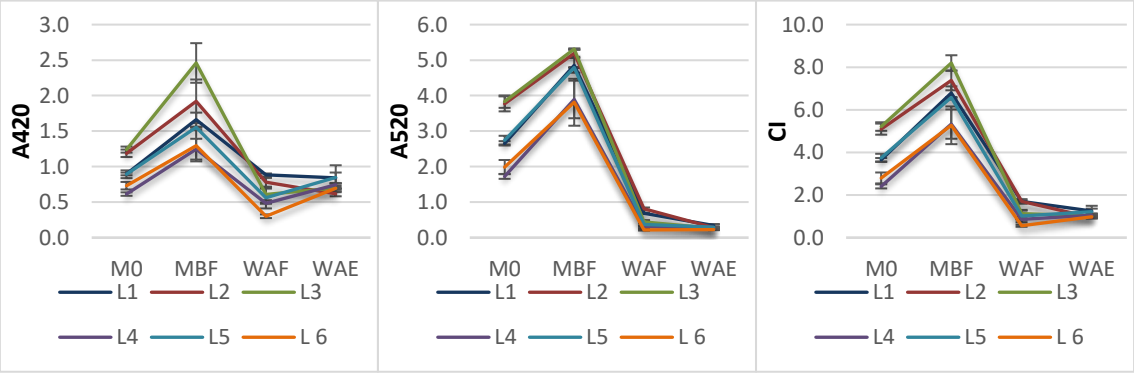

Figure 25 – Browning index (BI) and color intendity (CI) evaluation in different vinification and aging steps from Madeira wines produced by Tinta Negra grapes from different regions locations (South Location: L1, L2 and L3; North Location: L4, L5 and L6).

The BI was evaluated by measuring the absorbance at 420 nm. All samples showed similar trends in the color evolution during the vinification and *estufagem* processes. In M0, musts produced with Tinta Negra grapes collected from the southern vineyards revealed higher BI (0.90 to 1.24 AU) that those produced with grapes from northern locations (0.61 to 0.89 AU). Indeed, this fact may be correlated with the TP levels since this parameter, and in specific some individual phenolic compounds, such as *+(-)*catechin, gallic acid, chlorogenic acid, catechol, and rutin, were reported to correlate with the BI (229). Additionally, the BI was similar in all WAE samples, revealing that the grapes geographic origin did not influence the BI final Madeira wines. Similar to results from Carvalho *et al*. (2015) (201), the fermentation process promoted an increase in the BI; however, higher values were found in the present study, with a BI of 2.46 AU in L3 samples. This is considered intense browning (>0.5AU), according to the categories defined by Fernández-Zurbano *et al.* (1998) (230) and can be associated with enzymatic browning due to the oxidation of flavonols (47, 201). While the fortification step promoted a decrease in BI in all samples, down to a maximum of 0.30 AU in L6 samples, *estufagem* increased the BI by 0.74 AU, on average.

Regardless of the vineyard location, the values of CI were similar in all WAE samples, with an average value of 1.08 AU and no significant differences (p>0.05) between samples. These results were lower than those reported by other authors after the *estufagem* of dry Tinta Negra Madeira wines (198). So, although the BI and CI of the initial grape juice varied according to the vineyard region, the vinification and mainly the *estufagem* process tends to standardized the final color of the wines.

The CIELab chromatic coordinates L^*, a^* and b^* were also determined in M0, MBF, WAF, and WAE samples and are represented in Fig. 26.

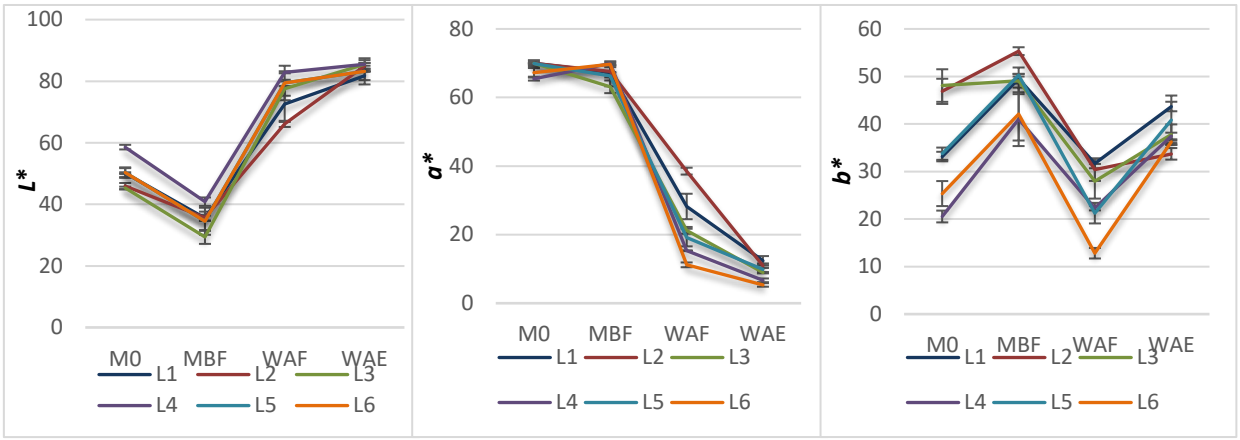

Figure 26 - CIELab coordinates (*L, *a**, and *b**) determined throughout the winemaking and aging processes of Tinta Negra Madeira wines and musts produced with grapes from different vineyard locations (South Location: L1, L2 and L3; North Location: L4, L5 and L6).**

The results demonstrated that the color of all wines tends to evolve towards the same chromatic characteristics, regardless of the grape's geographic origin. During fermentation, *L** values decreased; however, after fortification and *estufagem*, it increased as the wines became lighter due to anthocyanin polymerization (198, 231). Moreover, the predominance of red tones (positive values of *a**) in the M0 stage decreased during fermentation and aging, while the yellow tones (positive values of *b**) increased from WAF to WAE stages, which may be associated with the presence of yellow pigments derived from Maillard reactions and the degradation of sugars (194, 201, 231). Furthermore, there was no clearly tendency regarding the vineyard locations with the color evolution during the vinification and aging process. Indeed, the geographic origin of Tinta Negra grapes did not seem to influence the chromatic parameters in WAE samples. After *estufagem* process, *a** values ranged from 5.33 to 12.41 and the *b** values from 33.72 to 43.68. Similar results were previously reported for dry Tinta Negra Madeira wines (201).

4. Conclusion

This study demonstrated that Madeira wines chemical composition can be affected by the location from where the grapes are collected. In fact, sugars (up to 196.32 g/L), the total phenolic content (281.09 mg GAE/L), and the antioxidant potential (163.99 mg Trolox/L) were higher in Tinta Negra grape juices produced with grapes from locations in the south region (L1, L2, and L3) compared to those from the

north region (L4, L5, and L6). Even though these compounds showed a similar trend throughout fermentation and aging, the final wine composition seems to correlate with the initial grapes chemical composition.

The concentration of organic acids was not influenced by the vineyards location, except citric acid, which revealed higher concentrations in grape juices produced with grapes from northern locations. A similar trend for organic acids was observed in all samples during vinification and *estufagem* since wine samples showed similar compositions regardless of the origin of the grapes. Despite BI and CI of the initial grape juice showed higher values for grapes from the south location, the vinification and mainly the *estufagem* process tends to standardized the final color of all wines, by increasing the yellow tones and a decrease in red tones.

In this sense, the south vineyards location seems to improve some of significant chemical parameters that influence the wine final quality. So, this study provides relevant information to winemakers to enhancing the Madeira wines characteristics and improved its quality.

Chapter 3

Impact of non-*Saccharomyces* yeasts fermentation in the Madeira wine chemical composition

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This chapter is based on the following publications:

Leça JM., Pereira V., Miranda A., Vilchez JL., Malfeito-Ferreira M., Marques JC. Impact of Indigenous Non-*Saccharomyces* Yeasts Isolated from Madeira Island Vineyards on the Formation of Ethyl Carbamate in the Aging of Fortified Wines. Processes. 2021; 9: 799-809.

Impact of non-*Saccharomyces* yeasts fermentation in the Madeira wine chemical composition. Miranda A., Pereira V., Jardim H., Malfeito-Ferreira M., Marques JC. (In revision)

Abstract

Non-*Saccharomyces* yeast species are currently being used as starter cultures in wine production. Madeira wine is produced by spontaneous alcoholic fermentation arrested by ethanol addition. The increasingly demand of wine market lead to need to standardize the winemaking process. In this sense, this study focus in identify the microbiota of indigenous yeast present during the fermentation of Madeira wines and then evaluate the impact of selected indigenous non-*Saccharomyces* as starter pure culture in the chemical and phenolic characterization of Madeira wine production. Studies about the indigenous yeast microbiota involved in the Madeira wine production set allowed the identification of 11 yeasts species from wineries (*Hanseniaspora uvarum, Starmerella bacillaris, Pichia kluyveri, Pichia fermentans, Saccharomyces cerevisiae, Torulaspora delbrueckii, Candida apicola, Cystobasidium minutum, Pichia terricola, Cystobasidium slooffiae, and Wicheramomyces anolalus*) and 6 yeasts species from vineyards (*Hanseniaspora uvarum, Starmerella bacillaris, Pichia kluyveri, Pichia fermentans, Saccharomyces cerevisiae, and Hanseniaspora opuntiae*). *Hanseniaspora uvarum, Starmerella bacillaris, Pichia terricola, Pichia fermentans, and Pichia kluyveri* were inoculated and used as starter pure culture. Results showed that the phenolic content of the wines was influenced by yeast species, with higher levels found in wines produced by *Pichia* spp. (ranging from 356.85 to 367.68 mg GAE/L in total phenols and 50.52 to 51.50 mg/L in total individual phenols by HPLC methods). On the other hand, the antioxidant potential was higher in wines produced with *Hanseniaspora uvarum* (133.60 mg Trolox/L) and *Starmerella bacillaris* (137.61 mg Trolox/L). Also, *Starmerella bacillaris* stands out due to the sugar consumption during fermentation (the totality of fructose and 43% of glucose) and 15.80 g/L of total organic acids compared to 9.23g/L (on average) for the other yeasts. This knowledge can be advantageous to standardizing the winemaking process, resulting in the production of high-quality wines.

1. Introduction

Wine spontaneous fermentation is a complex biochemical process that involves mainly the interaction with yeasts and bacteria. Although *Saccharomyces cerevisiae* is the primary yeast involved in alcoholic fermentation, non-*Saccharomyces* yeasts species have been increasingly used to ensure the wine's final quality (232, 233).

The microbiology of wine, as well as the indigenous yeast, has been extensively studied. Non-*Saccharomyces* species originate mainly from the vineyard environment, being present in the soil and on the surface of the grape and vine. Non-*Saccharomyces* species and its concentration are conditioned by several factors such as grape variety, geographical and climatic conditions, and viticulture practices (234). The yeasts found in the vineyard are divided into three categories according to their fermentation performance (235). The most common oxidative yeasts initially isolated in grapes belong to the first category – *Cryptococcus*, *Rhodotorula*, and *Aerobasidium* genera. Their concentration decreases in the middle and late grape ripening stages due to the competition for nutrients (236). In the initial stage of the spontaneous fermentation, the semi-fermentative non-*Saccharomyces* yeast genera are dominant, such as *Hanseniaspora*, *Candida*, *Pichia*, and *Metschnikowia* (237, 238). As fermentation continues and the level of ethanol increases, these species are replaced by highly fermentative yeasts such as *Saccharomyces*, *Lachancea*, and *Torulaspora* (235). Another origin of indigenous yeasts that affects the wine flavor and its final quality is the microbial communities inhabiting the winery environment, such as the floor, air, and equipment. Even though non-*Saccharomyces* yeasts represent the major winery inhabitants, namely from the genera *Hanseniaspora*, *Candida*, *Pichia*, *Aureobasidium*, *Metschnikowia*, *Cryptococcus*, *Torulaspora*, and *Williopsis*, many studies have found *S. cerevisiae* in wineries (119, 234, 239). Also, several studies show that both yeasts species (*Saccharomyces* and non-*Saccharomyces*) tend to persist in the wineries over consecutive vintages and, for that reason, these resident microbial communities have an important role during both the fermentation process and in the wine's final quality (115, 240). Besides influencing the wine physicochemical parameters and its volatile composition, non-*Saccharomyces* species have been reported to influence the non-volatile composition, namely the final concentration of acids in wines, depending on the species and strains involved (170, 241), and the phenolic composition as well (35). Indeed, some non-*Saccharomyces* yeasts are described to enhance the phenols composition and concentration due to the metabolic activities of

yeast or by the enzymatic reactions during the fermentation process. The β-glycosidase is the main responsible for hydrolyzed the glycoside bonds and release the phenols aglycones (31, 51, 242). Additionally, non-*Saccharomyces* present a significant interest in the oenological properties since these yeasts when used in pure or mixed culture with *S. cerevisiae* can modulate the wine acidity (243).

Nowadays, Madeira winemakers aim to improve the fermentation process standardization, upgrading the quality of certain wines. In order to respond to this challenge, there is an increasing interest in identifying and studying the indigenous yeast populations, in particular the non-*Saccharomyces* species, since these can positively influence the enological parameters such as color, acidity, aroma, and also food safety (244). So, this work aimed to isolate, identify, and characterize the non-*Saccharomyces* native yeast population in musts of different wineries and vineyard locations of Tinta Negra variety. And then, study the impact of the inoculation of five selected indigenous non-*Saccharomyces* yeasts in the wine chemical composition, particularly the phenolic compounds.

