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Microbial Characterization of Late Harvest Wines

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À minha Mãe, Irmã e Carlos Faim

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RESUMO

A superfície das bagas da uva é habitada por uma grande diversidade de microrganismos, incluindo leveduras, bactérias e fungos filamentosos que desempenham um papel importante na produção de vinho, contribuindo significativamente para processo fermentativo e para propriedades aromáticas finais do vinho resultante. O fungo *Botrytis cinerea* é um dos microrganismos patogénicos mais importantes na viticultura, uma vez que provoca frequentemente danos na uva (podridão cinzenta), mas sob condições climatéricas específicas leva à podridão nobre pela perda de humidade uva e concentração de açúcar.

O objetivo deste estudo foi avaliar e comparar as comunidades microbianas presentes em uvas infetadas com *B. cinerea*, com base no estado fitossanitário e na casta. Desta forma, foram aplicados métodos dependentes de cultivo e técnicas moleculares para determinar a heterogeneidade das populações microbianas os dois tipos de podridão. Neste trabalho foram isolados e identificados 187 microrganismos, onde se destacaram os géneros *Glucanobacter e Acetobacter* na comunidade procariota, e as espécies *Hanseniaspora uvarum*, *Metschnikowia pulcherrima* e o género *Pichia* na comunidade eucariota.

Os resultados demonstraram que os sintomas de podridão nobre não parecem ser causados por diferenças na diversidade das populações microbianas em relação à podridão cinzenta. Em vez disso, parecem ser dependentes da densidade da população microbiana, o que tem consequências para a produção de vinhos de colheita tardia, nomeadamente para a produção de vinhos botritizados. Contudo, devem ser realizados estudos com intuito de desvendar as interações entre os microrganismos e as interações destes com as uvas.

Palavras-chave: *Botrytis cinerea*, Podridão cinzenta, Podridão nobre, Métodos Moleculares, Análise estatística

ABSTRACT

Grape berry surface is inhabited by a wide diversity of microorganisms including yeasts, bacteria and filamentous fungi that play an important role in winemaking, contributing significantly to fermentative process and final aromatic properties of the resulting wine. *Botrytis cinerea* is among the most important pathogens in viticulture since that causes frequently grape damage (grey rot), but under specific climacteric conditions leads to noble rot by the loss of grape moisture and sugar concentration.

The aim of the current study was to assess and compare the microbial communities presents on *Botrytis*-infected grapes, based on different health status and variety. Cultivation-based and molecular methods were applied to determine the heterogeneity in microbial populations of the two types of rot. In this work were isolated and identified 187 microorganisms. The prokaryotic community was highlighted by the presence of the *Glucanobacter* and the *Acetobacter* genera, and eukaryotic community was highlighted by the presence of the *Hanseniaspora uvarum* and *Metschnikowia pulcherrima* species, and the *Pichia* genus.

The results demonstrate that noble rot symptoms do not seems to be caused by different microbial population than grey rot, but instead it seems to be dependent of the microbial density, which has consequences for the late harvest, namely botrytized wines production. However, should be carried out studies designed to unveil the interactions between microorganisms and their interactions with grapes.

Key-words: *Botrytis cinerea*, Grey rot, Noble rot, Molecular methods, Statistical analysis

SYMBOLS AND ABBREVIATIONS

AAB	Acetic Acid Bacteria
AG	Variety A Grey rot
AN	Variety A Noble rot
BA	Beerenauslese
BG	Variety B Grey rot
BN	Variety B Noble rot
BLASTn	Basic Local Alignment Search Tool Nucleotide
CFU	Colony-Forming Unit
CTAB	Cetyl Trimethylammonium Bromide
DNA	Desoxyrribonucleic Acid
dNTPs	Deoxynucleotide triphosphates
EDTA	Ethylene Diamine Tetra Acetic Acid
ITS	Internal Transcribed Spacer
LAB	Lactic Acid Bacteria
M	Molar
Mb	Megabases (millions of base pairs)
mM	Milimolar
NGS	Next Generation Sequencing
OIV	International Organisation of Vine and Wine
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
rRNA	ribosomal Ribonucleic acid
rpm	Revolutions Per Minute
SD	Standard Deviation
TAE	Tris – Acetate - EDTA
TBA	Trockenbeerenauslese
TE	Tris - EDTA
YPD	Yeast extract Peptone Dextrose
µl	Microliter
µM	Micromolar

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CHAPTER I – INTRODUCTION

1. FERMENTED BEVERAGES AND WINE ORIGIN

Fermentation is one of the oldest known forms of food biotechnology and has played a significant role in most civilizations worldwide. Fermented foods and beverages can be defined as edible products, whose production involves biochemical changes, through a wide range of microbial or enzymatic process naturally present or intentionally added to achieve the desired characteristics of final products. Indeed, fermentation processes also contribute to food safety, to the improvement of organoleptic characteristics or nutritional properties, and ultimately may contribute to promotion of health [1, 2]. Therefore, it is of economic and cultural importance that the development of fermentation technologies is strongly implemented.