2. Material and methods

2.1. Spontaneous fermentations for yeast isolation

2.1.1. Winery samples

The spontaneous alcoholic fermentation of winery samples was performed by sampling grape musts from Tinta Negra red grape variety (*Vitis vinifera* L.) collected from three different local wineries (W1, W2, and W3) in the harvest of 2017. The samples collection was described in the Chapter 1 (Section 2.1). For each winery, two samples were collected in the M0 (grape juice) and MBF (must before fortification) stages.

2.1.2. Vineyard samples

The spontaneous alcoholic fermentation of Tinta Negra grapes collected from two different geographical locations on Madeira Island: Câmara de Lobos (L1, L2, and L3) and São Vicente (L4, L5, and L6) were also performed. The microvinification procedure

is described in Chapter 2 (Section 2.1). For each location, two samples were collected in the following fermentation steps: grape juice (M0) and must before fortification (MBF).

2.2. Yeast counting and isolation

The indigenous yeast strains used in the current study were isolated from the samples taken from the trials performed in the wineries and vineyards at the different fermentation steps (M0 and MBF). Must samples were serially diluted (10^{-1} to 10^{-3}) in peptone water (Merck, Darmstadt, Germany), and 100 µL were surface plated onto different culture media (in duplicate). The yeasts isolates were obtained using general glucose–yeast–peptone (GYP) medium composed of 20 g/L of glucose (Scharlab, Barcelona, Spain), 5 g/L of peptone, 20 g/L of Nutrient Agar, and 5 g/L of yeast extract (Himedia, Einhausen, Germany) with 1 mL of Biphenil solution (0.075 g/mL) and 1 mL of Chloramphenicol (0.01 g/mL) from Fisher Scientific (Lisbon, Portugal). The incubation was performed at 25 °C for 5 days. For the isolation of non-*Saccharomyces*, an identical GYP medium was prepared with the addition of a cycloheximide solution (10 µg/L) (Merck, Darmstadt, Germany). The *Dekera/ Brettanomyces* isolates were obtained following the procedures described by Rodrigues *et al.* (2001) (245), using the selective *Dekkera/Brettanomyces* Differential Medium (DBDM) with samples being incubated at 25 °C for 12 days. *Zygosaccharomyces* species were isolated in a *Zygosaccharomyces* Differential Medium (ZDM), with samples being incubated at 28 °C for 48h, according to the Schuller *et al.* (2000) (246) work. The total yeast counting was obtained by recording the number of colony-forming unit (cfu) counts. Different colony morphologies were registered and two to five representative isolates were selected and purified on GYP plates.

The fermentative yeast species were selected using the urease and fermentation tests (Glucose). For the urease test, 1 L of Christensen's medium was prepared with 1 g of peptone (Himedia, Einhausen, Germany), 1 g of glucose (Himedia, Einhausen, Germany), 5 g of NaCl, 2 g of KH_2PO_4, and 0.012 g of red phenol from Merck (Darmstadt, Germany) and pH adjusted to 6.8. Solutions of 20% urea (Panreac, Barcelona, Spain) were prepared, before being added (0.5 mL) to tubes previously loaded with 4.5 mL of Christensen's medium. The colonies were inoculated into the tubes and then incubated for 3 days at 25 °C. The development of a yellow color was considered as positive result. The fermentation test was prepared with 1 L of distilled water, 10 g/L of

yeast extract (PVL, Lisbon, Portugal), and 20 g/L of glucose monohydrated (Merck, Darmstadt, Germany). Ten milliliters of this solution were placed into Durhan tubes and sterilized at 121 ℃ for 15 min. The colonies were inoculated for 2 to 4 days at 25 °C. The gas formation was considered as positive result. Fermentative yeast species were those colonies that had a positive response for both urease and fermentation tests.

2.2.1. Yeast identification

The isolates were collected from fresh yeast colonies, and then DNA extraction was performed by thermal shock (95 °C for 15 minutes, -80 °C for 15 minutes, and then 95 °C for 15 minutes). The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was carried out using an ITS1 primer (50-TCCGTAGGTGAACCTGCGG-30) and an ITS4 primer (50-TCCTCCGCTTATTAGATATGC-30) from STABVida (Lisbon, Portugal) and the digestive enzyme NZYTaq II DNA Polymerase from NZYTech (Lisbon, Portugal) was used. The PCR conditions were as follows: initial denaturation at 95 °C for 5 min; 35 cycles of denaturing at 94 °C for 1 min, annealing at 55.5 °C for 2 min, and extension at 72 °C for 2 min; and a final extension at 72 °C for 10 min. Agarose at 1.5% in 1 x TAE buffer (Cleaver Scientific, Warwick, United Kingdom) was used to separate DNA products and their restriction fragments. The PCR amplified fragments were sequenced by STABVida (Lisbon, Portugal), and then a blast analysis was performed (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). ITS1 and ITS4 sequences were considered for accurate results, and the identification was considered valid when at least 98% of correspondence was revealed.

2.2.2. Yeast inoculation and wine production

Five non-*Saccharomyces* yeasts strains were selected to be studied as starter pure cultures: *Hanseniaspora uvarum*, *Starmerella bacillaris*, *Pichia terricola*, *Pichia fermentans*, and *Pichia kluyveri*. All fermentations used Tinta Negra grapes. The inoculation was performed at laboratorial scale and the procedure was based on Benito *el al.* (2017) (247). The initial must, which had a density of 1.075 g/L (at 20 °C), was pasteurized at 105 °C for 5 min. After cooling, yeasts were individually inoculated using the GYP medium, containing 10^9 CFU/mL (measured by the Thoma cell counting

chamber method), at 25 °C for 24h and then added 3 mL of the solution to 3 L of must. The fermentations were performed in duplicate. The alcoholic fermentation was conducted under a controlled temperature (20 ± 3 °C), and the density was measured using a pycnometer. After fermentation, the samples were fortified by adding natural grape spirit (95% (v/v) of ethanol), raising the alcohol content up to 17% (v/v). After vinification, all wines were kept at 45 °C for 120 days (*estufagem* aging simulation). Samples were collected, in duplicate, at different stages of the winemaking and aging: M0 (grape juice), MAI (must after 24h of inoculation), MBF (must before fortification), WAF (wine after fortification), and WAE (wine after *estufagem*).

2.3. Characterization of the selected non-*Saccharomyces* species

2.3.1. Ethanol, reducing sugars and organic acids

The concentration levels of ethanol, fructose, glucose, glycerol, and organic acids were quantified based on the procedure described in the Chapter 2 (Section 2.2) followed the methodology proposed by Miranda *et al.* (2017) (22) through the HPLC -DAD-RID.

2.3.2. Phenolic composition and antioxidant potential

Samples were assayed for the TP through the Folin-Ciocalteu's method and for the AP by the DPPH assay. Both procedures were described in Chapter 1 (Section 2.3). Samples were analyzed in triplicate.

The identification and quantification of individual phenols were based on Pereira *et al.* (2013) (217). Briefly, 20 µL of each sample were directly injected into an HPLC-DAD from Waters Corporation (Santa Clara, CA, USA) equipped with an auto-injector (Waters 2695 separation module), photodiode array detector (Waters 2996), and the Empower Pro Software from Waters Corporation. The phenolic compounds were separated in an Atlantis T3 column (250 x 4.6 mm, i.d; 5 µm, from Waters, Milford, MA, USA) using three mobile phases for the chromatographic separation: A (10 mM of phosphate buffer adjusted to pH 2.7 with phosphoric acid), B (acetonitrile) and C (methanol). The column temperature and the flow rate were set to 30 °C and 1.0 mL/min, respectively. The gradient program varied from 100% aqueous mobile phase (Phase A) to 60% organic phase (Phase B in 58 minutes and then 12 minutes of re-equilibration. All

standards and wine samples were injected in triplicate. The analytes were identified based on their retention time and UV-Vis spectra (between 200 – 780 nm) and by spiking samples with a mixture of pure standards. All standards and samples were previous filtered through 0.20 μm PP Chromafil Xtra syringe filters (from Macherey-Nagel, Düren, Germany). The quantitative determination was performed according to the external standard calibration method. Wavelengths used for quantification were 210 nm (flavan-3-ols, hydroxybenzoic acids, and hydroxybenzaldehydes), 315 nm (*trans*-resveratrol and hydroxycinnamic acids), and 360 nm (flavonoids and ellagic acid).

2.4. Data processing

All results were presented as mean ± standard deviation (SD), and the significant differences were evaluated by the analysis of variance (One-way ANOVA, Holm-Sidak method) for 95% of probability, using the statistical software SigmaPlot, version 12.0.

3. Results/ Discussion

3.1. Identification of non-*Saccharomyces* derived from wineries and vineyards

Madeira wine fermentative yeasts of musts from three different wineries were identified during the fermentation process (M0 and MBF stages), as well as those of musts produced from the two main vineyard regions of the Madeira wine appellation. Only the fermentative yeasts species with positive response for both glucose and urease test were taken into account for the identification. A total of 287 isolates were identified from the spontaneous fermentation of Tinta Negra wines (154 isolates in the samples collected from wineries and 133 in the samples collected from the different vineyards).

Table 9 reports the distribution of the 11 yeasts identified in the studied wineries: *Hanseniaspora uvarum, Starmerella bacillaris, Pichia kluyveri, Pichia fermentans, Saccharomyces cerevisiae, Torulaspora delbrueckii, Candida apicola, Cystobasidium minutum, Pichia terricola, Cystobasidium slooffiae,* and *Wicheramomyces anolalus*. In turn, 6 yeasts species were identified in the samples derived from the different vineyards: *Hanseniaspora uvarum, Starmerella bacillaris, Pichia kluyveri, Pichia fermentans, Saccharomyces cerevisiae,* and *Hanseniaspora opuntiae*).

Dekkera/Brettanomyces and *Zygosaccharomyces* species were not detected in any sample.

Table 9 - Distribution (%) of the yeasts identified during the fermentation (M0 and MBF stages) of the must samples collected in the different wineries and vineyards locations.

	Wineries			Vineyard Locations					
Yeast strain	W1	W2	W3	L1	L2	L3	L4	L5	L6
M0 stage									
Pichia terricola	18								
Starmerella bacillaris	32	43	9	7			5	6	
Pichia kluyveri	12			2		13			
Candida apicola	14	19							
Cystobasidium minutum	3								
Hanseniaspora uvarum	21	32	83	91	98	86	95	94	100
Pichia fermentans	18	6			2	1			
Cystobasidium slooffiae			8						
Total Ascomycetes	79	100	92	100	100	100	100	100	100
MBF Stage									
Pichia fermentans	3				1				
Saccharomyces cerevisiae	94	68	85	72		5	10	3	11
Wicheramomyces anomalus	3								
Hanseniaspora uvarum		31	15	28	99	95	90	38	89
Torulaspora delbrueckii		1							
Hanseniospora opuntiae								59	

Wineries: W1, W2 and W3. Locations: South Locations (Câmara de Lobos – L1, L2 and L3); North Locations (São Vicente – L4, L5 and L6). Stages from grape collection: grape juice (M0) and must before fortification (MBF)

The yeast microbiota varied between wineries, demonstrating that each winery has its own native yeast culture (M0 stage). Most of the yeasts found in Madeira wine wineries belong to the *Ascomycota* phylum, ranging between 79% and 100% in the M0 stage and 100% in the MBF stage. The yeasts belonging to *Basidiomycota* phylum were only *C. minutum* and *C. slooffiae*. Similar to other studies (232, 239), the proportion of non-*Saccharomyces* yeasts in wine cellars at the initial stage (M0) was higher compared to *Saccharomyces*. At this stage, *S. bacillaris* and *H. uvarum* species were present in all wineries, representing about 73% of the total yeasts. Both yeasts are recognized for persisting in cellar environments and are capable of reimplantation, during the next vintage, becoming an integral part of the winery's yeast flora (115). As expected, *S.*

cerevisiae took over the process at the end of the fermentation (MBF stage), representing about 82% of the total yeast in all wineries. Also, at the MBF stage, *H. uvarum* was detected in wineries 2 and 3 (15%), followed by other non-*Saccharomyces* species such as *P. fermentans*, *W. anomalus*, and *T. delbrueckii*. The diversity and distribution of yeasts in wineries can vary depending on the winery and antiseptic conditions (115, 239).

H. uvarum was the predominant non-*Saccharomyces* species isolated from the grape musts collected in the main Madeira wine vineyards (M0 stage), in agreement with previous studies performed in other vineyards involved in the production of fortified wines (241) and others (248-250). Although grape samples belong to different vineyard locations, the yeast microbiota found at the M0 stage was practically similar in all locations. At this stage, other yeast species such as *S. bacillaris*, *P. kluyveri*, and *P. fermentans* were also present in lower concentrations. These yeasts were previously identified in grape vineyards in several studies (112, 170, 251). *S. cerevisiae* emerged in the MBF stage in five vineyards. Even though *S. cerevisiae* is extremely rare on grapes or vineyards (104, 119, 252), this yeast can be found during the spontaneous alcoholic fermentation in sterilized vessels (253) or even on damaged berries (254).

3.2. Analytical characterization

The selection and utilization of indigenous cultures is a topic of interest in regions with oenological tradition, such as in the case of Madeira. In this sense, five non-*Saccharomyces* yeasts were selected and individually inoculated in the grape must for evaluating their role in the chemical composition to produce Madeira wines, namely: *H. uvarum, S. bacillaris, P. terricola, P. fermentans,* and *P. kluyveri*. The fermentation process was monitored through the control of the must density and the ethanol produced (Fig. 27).

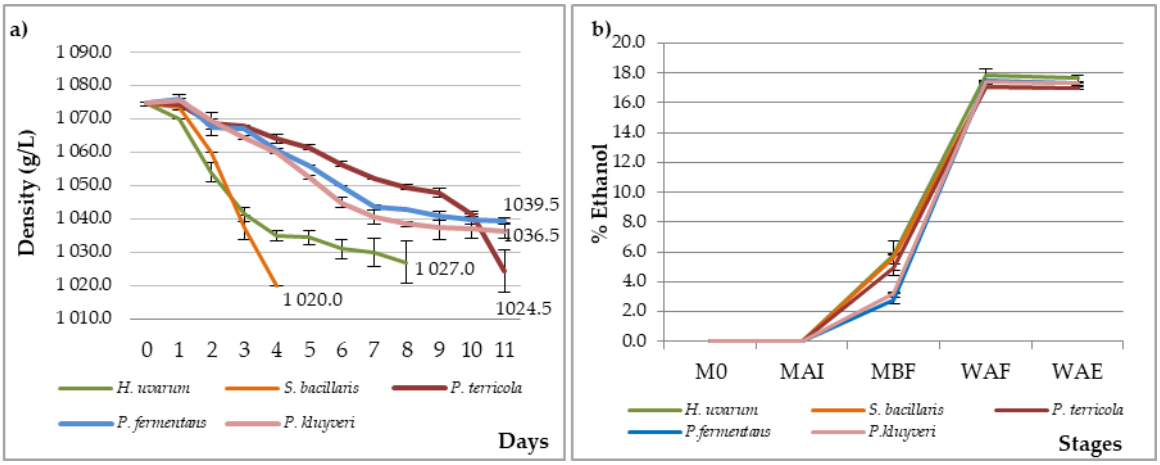

Figure 27 - Density depletion (a) and the ethanol formation (b) during the Madeira winemaking using different selected non-*Saccharomyces* yeasts (*H. uvarum, S. bacillaris, P. terricola, P. fermentans*, and *P. kluyveri*) as starter cultures.

The fermentative performance varied according to the yeast species. Figure 27 depicts that *S. bacillaris* showed higher fermentative capacity, reaching a density of 1020 g/L within 4 days, within the range allowed for dry wines. Indeed, most of the strain of this non-*Saccharomyces* specie are tolerant to relative high levels of ethanol and can persist up to the middle-end phase of the fermentation (255). *H. uvarum*, and *P. terricola* also showed fermentation capacity to produce this wine style, reaching a density of 1027 g/L and 1024.5 g/L within 8 and 11 days, respectively. In contrast, *P. fermentans* and *P. kluyveri* species has presented low fermentative capacity (density reached 1039.5 g/L and 1036.5 g/L, respectively), characteristic of *Pichia* species (181) and, therefore, can only be considered for the sweet wines production. In line with these findings, ethanol production before fortification (Fig. 27b) in inoculates of *H. uvarum* (5.8 %), *S. bacillaris* (5.6 %), and *P. terricola* (5.0 %) was also higher than those found in *P. fermentans* (2.7 %) and *P. kluyveri* (3.2 %). Some studies previously demonstrated that ethanol production was 4% in wines produced by *H. uvarum*, 10% for *S. bacillaris*, and up to 7% for those produced by *Pichia* species (181, 241, 256).

Individual sugars (fructose and glucose) and glycerol were also quantified to evaluate the impact of the non-*Saccharomyces* species during the vinification and aging processes (*estufagem*) of Madeira wine production (Fig. 28).

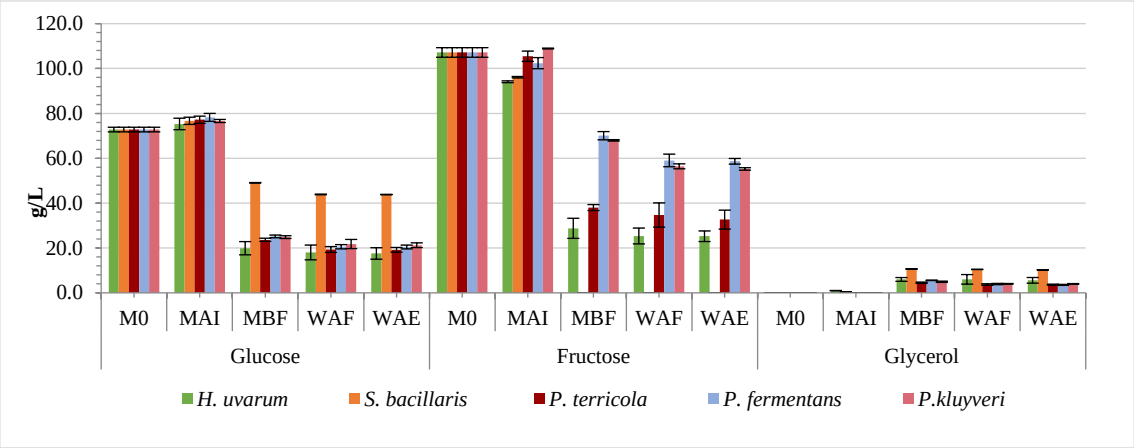

Figure 28 - Glucose, fructose, and glycerol amounts during the Madeira winemaking using different selected non-*Saccharomyces* yeasts (*H. uvarum, S. bacillaris, P. terricola, P. fermentans*, and *P. kluyveri*) as starter cultures.

H. uvarum showed similar preference for both individual sugars (glucose consumed 76% and fructose about 77%). In accordance with findings by Junior *et al.* (2019) (257), fructose was completely consumed in the presence of *S. bacillaris*, while only about 43% of glucose was consumed. On the other hand, as reported previously (181), *Pichia* species revealed a preference for glucose (on average 74%). Regarding Pereira *et al.* (2017) studies, the presence of fructose and its degradation mechanisms during the *estufagem* process greatly contributes for the development of typical features of Madeira wines such as color and aroma (194). The increase in glycerol concentration during fermentation was observed in all non-*Saccharomyces* species. However, *S. bacillaris* stood out, reaching a concentration of 10.3 g/L of glycerol. This yeast tends to improve the wine softness and body through the increase of glycerol (up to 14 g/L), as previously reported Junior *et al.* (2019) (257).

The main individual organic acids found in Madeira wine samples in the WAE stages are described below in Fig. 29. Formic acid were not quantified in the present study.

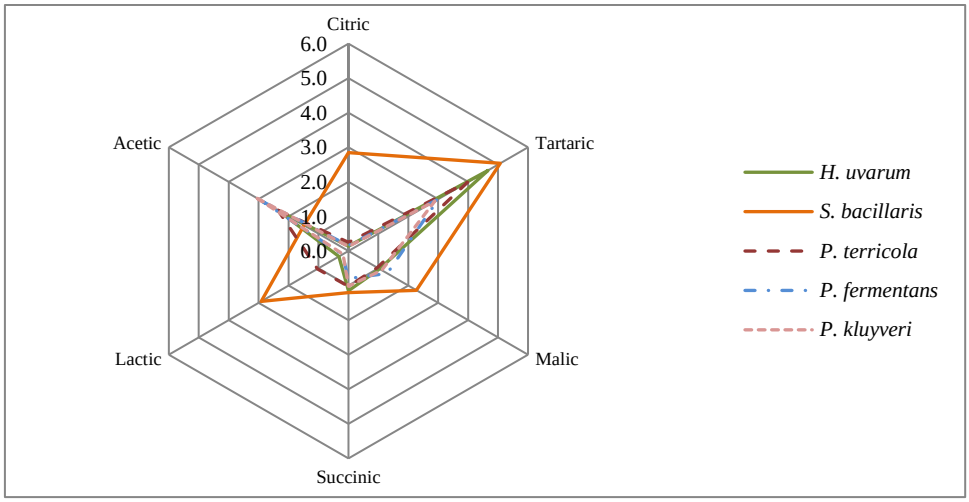

Figure 29 - Concentration of individual organic acids (g/L) in Madeira wines produced using different selected non-*Saccharomyces* yeasts (*H. uvarum, S. bacillaris, P. terricola, P. fermentans,* and *P. kluyveri*) as starter cultures, at the WAE stage (wine after *estufagem*).

In accordance to a previous study by Pereira *et al.* (2010) (258), tartaric, malic, and lactic acids were the main organic acids found in the current Madeira wine samples. Tartaric acid ranged from 2.88 to 5.07 g/L, malic acid from 0.97 to 2.27 g/L, and lactic acid from 0.31 to 2.92 g/L. *S. bacillaris* stood out from the other non-*Saccharomyces* species showing higher levels of tartaric, malic, lactic, and citric acids. The total concentration of organic acids for wines produced by *S. bacillaris* and the other yeasts was 15.80 g/L and 9.23 g/L (on average), respectively. This non-*Saccharomyces* species acts as a natural acidification agent, increasing the total acidity of wines by reducing the pH (255). Regarding the main acid involved in the volatile acidity of wines, acetic acid concentration varied between 1.49 g/L (*S. bacillaris*) and 3.04 g/L (*P. kluyveri*). Despite high levels of this compound can contributes negatively for the wine aroma with vinegar notes, Miranda *et al.* (2017) (22) reported that the odor rejection thresholds for acetic acid in Madeira wines can range between 1.96 and 5.72 g/L depending on the evaluation panel (regular or non-regular wine consumers) and the age and sweetness degree of the wine. Other study by Pereira *et al.* (2010) (258) showed that the concentration of this organic acid ranged from 0.67 to 2.21 g/L in Madeira wine samples. Even though some studies report no significant differences in acetic acid production on fermentations performed by *P. kluyveri*, the concentration of acetic acid produced may depend on the strain involved (181).

3.3. Antioxidant potential and total phenolic content

Table 10 summarizes the TP and antioxidant potential (DPPH) results found during the winemaking process of the Madeira wines produced from the different non-*Saccharomyces* yeasts.

Table 10 - Total phenols (TP) and antioxidant potential (DPPH) during the Madeira winemaking using different selected non-*Saccharomyces* yeasts (*H. uvarum, S. bacillaris, P. terricola, P. fermentans*, and *P. kluyveri*) as starter cultures.

	H. uvarum	*S. bacillaris*	*P. terricola*	*P. fermentans*	*P. kluyveri*
TP (mg GAE/L)					
M0	398.52±0.00***a***	398.52±0.00***a***	398.52±0.00***a***	398.52±0.00***a***	398.52±0.00***a***
MAI	255.94±3.74***b***	248.44±6.82***ab***	269.27±8.31***a***	252.30±0.00***ab***	217.00±3.65***c***
MBF	174.35±0.00***e***	215.48±2.65***a***	186.09±0.00***c***	177.98±0.00***d***	186.17±0.00***b***
WAF	201.92±0.00***c***	185.94±0.00***e***	212.15±0.00***a***	201.09±0.00***d***	205.94±0.00***b***
WAE	294.95±2.04***c***	263.74±3.93***d***	356.85±1.70***b***	361.85±3.65***ab***	367.68±8.10***a***
DPPH (mg Trolox/L)					
M0	209.91±0.00***a***	209.91±0.00***a***	209.91±0.00***a***	209.91±0.00***a***	209.91±0.00***a***
MAI	159.18±0.00***d***	145.21±0.00***e***	161.48±0.00***b***	159.87±0.00***c***	167.56±0.00***a***
MBF	141.37±0.00***b***	147.28±0.00***a***	136.03±0.00***d***	136.77±0.00***c***	131.60±0.00***e***
WAF	126.15±0.00***b***	111.58±0.00***e***	121.46±0.00***d***	124.98±0.00***c***	134.15±0.00***a***
WAE	133.60±0.00***b***	137.61±0.00***a***	120.95±0.00***e***	129.96±0.00***c***	125.27±0.00***d***

* Different letters in the same row denote statistically significant differences at (p<0.05). Samples were collected in different stages: M0 (grape juice), MAI (must after 24h of inoculation), MBF (must before fortification), WAF (wine after fortification and WAE (wine after *estufagem*).

One can observe that all fermentations (from M0 to MBF) promoted a decrease on TP regardless of the non-*Saccharomyces* specie used, between 45% and 56% when wines were inoculated with *S. bacillaris* and *H. uvarum*, respectively. This decrease can be due to physical processes, mostly involving the reversible interaction between anthocyanins and the yeast walls by absorption (258, 259). On the other hand, the *estufagem* process (WAF to WAE stage) promoted an increase on TP, greater in the wines that underwent fermentation with the *Pichia* species (between 68%, using *P. terricola*, and 79% using *P. fermentans*).

Similarly, the AP decreased between 29.8 – 37.3% during the fermentation process of all wine samples (from M0 to MBF). *S. bacillaris* promoted the highest antioxidant potential in the final wines 137.61 mg Trolox/L. The final results were lower than those found in Madeira wines produced by spontaneous fermentation (305.52 mg Trolox/L for sweet wines and 409.66 mg Trolox/L for dry wines after the *estufagem*

process) (217). However, in the present study the vinification underwent without grape skins, contributing for lowering the initial AP levels.

3.4. Phenolic composition

Twenty-four individual phenols were identified and quantified in the wines produced by different non-*Saccharomyces* species (Table 11), including non-flavonoids (7 hydroxybenzoic acids, 8 hydroxycinnamic acids, and 1 stilbene) and flavonoids (4 flavonols and 4 flavan-3-ols).

Table 11 - Individual phenols (mg/L) in Madeira wine samples produced by different non-*Saccharomyces* species in the initial must (M0) and in the final wine samples (WAE).