Evidence of the production of fermented beverages was found in China as early as 7000 B.C. [3] with rice, honey and fruit as the subjects of the process [4]. The earliest evidence of wine residues was discovered at Hajji Firuz Tepe in the northern Zagros Mountains of Iran, dated to ~ 5400 B.C. [4, 5] and the cultivation of grapevine and the production of wine was extended all over the Mediterranean Sea towards Greece (2000 B.C.), Italy (1000 B.C.), Northern Europe (100 A.D.) and America (1500 A.D.) [3].

1.1. Wine sector

Currently, the vineyards of the European Union account for about 45% of the area and 60% of the volume of world production, and almost for 60% of world wine consumption. Indeed, the wine sector is one of the most important sectors of agricultural production in the European Union, where it represents a vital economic activity [6]. Nevertheless, its contribution to the economy varies significantly from one region to another. About 51% of the world's wine is produced by four countries, i.e. France (17%), Italy (16%), Spain (15%) and Germany (3%) [7].

1.2. Grapes, the Base of Winemaking

The grape is the fruit of the vine, a plant of Vitaceae family. It is often used to make juice, jam, raisins and wine or to eat as fresh fruit. Grapes used in wine production belong to the *Vitis vinifera* species.

The grape bunch (Figure 1) is made up of both a woody part and berries. The woody part represents 3-7% of the bunch weight and its structure consists of main axis (rachis) connected to the peduncle, and shorter branches (pedicel) that supports the berries. The stems contain water, small amounts of sugar, is rich in potassium and has low acidity [8, 9].

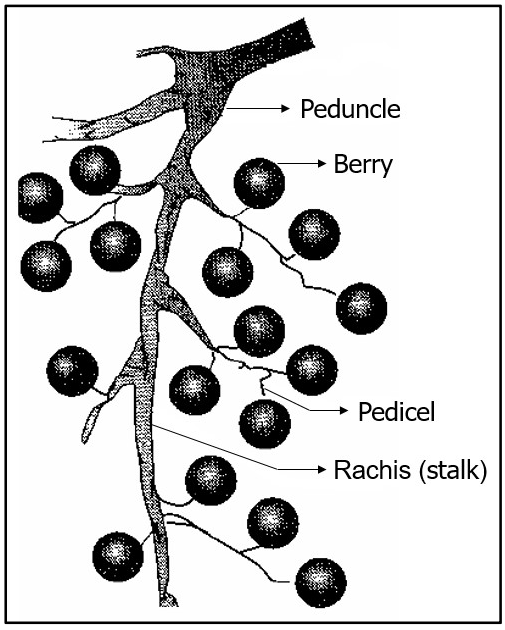

Figure 1 | Structure of a bunch of grapes [9].

The grape berries represent 91-97% of bunch weight [9]. Each berry contains the seed surrounded by a range of tissues, known as pericarp. The pericarp is divided into exocarp (skin), endocarp, and mesocarp (pulp), which is used to produces juice or wine [8]. The skin is a membrane that accompanies the berry development and forms a heterogeneous surface divided into: cuticle, epidermis and hypodermis. The cuticle is the interface between the plant tissue and the environment and its function is to protect the berry against dehydration and fungal pathogens, and to control the gas exchange [10].

1.3. Types of wines

Although no internationally agreed overall system exists, the classification of wine can be made according to various methods based on geographic origin, vinification methods and style, sweetness, vintage or variety used. However, wines are frequently divided into still, sparkling, fortified and sweet wines [4], which recognizes significant differences in production methods (Figure 2).