	M0 stage	**WAE stage**				
		H. uvarum	***S. bacillaris***	***P. terricola***	***P. fermentans***	***P. kluyveri***
Non-flavonoids						
Hydroxybenzoics						
Gallic	0.46±0.04***f***	0.62±0.00***e***	1.17±0.11***c***	1.50±0.00***a***	1.21±0.02***b***	0.83±0.04***d***
Protocatechuic acid	1.09±0.05***b***	1.95±0.03***a***	2.10±0.03***a***	1.85±0.18***a***	1.90±0.08***a***	2.03±0.00***a***
Syringaldehyde	n.q.	1.49±0.14***a***	1.09±0.04***c***	0.40±0.01***e***	0.68±0.00***d***	1.29±0.00***b***
Syringic	n.q.	4.48±0.24***d***	4.19±0.01***e***	4.68±0.15***c***	5.19±0.01***b***	5.89±0.07***a***
Vanillic acid	n.q.	1.92±0.04***d***	2.36±0.18***c***	3.21±0.15***a***	2.72±0.14***b***	3.29±0.06***a***
p-hydroxybenzoic acid	n.q.	1.18±0.01***c***	0.44±0.00***e***	0.97±0.09***d***	2.53±0.25***a***	2.20±0.13***b***
Ellagic	n.d.	0.99±0.09***b***	1.07±0.08***a***	0.88±0.03***c***	0.88±0.02***c***	n.q.
Total	**1.55**	**12.63**	**12.42**	**13.49**	**15.11**	**15.53**
Hydroxycinnamates						
Caffeic acid	n.q.	1.08±0.08***c***	1.28±0.08**b**	1.54±0.01***a***	0.89±0.06***d***	1.05±0.07***c***
trans-caftaric acid	4.89±0.04***a***	3.55±0.00***c***	3.68±0.10**b**	3.69±0.09***b***	3.26±0.07***d***	3.14±0.06***e***
Ferrulic acid	0.78±0.06***c***	0.98±0.08***b***	1.26±0.01**a**	0.86±0.04***c***	0.85±0.04***c***	0.80±0.00***c***
Sinapic acid	n.d.	0.23±0.01***c***	0.36±0.01**a**	0.24±0.02***c***	0.26±0.02***b***	n.q.
p-coumaric acid	n.q.	n.q.	0.41±0.01**b**	0.64±0.00***a***	0.40±0.04***b***	0.41±0.00***b***
cis-coutaric	0.13±0.01***c***	0.14±0.00***b***	0.14±0.00**b**	0.15±0.01***a***	0.11±0.00***d***	0.14±0.00***b***
trans-coutaric	1.66±0.01***d***	2.05±0.08***c***	2.22±0.04**a**	2.01±0.06***c***	2.01±0.02***c***	2.12±0.00***b***
trans-fertaric	0.43±0.02***b***	0.44±0.04***b***	0.47±0.00**a**	0.23±0.00***d***	0.38±0.00***c***	0.39±0.00***c***
Total	**7.89**	**8.47**	**9.82**	**9.36**	**8.16**	**8.05**
Stilbene						
trans-resveratrol	n.q.	0.34±0.00***b***	0.27±0.00***d***	0.29±0.01***c***	0.34±0.00***b***	0.36±0.02***a***
Flavonoids						
Flavan-3-ols						
(+)-catechin	1.16±0.07***e***	14.33±0.8***c***	10.79±0.40***d***	19.71±0.62***a***	17.44±1.71***b***	17.22±1.62***b***
(-)-epicatechin	6.84±0.23***a***	1.54±0.10***b***	1.36±0.08***b***	1.28±0.01***b***	1.60±0.14***b***	1.76±0.06***b***
(-)-epigallocatechin	0.15±0.01***d***	2.11±0.11***a***	1.36±0.06***c***	2.37±0.05***a***	1.15±0.05***c***	1.64±0.10***b***
(-)-epigallocatechin gallate	1.68±0.04***c***	1.85±0.05***b***	2.03±0.12***a***	1.40±0.01***d***	1.70±0.02***c***	1.45±0.02***d***
Total	**9.83**	**19.82**	**15.54**	**24.75**	**21.88**	**22.07**
Flavonols						
Kaempferol	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
Myricetin	n.q.	0.30±0.03***a***	0.32±0.02***a***	0.24±0.01***b***	0.29±0.01***c***	n.q.
Quercetin	n.d.	1.01±0.03***c***	0.57±0.01***d***	0.24±0.01***e***	1.21±0.02***b***	1.45±0.02***a***
Rutin	n.d.	0.66±0.05***a***	0.65±0.01***a***	0.50±0.01***c***	0.54±0.01***b***	0.51±0.00***c***
Total	**--**	**4.98**	**4.59**	**3.61**	**5.03**	**4.99**
Total phenols	**19.27**	**46.24**	**42.64**	**51.50**	**50.52**	**51.00**

n.q. – not quantified, below LOQ; n.d. – not detected, below LOD; Different letters in the same row denote statistically significant differences at (p<0.05).

According to Table 11, the concentration of total individual phenols increased from the M0 to WAE stage in all samples produced by non-*Saccharomyces* species, from 19.27 mg/L up to 51.50 mg/L (*P. terricola*). The proportion of non-flavonoids (53.39 %) was similar to flavonoids (54.76%). Although the total individual phenols contents were similar for all non-*Saccharomyces*, *Pichia* species produced a slightly higher concentration. (+)-catechin was the most abundant compound found in all final wines, varying from 10.79 mg/L (*S. bacillaris*) to 19.71 mg/L (*P. terricola*). This phenol showed the highest concentration in Madeira wine samples previously studied (337 µg/mL) (260). Catechin can affect the quality of wines, conferring flavor (bitterness) and oxidation resistance (261). Regarding the hydroxybenzoic acids, syringic and vanillic acids were the ones that stood out, mainly in wines produced by *P. kluyveri* (5.89 mg/L and 3.29 mg/L, respectively). These compounds showed similar concentrations to those previously described in Gonçalves *et al.* (2013) (196) for commercial table wines, namely 0.2 to 1.0 g/L for vanillic acid and 2 to 4 mg/L for syringic acid. The most abundant hydroxycinnamic acid was *trans*-caftaric acid, showing higher levels in wines produced by *S. bacillaris* (3.68 mg/L) and *P. terricola* (3.69 mg/L). The heating process promoted a decrease of this compound by at least 28%, which is in agreement with the findings by Pereira *et al* (2010) (258). *trans*-resveratrol concentration increased from M0 to WAE stage, varying according to the yeast species used from 0.27 mg/L (*S. bacillaris)* to 0.36 mg/L (*P. kluyveri*). This bioactive compound is usually found in wine at concentrations ranging from undetectable to 14.3 mg/L depending on the type of the wine (50, 262). The levels of resveratrol tend to be higher when grapes are exposed to biotic or abiotic stress or by the yeasts-endowed *β*-glucosidase activity (35, 50). Gaensly *et al.* (2015) (50) demonstrated that four strains of *H. uvarum* increased free resveratrol after alcoholic fermentation of *V. labrusca* without modifying its composition or sensorial properties. Flavonols have an important role in the color and sensory perception of wines. According to Table 11, quercetin was the flavonol with the highest concentration, ranging from 0.24 mg/L in wines inoculated with *P. terricola* to 1.45 mg/L in wines produced by *P. kluyveri*. Although skin maceration was not carried out in this study, some wine samples showed higher levels of quercetin when compared to other Madeira wine studies (0.65 mg/L) (258). Quercetin levels varied between wine samples (WAE) according to the yeast species used, revealing that yeast species can influence its concentration in wines during the fermentation process, consistent with results from other studies (263).

4. Conclusion

The current study revealed the non-*Saccharomyces* indigenous yeasts involved in the production of Madeira wines, with *H. uvarum, S. bacillaris, P.terricola, P. fermentans,* and *P. kluyveri* being the most representative species. *H. uvarum* and *S. bacillaris* represented about 73 % of the total yeast in grape juices in wineries. Only *H. uvarum, S. bacillaris,* and *P. terricola* showed fermentation capacity to produce dry Madeira wines. The different non-*Saccharomyces* species promoted great variability on the wine characteristics. Wines produced with *S. bacillaris* evidenced higher acidity (15.80 g/L of total organic acids) when compared to the ones produced with other non-*Saccharomyces* yeasts. The contents of phenols in wines were influenced by the yeast species and were higher in the *Pichia* inoculates, reaching 51.50 mg/L of the total phenols in wines produced with *P. terricola*. On the other hand, the antioxidant potential was higher in musts inoculates with *H. uvarum* and *S. bacillaris*. Even though the evaluated non-*Saccharomyces* species show promising results to be used as starter cultures in the production of Madeira wines, additional information is needed to evaluate the sensorial properties of these wines.

Chapter 4

Volatile profile, chromatic characteristics and sensorial analysis of Madeira wines produced by selected indigenous non-*Saccharomyces* yeasts

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This chapter is based on:

Volatile profile, chromatic characteristics and sensorial analysis of Madeira wines produced by selected indigenous non-*Saccharomyces* yeasts
Miranda A., Pereira V., Jardim H., Malfeito-Ferreira M., Marques JC.
(Prepared to be submitted)

Abstract

In a previous work, an extensive collection of indigenous yeasts in Madeira wine wineries and vineyard locations was established and analyzed. In the current study, 5 selected non-*Saccharomyces* pure yeast cultures (*Hanseniaspora uvarum*, *Starmerella bacillaris, Pichia terricola, Pichia fermentans*, and *Pichia kluyveri*) were inoculated in Tinta Negra grape juice to produce dry Madeira wines. The aim of the present work was to evaluate the impact of these non-*Saccharomyces* yeasts, used as starter cultures, in the volatile composition, chromatic characteristics, and sensory properties of the wines. The results showed that the higher proportion of total volatile organic compounds (VOCs) were presented in wines produced by *P. kluyveri* yeast with 24.50%. Esters were the main chemical family in all wines, representing between 50.23% (*S. bacillaris)* and 77.35% (*P. kluyveri*) of the total VOCs, being ethyl acetate and diethyl succinate the most abundant esters in all wine samples. Alcohols were more abundant in wines produced by *P. terricola* (25.73% of total alcohols), being 3-methyl-1-butanol and phenylethyl alcohol the most representative alcohols in all samples. The levels of carbonyl compounds stood out in wines produced by *S. bacillaris* (21.26%), being benzaldehyde and acetaldehyde the most abundant carbonyl compounds in all wines. *S. bacillaris* produced the wines with the lowest total concentration of VOCs, particularly in terms of esters. Regarding wine color, the Browning Index (BI) was higher in wines produced by *P. fermentans* (0.962 AU). The sensory descriptors indicated a high intensity of nutty aromas in wines produced by *H. uvarum* and *P. terricola*, floral notes in the wine produced by *S. bacillaris*, herbaceous notes in wines produced by *S. bacillaris* and *P. terricola*, and caramel notes in wines produced by *P. fermentans* and *P. kluyveri*. The wine consumer's preference was about 37% for wines produced with *P. kluyveri*. These findings suggest that *P. kluyveri*, which produced a wine with an interesting volatile profile and organoleptic characteristics, may be used as a starter culture for the production of Madeira wines, by improving the wine aroma.

1. Introduction

The wine's volatile composition significantly impacts the wine quality since it can influence the wine tasting and determine its acceptance or rejection by consumers (3, 27). The wine aroma consists of a combination of several hundred volatile organic compounds (VOCs) that are mainly present in trace amounts (222).

The traditional winemaking process results from the biological interactions among microorganisms (yeasts, bacteria, and fungi) naturally present on grapes and wine cellar equipment, which leads to alcoholic (103). Alcohols, esters, fatty acids, carbonyl compounds, and lactones (3, 27) are amongst the most important wine secondary aromas. These VOCs are formed by the grapes microbiota, which includes different *Saccharomyces* and non-*Saccharomyces* yeast species (78, 79).

Fermentation temperature and pH, must nutrients, and the strain and type of yeast involved in the process determine the volatile composition of wines (3, 27). Usually, spontaneous fermentation is initiated by heterogeneous non-*Saccharomyces* yeast species, while *Saccharomyces* species, mainly *S. cerevisiae*, are predominant in the final phase of the fermentative process (103, 105, 264). Indeed, in the past, non-*Saccharomyces* species have been generally associated with wine off-flavors and spoilage (169, 264). At the time, producers started using commercial *Saccharomyces* yeast strains to reduce the risk of spoilage, accelerating and standardizing alcoholic fermentation. This resulted in the decrease of indigenous yeast strains diversity and in the loss of wine aromatic complexity (265).

Nowadays, non-*Saccharomyces* yeast species are positively associated with wine organoleptic characteristics, enhancing the aroma complexity by forming volatile compounds due to their capacity to secrete enzymes, such as β-glycosidases, esterases, lipases, proteases, and others (169, 266). For that reason, the use of indigenous non-*Saccharomyces* yeasts is an approach with growing importance to the wine industry. Indeed, this trend can be very important in the modern and highly competitive wine market, where winemakers aim to produce wines with unique and distinguished flavors and aromas (169, 267).

After the isolation and selection of yeast strains with a desirable phenotype and technological characteristics, they can be employed as a starter culture (169, 267). Some non-*Saccharomyces* yeasts, such as *Pichia kluyveri*, *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, and *Lachancea thermotolerans*, are already being

commercialized as starter cultures to be sequentially combined with *S. cerevisiae*. Other species, such as *Hanseniaspora uvarum*, *Starmerella bacillaris*, and *Pichia* spp., are considered significantly promising species to be applied in the wine industry due to their contribution to the wines final aroma (166).

In fact, using a mixed culture of *H. uvarum* and *S. cerevisiae* in wine fermentation has been shown to increase the production of primary metabolites (glycerol and acetaldehyde) and also secondary metabolites, such as terpenes, C_{13}-norisoprenoids, ethyl esters, acetate esters, fatty acids, and higher alcohols (167-169). Regarding the use of *S. bacillaris*, it has been shown that this yeast has a strong fructophilic character and produces less ethanol during the fermentation process when compared with *S. cerevisiae*. Additionally, the combination of *S. bacillaris* with *S. cerevisiae* in a starter mixed culture showed great potential for improving the wine's chemical and aromatic profile by producing higher levels of esters and thiols in Sauvignon blanc wines and reducing volatile fatty acids that negatively contribute to red wine quality (177, 178). Furthermore, *S. bacillaris* can enhance the wine's aroma by increasing terpenes, ethyl esters, and higher alcohol levels (264). Also, several *Pichia* species, such as *P. kluyveri*, *P. fermentans*, *P. terricola*, *P. membranifaciens*, and *P. manshurica*, have been reported to contribute to wine flavor by promoting the formation of thiols, terpenes, fruity esters, volatile phenols, and higher alcohols (181, 264). Regard the volatile profile, mixed cultures of non-*Saccharomyces* yeasts with *S. cerevisiae* are known to stabilized the wine color through the synthesis of vitisins (stable pigments) due to the increased of specific precursor compounds such as acetaldehyde and pyruvic acid during the fermentation process (268).

Madeira wine, known for its intense and complex aroma, stands out due to its characteristic winemaking process, in which indigenous yeasts spontaneously ferment grape must, followed by a peculiar aging process (*canteiro* and *estufagem*). During *estufagem*, fortified wines are usually heated to about 45 ºC for 120 days in *estufas* and then continue to age in oak casks (192, 222). Madeira wines volatile composition has been the topic of several studies regarding the varietal (e.g., terpenoids, norisoprenoids, aldehydes) (3, 97, 189), fermentative (such as alcohols, esters, carbonyl compounds, and acids) (3, 96, 97, 187, 189, 205), and aging aroma compounds (such as volatile phenols and lactones) (90, 92, 192, 208).

As far as we are aware, there are no studies about the role of non-*Saccharomyces* yeasts on the organoleptic characteristics of Madeira wines. In a previous work, an

extensive collection of indigenous yeasts in Madeira wine wineries and vineyard locations was established and analyzed. The most 5 representative non-*Saccharomyces* were selected (*H. uvarum, S. bacillaris, P. terricola, P. fermentans*, and *P. kluyveri*) to be used as starter pure culture in the production of Madeira wines. Therefore, the aim of this study was to assess the impact of these selected non-*Saccharomyces* yeasts in the volatile composition, chromatic and sensory characteristics, throughout the production of Tinta Negra Madeira wines.

2. Material and methods

2.1. Microorganisms

Tinta Negra *Vitis vinifera* L. is the grape variety used in about 80 to 85% of the Madeira wine production. The non-*Saccharomyces* yeast species (*Hanseniaspora uvarum, Starmerella bacillaris, Pichia terricola, Pichia fermentans*, and *Pichia kluyveri*) used in the current study were identified, isolated, and selected for being the most representative in the must juice during the spontaneous fermentation that took place in different wineries or from grapes juice collected from different regions (south and north coast) of Madeira Island (Portugal). The procedure was previously described in Chapter 3 (Section 2.2).

2.2. Inoculation and wine production

The selected non-*Saccharomyces* yeasts were inoculated in grape juice from Tinta Negra grapes according to the procedure described in Chapter 3 (Section 2.2). Briefly, the initial must (with a density of 1075 g/L) was pasteurized at 105 ºC for 5 min. After cooling, yeasts were individually inoculated at 25 ºC for 24h, using a glucose–yeast–peptone medium (GYP) composed of 20 g/L of glucose (Sharlab, Barcelona, Spain), 5 g/L of peptone, 20 g/L of Nutrient Agar, and 5 g/L of yeast extract (Himedia, Einhausen, Germany). Then, 3 ml of a solution of 10^9 cfu/mL were added to 3 L of must. The fermentations were performed in duplicate. The alcoholic fermentation was conducted under a controlled temperature (20 ± 3 ºC) and density was monitored. After fermentation, the samples were fortified by adding natural wine spirit (95% (v/v) of ethanol) until reaching 17% (v/v). Then, to simulate *estufagem*, about 750 mL of

each wine sample (in duplicate) were placed into 750 mL bottles with a small headspace volume and kept at 45±0.5 ºC for 120 days in a Memmert UFE 400 oven (Schwabach, Germany). Samples of the initial grape juice (M0) and wines after *estufagem* (WAE) were collected in duplicate.

2.3. Analysis of volatile organic compounds (VOCs)

2.3.1. Headspace-solid phase microextraction (HS-SPME)

The screening of VOCs was accomplished by following the SPME methodology proposed by Miranda *et al.* (2017) (22). Briefly, a solution of 500 mg/L of 3-octanol (97%, Acros Organics, Geel, Belgium) was prepared in synthetic wine to be used as internal standard (IS). In an SPME headspace vial containing 3 g of NaCl from Panreac Química S.A (Barcelona, Spain), 5 mL of sample, 5 mL of ultrapure water, and 2.5 µL of IS were added. The incubation time was 5 min at 60 ºC. The extraction was performed by a TriPlus autosampler (in SPME mode) from Thermo Scientific (Hudson, NH, USA), using a Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS, bipolar adsorbent) 50µm/30µm SPME fiber, from Supelco (Bellefonte, PA, USA), which was exposed to the sample headspace for 30 min at 60 ºC, followed by 5 min of desorption into the GC inlet at 260 ºC.

2.3.2. Gas chromatography-mass spectrometry (GC-MS) analysis

VOCs separation was carried out using a TRB-WAX column (60 m x 0.250 mm and 0.250 µm of film thickness) from Teknokroma Analítica S.A. (Barcelona, Spain), using helium, at 1 mL/min, as carrier gas. The oven temperature program started at 40 °C for 2 min, then increased to 240 ºC at 4 ºC/min, and then was kept at 240 ºC for 15 min. The transfer line and ion source temperatures were both kept at 240 ºC. The VOCs were analyzed using an ISQ single quadrupole mass spectrometer (in electronic impact ionization mode) in the mass range 30-300 m/z.

The Kovats Index (KI) was calculated based on a C_7-C_{30} *n*-alkanes mixture from Supelco (Missouri, EUA). The VOCs were identified by comparing their mass spectra with those in the NIST08 and Wiley 6.0 MS library databases and comparing the obtained KIs with those from authentic compounds or reported in the NIST Chemistry

WebBook online database (269). The amount of each compound was estimated based on the following equation: Semi-quantitative concentration = (peak area/IS area) x IS concentration (192). The coefficient of variation (% CV) was below 10% between sample duplicates.

2.4. Color

The color determination was performed by the Glories method and the CIELab coordinates system, according to procedures previously described in Chapter 1 (section 2.4). The BI was evaluated by measuring the absorbance at 420 nm, and the Glories parameters (OIV-MA-AS2-07B) by the determination of A_{420}, A_{520}, and CI (calculated by the sum of the absorbances at 420, 520, and 620 nm). The CIELab coordinates were determined according to the OIV methodology (OIV-MA-AS2-11-R2006). The coordinate L^* represents lightness (in which $L^*=0$ indicates black/dark and $L^*=100$ indicates white/light), a^* is the green/red color component (in which $a^*>0$ indicates the color red and $a^*<0$ indicates the color green), and b^* is the blue/yellow component (in which $b^*>0$ indicates the color yellow and $b^*<0$ indicates the color blue).

2.5. Sensory analysis

The sensory analysis was conducted by two tests: a sensory evaluation for wine descriptors by experienced Madeira wine tasters and a ranking preference test performed by regular wine consumers.

2.5.1. Sensory evaluation of Madeira wine descriptors

The wines from musts inoculated with the selected non-*Saccharomyces y*easts were evaluated through a blind tasting performed by 6 Madeira wine experienced tasters (5 females and 1 male) without any specific training before the tasting session. Thirty milliliters of each coded wine were presented in tasting glasses at 20 ± 2 ºC, and tasters were asked to rate the main Madeira wine descriptors on a scale of 0 (absent) to 5 (very intense). Six descriptors were assessed: floral, herbaceous, fruity, spicy, caramel, and nutty.

2.5.2. Ranking preference test

The aroma of the wines produced from musts inoculated with selected individual non-*Saccharomyces* yeasts was evaluated through a preference test performed by 41 regular wine consumers (15 females and 26 males). Thirty milliliters of 5 coded samples, kept at 20 ± 2 ºC, were presented to the tasters, who were asked to order them from the least preferred (score of 1) to the most preferred (score of 5). The average score of each wine was calculated, and a preference ranking was determined.

2.6. Data analysis

All results were presented as mean value ± SD, and significant differences were evaluated by the analysis of variance (One-way ANOVA, Holm-Sidak method) for 95% of probability, using the statistics software SigmaPlot, version 12.0.

3. Results/ Discussion

3.1. Volatile profile characterization

To study the influence of different non-*Saccharomyces* yeast species, as starter cultures, on the aroma of Tinta Negra wines, the VOCs of all samples were determined by HS-SPME-GC-MS. The analysis of the samples in this study allowed the identification of 68 volatile compounds, including 16 carbonyl compounds, 22 esters, 15 alcohols, 8 fatty acids, and 7 miscellaneous compounds. Table 12 summarizes the data of the individual volatile compounds found in Madeira wines produced with the selected non-*Saccharomyces* yeast species. Despite being rough estimates, these values demonstrate the influence of each yeast species on the wine aroma and elucidate whether these VOCs are perceptible in the wine final samples.

Table 12 – Individual volatile organic compounds (as µg 3-octanol/L) found in grape juice and wines after *estufagem* process from Madeira wines produced with selected non-*Saccharomyces* species.

#	Compound	KI	CAS-Number	M0 (µg/L)	*H. uvarum* (µg/L)	*S. bacillaris* (µg/L)	*P. terricola* (µg/L)	*P.fermentans* (µg/L)	*P.kluyveri* (µg/L)	Common Odour Descriptors
	Carbonyl compounds (16)									
1	acethaldeyde*	584	75-07-0	n.d.	57.48±7.09*b*	57.58±3.48*b*	113.18±2.27*a*	73.45±4.45*d*	85.86±0.05*c*	Ripe Apple
2	2-methylpropanal**	681	78-84-2	n.d.	12.20±0.84*b*	41.66±2.71*a*	14.06±0.18*b*	10.52±0.73*bc*	11.38±1.12*bc*	Fruity, Green, Burnt, Malty, Toasted, Pungent,
3	2-methylbutanal**	818	96-17-3	n.d.	19.50±2.19*a*	19.01±2.75*b*	9.64±0.59*d*	9.92±0.53*c*	10.38±0.42*c*	Green, , Cocoa, Strong Burnt, Malty, Almond
4	hexanal**	1091	66-25-1	68.90±2.55*a*	n.d.	n.d.	n.d.	n.d.	n.d.	Fatty, Grass, Tallow
5	acetoin**	1230	513-86-0	n.d.	30.00±5.00*b*	72.32±3.51*a*	13.31±1.70*c*	1.68±0.05*e*	7.41±1.31*d*	Butter, Flowery Cream,Wet
6	octanal**	1296	124-13-0	11.74±1.10*a*	n.d.	n.d.	n.d.	n.d.	n.d.	Rancid, Soapy, Citrus, Green, Flower, Fruity
7	(E)- 2-heptenal**	1331	18829-55-5	17.54±1.25*a*	n.d.	n.d.	n.d.	n.d.	n.d.	Green, Green Grass
8	nonanal**	1397	124-19-6	49.19±3.93*a*	25.41±3.20*b*	18.31±1.62*c*	16.92±2.52*d*	11.60±0.42*e*	15.31±0.39*d*	Green, Slightly Pungent
9	(E)-2-octenal**	1435	2548-87-0	37.17±6.72*a*	n.d.	n.d.	n.d.	n.d.	n.d.	Fatty, Nutty, Waxy, Green
10	decanal**	1501	112-31-2	18.26±2.17*b*	37.43±1.37*a*	27.51±5.43*c*	25.93±1.25*c*	23.36±1.91*c*	25.75±1.12*c*	Soap, Orange
11	benzaldehyde**	1529	100-52-7	n.d.	98.39±18.94*e*	161.12±1.56*a*	144.81±0.19*b*	132.49±7.91*c*	116.76±8.02*d*	Almond, Burnt Sugar
12	4-tert-butylcyclohexanone**	1642	98-53-3	16.55±1.62*a*	n.d.	n.d.	n.d.	n.d.	n.d.	--
13	(E)-2-decenal**	1648	3913-81-3	40.01±3.38*a*	n.d.	n.d.	n.d.	n.d.	n.d.	Green, Fatty, Orange
14	2,4-nonadienal**	1704	6750-03-4	8.05±0.16*a*	n.d.	n.d.	n.d.	n.d.	n.d.	--
15	dodecanal**	1713	112-54-9	n.d.	72.39±4.34*a*	39.81±7.96*b*	39.81±4.97*b*	29.84±1.84*c*	33.36±4.95*bc*	Herbal, Fatty, Waxy, Citrus
16	2-undecenal**	1756	2463-77-6	22.72±3.94*a*	n.d.	n.d.	n.d.	n.d.	n.d.	Fresh, Citrus, Fruity
	Total			**290.13**	**352.80**	**437.31**	**377.64**	**292.86**	**306.23**	
	Esters (22)									
17	ethyl acetate*	779	141-78-6	4.79±0.33*f*	8912.30±120.19*c*	1744.45±289.92*e*	7873.19±694.65*d*	10413.32±716.19*b*	13193.08±842.14*a*	Fruity, Solvent-Like
18	ethyl isobutyrate**	902	97-62-1	n.d.	180.47±10.75*d*	192.16±33.47*d*	333.11±7.99*a*	257.52±10.96*c*	297.84±0.01*b*	Fruity, Sweet, Strawberry
19	isobutyl acetate**	978	110-19-0	n.d.	62.47±12.42*b*	20.33±1.63*c*	119.50±1.59*a*	65.98±4.71*b*	63.05±4.45*b*	Fruity, Pear Flowery, Banana
20	ethyl butanoate**	1016	105-54-4	3.62±0.01*e*	14.25±1.99*d*	15.26±1.50*d*	21.52±2.44*c*	31.15±2.00*a*	26.20±2.28*b*	Caramel, Fruit, Bubblegum
21	ethyl -2-methyl butanoate**	1040	7452-79-1	n.d.	20.24±2.53*c*	20.53±2.96*bc*	36.37±1.32*a*	25.15±0.74*b*	22.66±1.86*bc*	Sweet, Fruity, Strawberry, Blackberry, Green Apple
22	ethyl isovalerate**	1065	108-64-5	n.d.	77.72±3.11*d*	9.53±0.51*e*	121.21±3.31*c*	227.15±20.44*a*	138.65±2.96*b*	Cashew, Fruity,Anise Sweet, Apple
23	isoamyl acetate*	1145	123-92-2	n.d.	349.51±62.74*a*	33.60±6.29*c*	267.77±13.59*b*	265.08±10.64*b*	335.34±41.12*a*	Banana, Fruit, Fresh