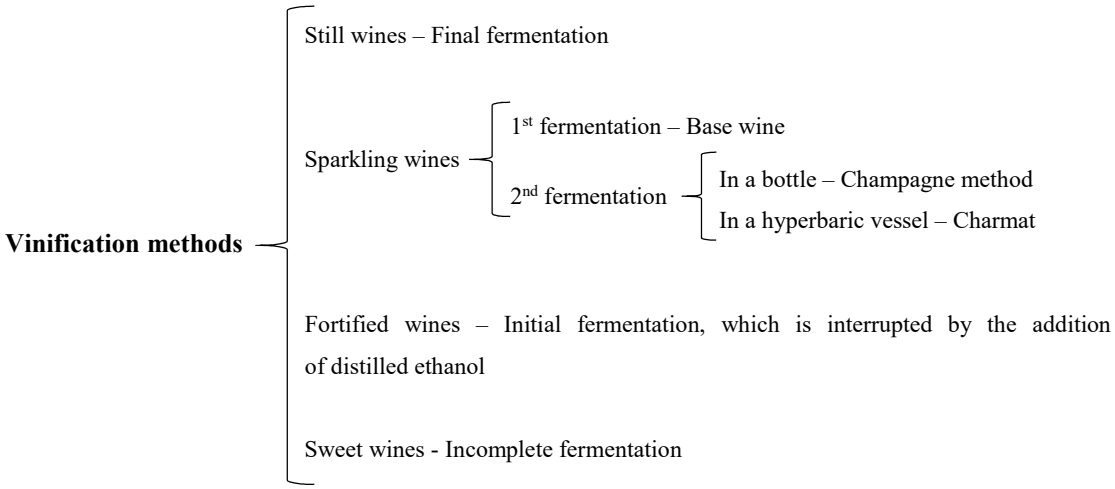

Figure 2 | Wine classification based on vinification methods [4, 8].

Sparkling wines contain carbon dioxide and their production involve the fermentation of a base wine, to which is then added sugar and a second fermentation is promoted. Therefore, these type of wines are often classified by the type of second fermentation method used: after bottling, where yeasts are added directly to the bolttle (traditional method – the *champagnoise*) or in bulk, where the second fermentation is carried out in a pressure-resistant vessel, and bottled at the end of the process (Chamart type wines). In contrast, Still wines are wines that have undergone a single fermentation step, and there is no effervescence.

In addition, wines are further classified according to their colour, namely red, white and rosé [4]. Within these subcategories, wines appear with different colours as well (Figure 3). Indeed, colour is an important characteristic of wine and it is related with some components, such as polyphenols, tannins and anthocyanins and is influenced by the pH of wine, total acidity, grape variety, the degree of maturity of the grapes and winemaking technology [11, 12].

Figure 3 | Wine colour chart [13].

Regarding Fortified wines, they derived from the partial fermentation of fresh grapes or grape juice, which is interrupted by the addition of alcohol. These wines were created in the past in response to technical problems encountered in warm regions, because sugar-rich grapes and elevated temperatures resulted in explosive fermentations, easily leading to stuck fermentations. Therefore, the addition of alcohol during fermentation is a simple way to stabilize the wine [8]. Consequently, these wines are characterized by their high concentrations of alcohol and sugar, and are produced in a wide range of styles: dry or bitter-tasting forms are normally consumed as aperitifs before meals, or examples like Sherries, Ports and Madeiras are mostly consumed after meals, or as a dessert substitute [4].

Finally, Sweet (non-fortified) wines with a sweet finish are intended to be consumed alone or to accompany or replace a dessert. This type of wines has an incomplete fermentation and a certain proportion of grape sugar is not transformed into alcohol, leaving behind varying residual sugar levels [8]. High-quality sweet wines are often made from grapes with their own concentrated sugars and this concentration process can occur in different ways: the classic method dries grape clusters on mats of straw in the sun, but some regions dry them under cover, on roofs, on racks, or hanging up the grapes, while other regions leave them naturally dehydrating on the vineyard (Late Harvest). In fact, Late Harvest wines are known as wines made from grapes left on the vineyard longer than usual, and the over ripening process leads to concentration of sugars. Therefore, leaving grapes dry on the vineyard, they become vulnerable to *Botrytis cinerea* infection, which allows the production of Botrytized wines.

2. BOTRYTIZED WINES

Botrytized wines are sweet white wines made from grapes infected with *Botrytis cinerea* [4, 8]. The grapes used in these wines are dried naturally in the vineyard, where they become over ripped, and naturally infected by the mould, as a result, these grapes are dehydrated and have very high sugar concentration. Consequently, *B. cinerea* infection is often referred to "*Noble-Rot"*, and permits the production of great sweet wines, and these exceptional wines can only be made in specific conditions and therefore, their production is limited [14]. However, *B. cinerea* is also the causing agent of grey rot, which results in the complete degradation of grapes, and only under unique climatic conditions is possible to obtain high-valuable noble rot grapes.

Although botrytized wines are very well-known sweet white wines, it is not known when the noble rotted grapes were first intentionally used for wine production. There are historical evidences that they were firstly produced in Tokaj region of Hungary in about 1560. In Germany, its production is reported to about 1750 and in France the production appears to have been well established in Sauternes between 1830 and 1850. Nowadays, botrytized wines are also produced worldwide, including Portugal [4].

2.1. Main Types of Botrytized Wines

There are different grapevine varieties used to produce botrytized wines, such as Chenin Blanc, Sauvignon Blanc, Semillon, or Riesling, but their transformation is always the same: overriped berries under specific climatic conditions and *Botrytis cinerea* development [15]. Due to the very specific climate conditions that allows the development of the noble rot, only a few regions in the world can produce this type of wines, namely: Tokajs (Hungary), Auslese, Beerenauslese (BA) and Trockenbeerenauslese (TBA) (German) and Sauternes (France) [16].