24	ethyl hexanoate*	1290	123-66-0	n.d.	313.72±10.73*a*	142.61±6.75*e*	237.83±13.44*b*	208.62±10.70*c*	169.91±2.44*d*	Caramel, Anise, Fruit, Wine
25	1-hexylacetate**	1333	142-92-7	n.d.	26.30±2.88*a*	27.94±3.41*a*	11.53±0.03*c*	15.30±1.38*b*	12.62±1.44*bc*	Fruity,Spicy,Herbal, Sweet Wine, Tobacco
26	ethyl lactate**	1346	97-64-3	n.d.	351.12±64.36*a*	24.02±4.56*b*	15.74±0.11*cc*	11.68±0.31*cd*	10.81±0.60*d*	Ethereal, Acidic, Fruit , Sweet
27	ethyl octanoate**	1439	106-32-1	5.31±0.33*f*	277.06±28.07*a*	74.99±12.29*e*	201.60±22.35*c*	129.41±4.25*d*	236.38±37.80*b*	Waxy, Must, Soap, Fruit, Sweet
28	ethyl-2-furoate**	1624	614-99-3	n.d.	8.44±0.40*d*	4.19±0.32*e*	11.08±1.49*c*	15.89±1.16*a*	12.39±036*b*	--
29	ethyl decanoate**	1641	110-38-3	n.d.	132.39±21.56*a*	47.27±1.16*c*	65.24±6.27*b*	53.04±3.31*bc*	69.85±5.52*b*	Waxy, Soap, Fruit, Pleasant, Sweet
30	dietyl succinate**	1675	123-25-1	n.d.	1445.45±49.15*b*	2004.21±336.55*a*	1698.48±89.21*c*	1064.72±82.15*d*	821.25±16.63*e*	Floral, Potato, Fruit, Lavender
31	ethyl 9 decenoate**	1691	67233-91-4	n.d.	5.72±0.06*c*	6.21±0.37*c*	5.42±0.13*c*	12.65±0.73*b*	28.63±1.14*a*	Fatty, Fruit
32	ethyl glutarate**	1778	818-38-2	n.d.	13.72±2.38*e*	35.45±6.79*a*	29.08±3.33*b*	22.46±1.89*c*	20.46±2.99*d*	--
33	ethyl phenylacetate**	1787	101-97-3	n.d.	63.15±9.99*b*	16.29±3.01*d*	52.48±5.87*c*	82.77±6.68*a*	64.30±7.77*b*	Fruity, Sweet, Honey-Like
34	phenethyl acetate**	1818	103-45-7	5.60±0.48*e*	151.54±28.99*b*	39.82±3.54*d*	157.98±9.76*b*	126.26±3.39*c*	164.08±3.91*a*	Rose, Fruity, Floral, Sweet
35	ethyl dodecanoate**	1845	106-33-2	n.d.	219.99±40.34*a*	21.96±3.07*b*	57.03±10.37*c*	113.76±6.34*b*	n.d.	Fruity, Cream
36	diethyl malate**	1971	626-11-9	n.d.	92.22±17.12*d*	197.73±33.78*b*	137.30±19.30*c*	256.03±14.03*a*	212.03±11.96*b*	Peach, Cut Grass
37	ethyl hexadecanoate**	2257	628-97-7	n.d.	90.32±4.92*a*	65.90±7.98*b*	40.41±0.11*d*	55.27±4.02*c*	69.03±10.10*b*	--
38	ethyl linoleate**	2523	544-35-4	n.d.	33.58±2.54*a*	27.32±0.59*b*	16.62±1.82*e*	22.51±1.17*c*	17.85±1.27*d*	Fatty, Fruity
	Total			**19.32**	**12841.69**	**4771.77**	**11510.51**	**13475.72**	**15986.40**	
	Alcohols (15)									
39	1-propanol**	1011	71-23-8	n.d.	33.37±1.22*a*	22.60±3.78*b*	20.89±1.39*b*	11.30±0.39*c*	10.73±0.08*c*	Fresh, Alcohol
40	isobutyl alcohol*	1095	78-83-1	n.d.	211.03±0.70*c*	558.06±104.48*b*	651.53±20.66*a*	188.27±1.93*c*	5.43±0.21*d*	Fusel, Alcohol
41	3-methyl-1-butanol*	1253	123-51-3	n.d.	1183.36±62.61*c*	768.52±2.09*e*	1538.92±23.69*a*	1346.79±12.82*b*	1109.97±49.69*d*	Balsamic, Astringent
42	1-hexanol*	1353	111-27-3	43.20±8.10*cd*	112.08±11.74*a*	97.14±19.07*b*	67.84±1.37*c*	43.13±1.98*d*	49.23±2.59*cd*	Floral, Toasty, Wood¸Green, Fruit, Herbal
43	(Z) - 3-henxen-1-ol**	1384	928-96-1	4.64±0.45*a*	n.d.	n.d.	n.d.	n.d.	n.d.	Green, Freh, Leaf, Grass
44	(E)-2-hexen-1-ol**	1403	928-95-0	18.33±1.92*a*	n.d.	n.d.	n.d.	n.d.	n.d.	Medicinal, Cooked butter, Leafy, Green
45	2-ethyl-1-hexanol**	1487	104-76-7	4.50±0.88*a*	n.d.	n.d.	n.d.	n.d.	n.d.	Citrus, Floral,Freash, Oil, Sweet
46	2,6-dimethyl-4-heptanol**	1538	108-82-7	n.d.	37.07±4.13*bc*	120.86±9.99*a*	35.28±5.85*c*	40.23±1.77*b*	20.89±1.79*d*	--
47	1-octanol**	1551	111-87-5	10.00±1.97*a*	n.d.	n.d.	n.d.	n.d.	n.d.	Citrus, Roses

48	1-nonanol**	1656	143-08-8	9.93±0.63*e*	52.77±2.60*a*	48.72±8.85*b*	15.39±0.15*d*	24.41±1.56*c*	28.38±5.47*c*	Fatty, Green
49	1-decanol**	1757	112-30-1	n.d.	59.21±1.96*a*	55.66±8.42*a*	34.99±2.94*c*	38.37±1.04*bc*	40.26±7.88*b*	Fatty
50	1-undecanol**	1756	112-42-5	n.d.	56.75±3.27*b*	40.26±4.02*d*	74.16±3.86*a*	45.93±2.98*c*	44.81±2.33*cd*	--
51	2-phenylethanol**	1905	60-12-8	6.78±0.16*f*	1103.90±11.82*c*	1863.74±74.20*a*	1598.45±50.03*b*	877.04±78.09*e*	949.65±11.71*d*	Flowery, Roses, Pollen, Perfumed,
52	dodecanol**	1933	112-53-8	8.97±1.54*e*	322.60±6.31*a*	104.27±20.04*d*	301.41±3.58*b*	263.76±19.56*c*	318.42±4.93*a*	Waxy, Fatty
53	1-hexadecanol**	2167	36653-82-4	n.d.	99.01±4.17*a*	30.75±2.46*e*	48.87±4.91*c*	78.17±0.06*b*	42.80±0.18*d*	Sweet, Oily
	Total			**106.34**	**3271.14**	**3710.59**	**4387.73**	**2957.41**	**2620.58**	
	Fatty acids (8)									
54	acetic acid*	1459	64-19-7	n.d.	486.72±95.81*b*	1.50±0.02*c*	4.69±0.32*c*	389.96±25.53*b*	758.05±144.60*a*	Vinegar, Sour, Pungent
55	isobutyric acid**	1582	79-31-2	n.d.	5.66±0.17*e*	8.47±0.04*d*	21.15±1.70*a*	10.30±0.67*c*	16.27±0.40*b*	Cheesy Rancid, Butter.
56	isovaleric acid**	1681	503-74-2	n.d.	14.80±2.36*d*	5.10±0.33*e*	33.94±4.59*b*	44.03±2.36*a*	27.75±1.87*c*	Fatty, Rancid
57	hexanoic acid*	1872	142-62-1	6.58±0.02*a*	n.d.	n.d.	n.d.	n.d.	n.d.	Cheesy, Rancid, Pungent, Sweaty
58	octanoic acid*	1993	124-07-2	10.90±1.55*e*	144.69±7.11*a*	48.89±8.89*d*	115.09±8.33*c*	121.22±10.32*b*	121.51±18.30*dc*	Cheesy, Freash, Fatty, Moss
59	nonanoic acid*	2180	112-05-0	20.44±3.24*d*	55.49±8.23*c*	59.57±2.23*bc*	68.62±8.78*b*	66.23±5.64*bc*	89.59±13.38*a*	Fatty, Rancid
60	decanoic acid**	2286	334-48-5	7.75±0.04*e*	0.38±0.01*f*	76.57±0.03*d*	113.65±0.78*a*	102.87±4.25*c*	110.27±3.70*b*	Cheesy, Fatty, Soap
61	dodecanoic acid**	2496	143-07-7	n.d.	155.92±5.08*a*	58.90±10.20*c*	76.35±0.71*b*	69.54±6.60*bc*	70.57±10.90*bc*	Soap, Waxy
	Total			**45.67**	**863.66**	**259.00**	**433.49**	**804.13**	**1194**	
	Miscellaneous Compounds (7)									
62	DL- limonene**	1248	138-86-3	n.d.	9.84±0.27*a*	0.83±0.10*d*	4.61±0.25*c*	7.30±0.18*b*	0.40±0.01*e*	Citrus, Wood, Fruit
63	furfural**	1467	98-01-1	n.d.	329.80±7.37*b*	111.17±16.39*e*	486.77±30.90*a*	232.11±1.06*c*	196.91±38.42*d*	Almonds, Sweet, Bread, Pungent
64	5-methyl-2-furfural**	1576	620-02-0	n.d.	20.42±20.42*c*	19.42±1.27*cd*	28.70±0.55*a*	19.31±1.55*d*	26.62±3.41*b*	Caramel, Almond, Spice, Toast
65	L-α-terpineol**	1695	10482-56-1	4.53±0.10*d*	18.86±0.53*c*	31.45±5.45*a*	23.82±0.02*b*	16.23±1.20*c*	23.44±0.06*b*	Floral, Fruit, Mint
66	*β*- damascenone**	1826	23726-93-4	9.48±0.25*d*	27.71±2.38*c*	26.56±4.41*c*	36.55±2.53*a*	24.97±1.98*c*	33.83±4.97*b*	Floral, Honey, Tobacco
67	(E)-geranylacetone**	1857	3796-70-1	6.67±0.27*a*	n.d.	n.d.	n.d.	n.d.	n.d.	Fruit, Floral,Green Wood, Waxy
68	2,4-di-*tert*-butylphenol**	2305	96-76-4	23.49±4.17*e*	263.31±1.74*c*	131.06±11.03*d*	264.03±17.07*c*	286.75±6.94*a*	280.20±5.17*b*	--
	Total			**44.17**	**669.94**	**320.50**	**844.48**	**586.68**	**561.39**	
	TOTAL VOCs			**505.63**	**17999.23**	**9499.17**	**17553.85**	**18116.80**	**20668.60**	

Different letters in the same row denote statistically significant differences at (p<0.05) according to Holm-Sidak test; n.d. (not detected);* MS data and Kovats index in agreement with those of authentic compound; ** MS data and Kovats index in agreement with those in literature; Odor descriptors and OT (3, 27, 96, 192, 270).

Before the fermentation process (M0), the main volatile fraction belong to the carbonyl compounds representing about 57%, followed by alcohols (21%) and fatty acids (9%). Indeed, these pre-fermentative aromas (expressed as C6 carbonyl compounds) are mainly present in grape and can derive from the membrane lipids through the lipoxygenase pathways, being formed during crushing, stemming, pressing and maceration processes (3, 271).

As expected, the concentrations of VOCs from all chemical families increased during the fermentation and aging process, and according to Table 12, the results showed that, after *estufagem*, the volatile fraction of each chemical family was strongly influenced by the selected non-*Saccharomyces* species starter pure culture. Furthermore, the proportion of the total VOCs concentration was higher in samples fermented with *P. kluyveri* (24.50 %), followed by those inoculated with *P. fermentans* (21.48 %) and *H. uvarum* (21.34 %). In fact, previous studies performed by others authors showed that fermentation with *P. kluyveri* resulted in wines with an enriched aromatic profile, essentially due to the formation of esters (181, 272). Interestingly, fermentation with *S. bacillaris* resulted in wines with lower proportion of total VOCs (11.26%) compared to the other species, which is in accordance with other studies that have demonstrated the reduction of total esters in wines that resulted from mixed fermentation with *S. bacillaris* and *S. cerevisiae* (177, 178). Additionally, the high fructophilic character of this yeast can greatly affect the formation of VOCs in the final wines since studies performed by Pereira *et al.* (2017) revealed that the presence of fructose and its degradation mechanisms during the *estufagem* process, greatly contributes for the development of Madeira wines aroma (194).

After *estufagem*, esters and alcohols were the main chemical families contributing to wine aroma in all the samples.

3.1.1. Esters

Esters are usually considered one of the most significant chemical families in wines, contributing positively to their aroma with fruity notes (82). The results showed that esters were the most abundant chemical family, representing 50.23 to 77.35% of the total VOCs in wines produced with *S. bacillaris* and *P. kluyveri*, respectively. Indeed, all the selected non-*Saccharomyces* yeast species in the present work were previously reported by other authors as contributing to the increase of ester content in

wines, especially in mixed starter cultures with *S. cerevisiae* (181, 255, 264, 273, 274). *P. kluyveri* stood out in regard to the production of total esters (27.28%), followed by *P. fermentans* (22.99%), and *H. uvarum* (21.91%). Similarly to other studies (177, 178), wines produced by the inoculation of *S. bacillaris* showed a lower percentage (8.14%) of total esters compared to wines produced with other non-*Saccharomyces* yeast species.

The most abundant esters in all wine samples were ethyl acetate and diethyl succinate. Ethyl acetate is one of the main esters found in wines, positively contributing to wine aroma with fruity notes when at concentrations lower than 150 mg/L, while higher levels are associated with unpleasant varnish odors (3, 178). Wines inoculated with *S. bacillaris* showed lower levels of this compound (1.74 mg/L), being in accordance with other authors, who reported that wines produced with yeast specie had low levels of ethyl acetate (177, 178). On the other hand, wines produced with *P. kluyveri* reached the concentration of 13.19 mg/L of ethyl acetate.

Diethyl succinate was present in all wine samples, ranging from 0.82 mg/L in samples produced with *P. kuyveri* to 2.00 mg/L in those produced with *S. bacillaris*. However, this VOC only effectively contribute to the aroma of these wines if its concentration were above its OT. Other minor esters, reported as important odorants for Madeira wines, were also found in the wines produced with the selected yeasts, namely ethyl butanoate, isoamyl acetate, ethyl hexanoate, and ethyl octanoate (96). These compounds might impart positive notes to the wine samples when detected above their OTs.

Regarding ethyl butanoate levels, significant differences ($p<0.05$) were found between the wines produced with *Pichia* species, with the highest levels (31.15 µg/L) detected in wines produced with *P. fermentans*. Indeed, this yeast species have previously being reported to increase the concentration of this odorant when mixed with *S. cerevisiae* (275).

Isoamyl acetate had the highest levels in wines produced with *H. uvarum* and *P. kluyveri* (no significant differences were found with $p>0.05$), namely 349.51 and 335.34 µg/L, respectively. On the other hand, the concentration of these compound in samples produced with *S. bacillaris* was clearly lower (33.60 µg/L). These results are in accordance with those described by other authors, who demonstrated that *S. bacillaris* produces wines with reduced levels of isoamyl acetate (140, 177, 178), while *H. uvarum*

and *P. kluyveri* have been described as yeasts with great potential for the formation of isoamyl acetate (152, 276).

Ethyl hexanoate is also an important odorant in Madeira wines, conferring caramel notes (96) when found above its OT. The concentrations of this compound were significantly different ($p<0.05$) between wines produced by the selected yeasts, ranging from 142.61 µg/L in wines produced by *S. bacillaris* to 313.72 µg/L in wines produced by *H. uvarum*. The same trend was observed for ethyl octanoate, reaching concentrations of 277.06 µg/L in the wine produced with *H. uvarum.* In fact, *H. uvarum* was described as having potential for the production of ethyl hexanoate and octanoate, while *S. bacillaris* pure cultures reported considerably lower levels of these compounds compared to samples produced by mixed fermentation with *S. cerevisiae* (167, 277).

3.1.2. Alcohols

Alcohols represent one of the most abundant groups of VOCs in all wine samples after the *estufagem* process. These secondary yeast metabolites are formed either through amino acid decarboxylation and deamination or by the catabolism of sugars (70). When present in concentrations below 300 mg/L, alcohols can impart a fruity character to wines, while concentrations above 400 mg/L can negatively affect the wines aroma, with a pungent smell and taste (112, 278).

The capacity to produce high levels of alcohols has been previously reported for strains of *H. uvarum*, *S. bacillaris,* and *Pichia* spp. (112, 181, 185, 278). In this case, wines produced by fermentation with *P. terricola* revealed a higher percentage of alcohols, representing about 25.73% of the total alcohols compared to wines produced with the other non-*Saccharomyces* species. *P. kluyveri* produced the lowest levels of alcohols (about 15.37%). In fact, wines produced by co-inoculation with *P. terricola* were reported to have an increased content of alcohols, whereas the *P. kluyveri* reduced by about 15% the content of alcohols in a mixed culture with *S. cerevisiae* (181, 185, 279).

Of the 15 alcohols identified, 3-methyl-1-butanol and 2-phenylethanol were by far the most abundant alcohols found in all wine samples. Also, the concentration of 3-methyl-1-butanol was higher in wines produced by *P. terricola* (1.54 mg/L), while 2-phenylethanol was more abundant in wines produced with *S. bacillaris* (1.86 mg/L).

3.1.3. Carbonyl compounds

Regarding the carbonyl compounds, the main fraction was verified in wines inoculated by *S. bacillaris* with 21.26% of the total carbonyls. This non-*Saccharomyces* species has been reported to be effective in increasing the content of carbonyl compounds when inoculated in mixed cultures with *S. cerevisiae* (277). Benzaldehyde and acetaldehyde were the most abundant carbonyl compounds found in all wine samples. Benzaldehyde concentrations showed significant differences ($p<0.05$) between the samples in this study. *S. bacillaris* was the yeast species that stood out with a concentration of 161.12 µg/L. Similar levels of acetaldehyde were found in wines produced with *H. uvarum* and *S. bacillaris* ($p>0.05$); however, the highest concentration of this compound was observed in samples produced with *Pichia* spp., especially *P. terricola*. Additionally, other minor aldehydes that may contribute to wine's aroma were nonanal and decanal. Wines produced by the inoculation of *H. uvarum* and *S. bacillaris* showed higher concentrations of these compounds, contrarily to results reported by Tofalo *et al.* (2016), where these aldehydes were not detected in wines inoculated with the same non-*Saccharomyces* yeasts species (280).

3.1.4. Fatty acids

According to Table 12, fatty acids concentrations were higher in wines inoculated with *P. kluyveri*, representing about 33.17 % of the total acids. On the other hand, in accordance to others studies (112), wines inoculated with *S. bacillaris* produced significantly lower levels of fatty acids (7.19 %). Acetic and octanoic acids were the main fatty acids found in all wine samples. Acetic acid levels were higher in Madeira wines produced with *P. kluyveri* (758.05 µg/L). Although this non-*Saccharomyces* yeast has been reported to produce no significant differences in acetic acid production during mixed fermentation with *S. cerevisiae*, higher strain variability was shown to greatly influence the production of this volatile compound (181). Additionally, in accordance with previous studies (170, 178), acetic, decanoic, and dodecanoic acids were found at lower levels in samples produced with *S. bacillaris* compared to the wines produced with the other non-*Saccharomyces* yeast species.

3.1.5. Miscellaneous Compounds

As shown in Table 12, 3 terpenes, 1 norisoprenoid, 1 volatile phenol, and 2 furan compounds were also identified. *β*-damascenone is a great odorant in Madeira wines, conferring floral, honey, and tobacco notes when above its OT. In the current study, wines produced with *P. terricola* (33.83 µg/L) and *P. kluyveri* (36.55 µg/L) revealed greater potential to form this compound. Although this odorant has previously been reported especially in sweet Tinta Negra Madeira wines (192), recent studies revealed that the yeast strain used in fermentation influences the concentration of *β*-damascenone in wines, through the enzymatic hydrolysis of different precursors, followed by acid-catalyzed transformations of the aglycon compounds during the fermentation process (281, 282).

3.2. Color

Color is a critical parameter in wine quality since it affects consumer acceptance. Madeira wines are characterized by their brownish color, resulting from the oxidative aging process, ranging from pale yellow to dark brown. The Glories method and the CIELab coordinates system were used to study the color of grape juice and the corresponding final wines produced by different non-*Saccharomyces* yeast species. The Glories parameters (A_{420}, A_{520}, and CI) are illustrated in Fig. 30.

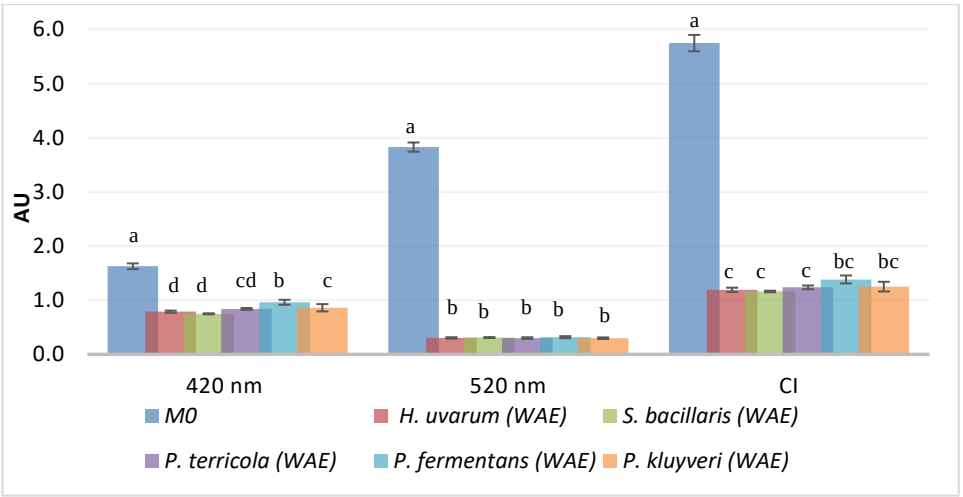

Figure 30 – Glories parameters (A_{420}, A_{520}, and color intensity -CI-) of musts (M0) and wines (WAE) produced by different non-*Saccharomyces* yeast species. Different letters in the same parameter denote statistically significant differences at $p<0.05$.

The BI evaluation was determined by measuring the absorbance at 420 nm. According to Fig. 30, the BI decreased in all samples, from M0 to WAE, by up to 54% in wines from musts inoculated with *S. bacillaris*. Indeed, fermentation and aging have previously been shown to affect the BI of these fortified wines (198, 201). After the *estufagem* process, all wines showed intense browning (>0.5AU), according to the categories defined by Fernández-Zurbano *et al.* (1998) (230). This can be associated with enzymatic browning due to the oxidation of flavonols (47, 201). Wines produced by *H. uvarum* and *S. bacillaris* showed BIs with no significant differences (p>0.05), with values of 0.788 and 0.749 AU, respectively. On the other hand, wines produced by *Pichia* spp. yeasts revealed higher BI values, mainly *P. fermentans,* which reached a BI of 0.962 AU. Regarding A_{520}, all wine samples showed no significant differences (p>0.05), ranging from 0.298 to 0.317 AU. Additionally, the CI values significantly decreased from M0 to WAE. Wines from musts inoculated with *H. uvarum*, *S. bacillaris*, and *P. terricola* showed no significant differences (p>0.05) in CI values which were, on average, 1.197 AU. The CI's highest value (1.384 AU) was found in wines from musts inoculated with *P. fermentans*. However, these results were lower than those reported by other authors after the *estufagem* of dry Tinta Negra Madeira wines produced by spontaneous fermentation (198).

The CIELab chromatic coordinates *L**, *a**, and *b** were also determined in M0 and WAE samples and are represented in Fig. 31.

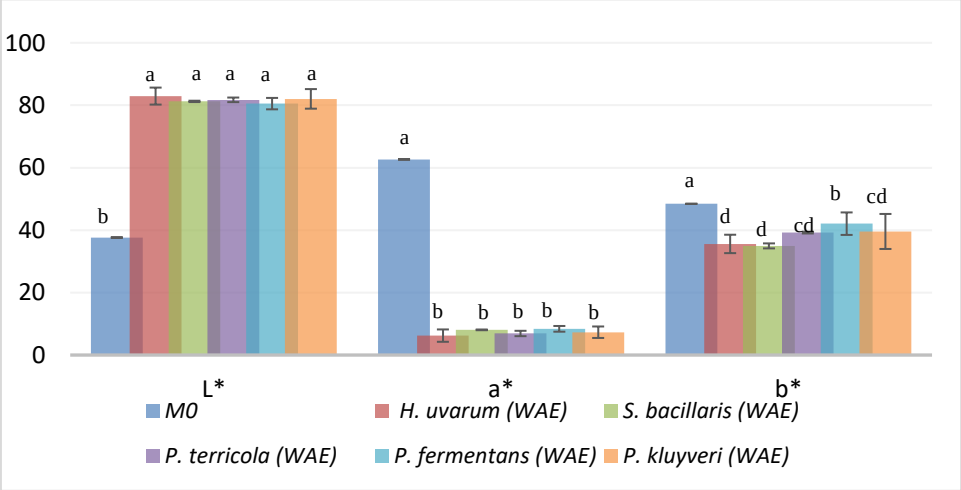

Figure 31 – CIELab parameters (*L, *a**, and *b**) of musts (M0) and wines (WAE) produced by different non-*Saccharomyces* yeast species. Different letters in the same parameter denote statistically significant differences at p<0.05.**

The results demonstrated that from M0 to WAE, the wines become lighter (L^* increased) in all samples, possibly due to anthocyanin polymerization during the vinification and aging processes (198, 231). There were no significant differences ($p>0.05$) in L^* between wines produced by different non-*Saccharomyces* yeasts. In M0 and WAE samples, the values of a^* and b^* were positive, which indicates the colors red and yellow, respectively. In WAE samples, the L^* was 81.699 on average, and in general, the yellow values (b^*) were higher than the red (a^*) in all wine samples. Moreover, the predominance of red in the M0 samples (62.650) decreased during fermentation and aging, being, on average, 7.380 in WAE samples, with no significant differences ($p>0.05$) between samples. Yellow decreased from M0 to WAE by 28% in wines inoculated with *S. bacillaris.* In WAE samples, similar to what was described for BI, wines from musts inoculated with *H. uvarum* and *S. bacillaris* revealed the lowest values of b^*, on average 35.277, with no significant differences at $p>0.05$. Wines from musts inoculated with *P. fermentans* stood out for having the highest values of yellow (42.076), possibly due to their phenolic composition since these compounds greatly affect the wines yellow color (229).

3.3. Sensory analysis

Sensory analysis is a crucial tool for assessing the sensory properties of wines. Wine sensory analysis was performed by two tests: a sensory descriptors test, in which the wines were evaluated by experienced Madeira wine tasters, and a ranking preference test, performed by regular wine consumers. The sensory descriptors test set determined the intensity of some descriptors, usually present in Madeira wines (floral, herbaceous, fruity, spicy, caramel, and nutty), in wines from musts inoculated with different non-*Saccharomyces* yeast species. The results are illustrated in Fig. 32.

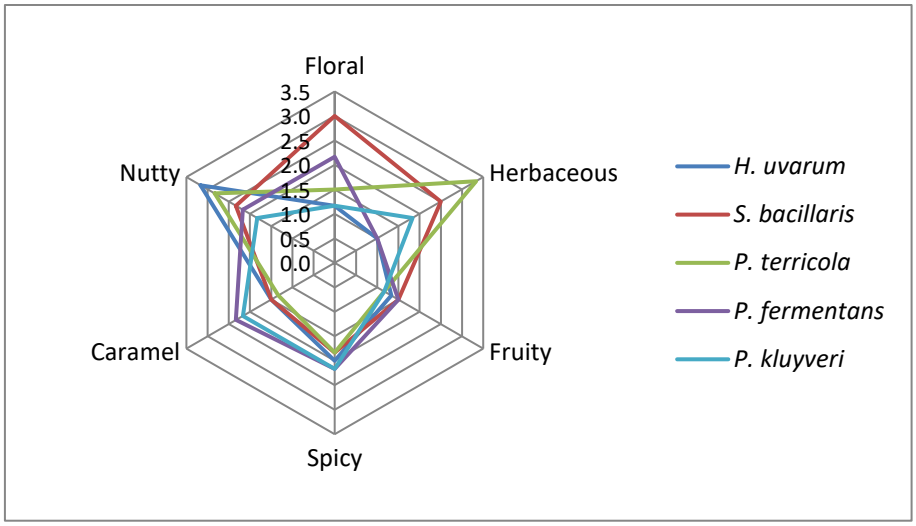

Figure 32 - Intensity of sensory descriptors of wines produced by starter non-*Saccharomyces* yeasts, evaluated by a panel of experienced Madeira wine tasters.

Fig. 32 clearly shows that the intensity of Madeira wine descriptors changed depending on the non-*Saccharomyces* yeast species involved in the fermentation process. The wine from the must inoculated with *S. bacillaris* revealed the highest intensity for floral (3.0) and herbaceous (2.5) notes. Nutty notes were more intense in the wine produced by *H. uvarum* (3.2). Additionally, the wine from the must inoculated with *P. terricola* stood out for its herbaceous (3.3) and nutty (2.8) notes, while wines produced with *P. fermentans* and *P. kluyveri* showed the highest intensity for caramel (2.3 and 2.2, respectively) and spicy descriptors (2.2 for both). Indeed, the sensory analysis for some VOCs identified in Table 12 showed higher levels of ethyl butanoate found in wines from musts inoculated with *P. fermentans* and *P. kluyveri*, which may confers caramel notes. Also, high levels of 2-phenylethanol was described in the wine from the must inoculated with *S. bacillaris*, that can promote the flowery aromas.

The olfactory preference test for the wines produced by different non-*Saccharomyces* yeasts were performed by 41 regular wine consumers and the results are illustrated in the Fig. 33.

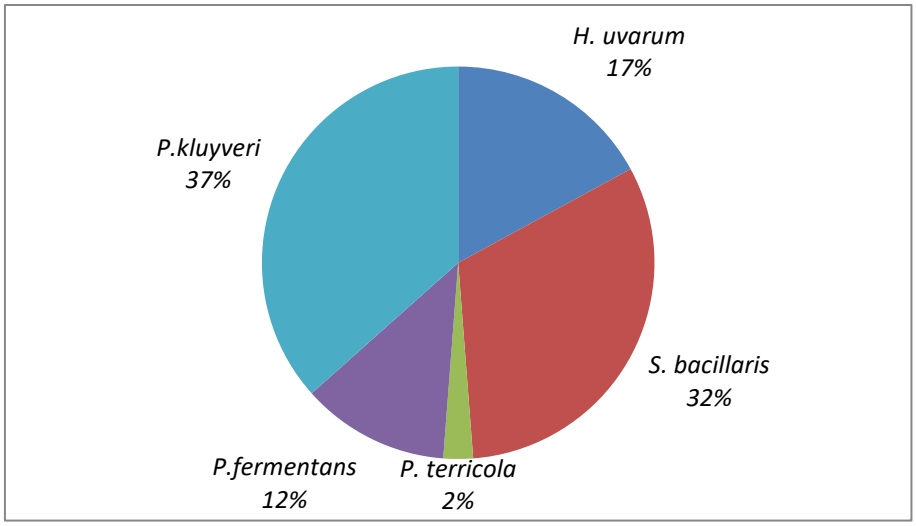

Figure 33- Olfactory preference test, performed by regular wine consumers, for wines produced by starter non-*Saccharomyces* yeasts.

According to Fig. 33, the wine from the must inoculated with *P. kluyveri* stood out in the olfactory preference test performed by the regular wine consumers with a preference score of 37%, followed by the wines produced with *S. bacillaris* (32%) and *H. uvarum* (17%). The wine produced with *P. terricola* scored the lowest, at only 2%.

4. Conclusion

The results of the present study demonstrated that the inoculation of the selected non-*Saccharomyces* yeasts in musts for the production of Madeira wines plays a decisive role in the wines volatile profile, color, and sensory properties. Indeed, wines produced with *P. kluyveri* stands out regarding the total VOCs proportion (24.50%), with the proportion of total esters and fatty acids higher in this particular wine. Alcohols were more abundant in wines from musts inoculated with *P. terricola*, while carbonyl compounds were mainly found in wines produced with *S. bacillaris*. Regarding the wine color, wines after *estufagem* process showed a similar tendency towards chromatic characteristics that are typical of these oxidative wines, namely the predominance of yellow compared to red. Furthermore, the BI was more notorious in wines from musts inoculated with *P. fermentans*. Finally, the sensorial analysis revealed that the intensity of descriptors typically associated with Madeira wines was greatly dependent on the selected non-*Saccharomyces* yeast specie. Wines produced by *P. kluyveri* showed about 37% of regular wine consumer preference, being described by experienced Madeira wine tasters as having notorious caramel and spicy notes, which are a significant

characteristic of Madeira wines. In this sense, *P. kluyveri* revealed great potential to be used as a starter culture in must fermentation for the Madeira wine production. However, further work is required regarding the improvement of wine quality by using mixed cultures and by analyzing its impact on the chemical characteristics and sensory properties of the final wine.

PART III – GENERAL CONCLUSIONS

The first aim of the present work was to evaluate the chemical composition of wines produced by different wineries and with grapes from different vineyard locations (south and north regions of Madeira Island) and to determine if there is variability in the wines and how it can compromised the standardization of the production process, which might affect the wine quality. The second and most important aim of the current study was to isolate, identify, and characterize the indigenous yeast microbiota of Tinta Negra grape musts from the previously mentioned wines and to evaluate the impact of five selected non-*Saccharomyces* yeasts species, used as pure starter culture, in the chemical composition (sugars, phenols, organic acids, color and volatile profile) and sensory properties of Madeira wine. The main conclusions were as follows:

- During the fermentation process, the volatile profile, phenolic content, antioxidant potential, and color vary between samples from different Madeira wine wineries. This variability started in the grape juice and continues throughout the fermentation process, affecting the reproducibility between different batches of must from the same winery. Several factors may be involved, most likely related to the grape characteristics (e.g., grape maturation and geographic origin), the yeast strains involved in the fermentation, and the winemaking practices of each winery.
- The chemical composition of Madeira wines can be affected by the location from where the grapes are collected. The vineyards in the south region produce grapes with a chemical composition that seems to positively influence the wine's final quality by increasing the sugar content, total phenolic content, and antioxidant potential. Even though these compounds showed a similar trend throughout fermentation and aging, the final wine composition seems to correlate with the grape's initial chemical composition.
- Regarding the indigenous yeasts microbiota involved in Madeira wine production, it was possible to identify 11 yeast species from wineries musts (*Hanseniaspora uvarum, Starmerella bacillaris, Pichia kluyveri, Pichia fermentans, Saccharomyces cerevisiae, Torulaspora delbrueckii, Candida apicola, Cystobasidium minutum, Pichia terricola, Cystobasidium slooffiae,* and *Wicheramomyces anolalus*) and 6 yeast species from vineyards musts (*Hanseniaspora uvarum, Starmerella bacillaris, Pichia kluyveri, Pichia fermentans, Saccharomyces cerevisiae,* and *Hanseniaspora opuntiae*).

- The studies regarding the inoculation of musts with selected non-*Saccharomyces* species (*H. uvarum, S. bacillaris, P. terricola, P. fermentans,* and *P. kluyveri*), used as pure starter cultures to produce Madeira wines, revealed that only *H. uvarum, S. bacillaris,* and *P. terricola* possessed the fermentation capacity to produce dry Madeira wines. The phenolic content was higher in wines produced by *Pichia* spp., while the antioxidant potential was more evident in wines from musts fermented with *H. uvarum* and *S. bacillaris.* Also, the wine produced by *S. bacillaris* showed higher acidity when compared to wines produced by the other non-*Saccharomyces* species.
- The volatile profile of wines produced from musts inoculated with the selected non-*Saccharomyces* yeasts showed that the wine produced by *P. kluyveri* stands out regarding the total VOC content, being the proportion of total esters and fatty acids higher in this wine. Alcohols were more abundant in the wine produced by *P. terricola*, while carbonyl compounds were mainly found in the wine produced by *S. bacillaris*.
- Regarding the wine color, the wines after *estufagem* showed a similar tendency towards chromatic characteristics that are typical of these oxidative wines, namely the predominance of yellow over red. However, the Browning Index (BI) was more notorious in the wine from the must inoculated with *P. fermentans*.
- The sensorial analysis revealed that the intensity of olfactory descriptors typically associated with Madeira wines greatly depends on the selected non-*Saccharomyces* yeast species involved in the fermentation process. About 37% of regular wine consumers showed a preference for the wine produced by *P. kluyveri,* described by experienced Madeira wine tasters as having notorious caramel and spicy notes, which are a significant and typical characteristic of Madeira wines.
- As results show that *P. kluyveri* produces high-quality wines and suggest that this yeast could potentially be used as a starter culture in the production of Madeira wines, especially for sweet wines in order to standardize the production process and reduce the variability between wines batches.

PART IV – FUTURE PERSPECTIVES

The main conclusions of this thesis represent an important advance in the knowledge of the microbiota involved in Madeira wine production and how some selected non-*Saccharomyces* yeast species can positively contribute to the wine's chemical composition and organoleptic characteristics. However, in the future, we consider it important to:

- Increase the knowledge about the impact of indigenous yeasts involved in the Madeira wine production, either *Saccharomyces* or non-*Saccharomyces* species, in the chemical composition and sensorial profile of wines;

- The use of *P. kluyveri* as a pure starter culture on an industrial scale in order to evaluate its real impact on the wine's chemical composition and sensorial characteristics;

- Extend the study about the influence of the selected non-*Saccharomyces* yeasts used in the present study to the production of Madeira wines from white grape varieties (Malvasia, Verdelho, Boal, and Sercial);

- Study the impact of mixed starter cultures on the chemical composition and sensorial characteristics of musts and wines during Madeira wine production;

- Develop and use an optical fiber sensor to measure must density in order to monitor and help standardize the fermentation process;

- Create a mixed of indigenous yeast culture representative of each winery and wine type (without compromising the genetic diversity and heritage) to be used as a starter culture in order to standardize the vinification process, improving Madeira wine quality.

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