2.1.1. Hungarian: Tokaji Aszú

Tokaji Aszú is known as the first wine intentionally made from noble-rotted grapes. Tokaj is the name of a town and a wine district in Hungary, consequently Tokaj is the official appellation in the European wine register system. However, the traditional local name Tokaji (meaning ‘‘of Tokaj’’) may be also officially used [16].

The unique Tokaj *terroir*[1] characteristics, the cultivated varieties, a late harvest (from the beginning of October to the end of November) and *Botrytis cinerea* infection, enable the noble rot development almost every year. The soil is constituted of red clay to loess soil with some volcanic debris. The climatic conditions are influenced by the Zemplén hills and the humidity from Tisza and Bodrog Rivers. Only six white grapevine varieties are permitted to Tokaji production: Furmint, Hárslevelű and Sárga Muskotály (Muscat Lunel) varieties, but sometimes Kövérszőlő, Zeta and Kabar, and typically, these wines are made as blends.

Although almost no fungal mycelium and conidia are visible or are totally lacking on the surface of the skin, the "noble rot" fungus is responsible for dehydrate the grape berries, concentrating their sugars. Optimally botrytized berries are brown, with violet tones and fully shrivelled. The result is the production of sweet wines with balance of fruit, acidity and residual sugar [16, 17], and the alcohol content may reach 14% [4]. Indeed, these wines can have a range of sweetness levels (60, 90, 120 and 150 g/l sugar content) based on the amount of botrytized fruit added to the must. This distinction is made on the bottle label by the number of *puttonyos*, ranging from 3 to 6. During grapes storage, a small portion of juice is collected under the perforated bottom of the storage container that flows out of the fruit by gravity. This juice is called Eszencia or Essence and represents the highest quality specialty of Tokaj wine, and is very different from Tokaji Aszú. Tokaji Eszencia has extremely high sugar content, flavour intensity and the alcohol content is low (usually below 5%, v / v) [4, 16].

Moreover, the winemaking technology of Tokaji Aszú is unique. The juice of the desiccated grapes cannot be extracted by pressing and a special maceration method has been applied since antiquity. The maturation occurs for at least 3 years, during which oak barrels are used for at least 2 years before bottling [16].

2.1.2. German: Auslese, Beerenauslese (BA) and Trockenbeerenauslese (TBA)

In Germany, the wine estate, located in Rheingau, in the Rhine valley, is famous for its high-quality Riesling vines and it is, from which the production of different styles

[1] The complete natural environment in which a particular wine is produced, including factors such as the soil, topography, and climate.

of botrytized wines extends to the Mosel-Saar-Ruwer wine district. However, the climatic conditions are not appropriate every year and thus these type of wines is not produced in a yearly basis. Despite relatively cold temperature conditions, the special microclimate and the morning mist of the Rhine River, are the factors that support the development of noble rot [16]. Regarding geology, it is made up of slate, quartzite, sandstone, gravel and loess. More than 80% of the soil is quartz containing other minerals, including small amounts of feldspath and mica [18].

Riesling is the predominant graoevine variety grown, but Gewürztraminer, Rulander (Pinot Gris), Scheurebe, Silvaner, and Huxelrebe are also prone to noble rot development [16]. Indeed, there are different categories of German botrytized wines, from which three of them involve *Botrytis cinerea*: Auslese, Beerenauslese (BA) and Trockenbeerenauslese (TBA). However, TBA derives from botrytized grapes only, whereas in the other cases, healthy and botrytized grapes are processed in simultaneous [4, 18].

The vinification technology of German botrytized wines is characterized by a short maceration of the must on the skins, with a gentle pressing. The fermentation might terminate spontaneously or can be interrupted by sulphite addition and filtration. Usually, these types of wines have low alcohol contents and are rarely matured in oak barrels. The wines are characterized by flavours like a slight taste of apricot, honey, caramel, and dried fruit [16].

2.1.3. French: Sauternes

Sauternes is located along the Garonne River and its tributary, the Ciron in southeast of Bordeaux region, which is the appellation for the most known botrytized wines produced in France [4]. There are four grapevine varieties used in these type of wines: Sémillon, Sauvignon Blanc, Sauvignon Gris, and Muscadelle. Sémillon is the principal grapevine cultivated and is especially susceptible to noble rot, accounting for about 80% of a typical estate's vineyard [19]. When the autumn is warm and dry, the confluence of the two rivers, with different water temperatures, normally generates fog in the morning, promoting the climatic conditions that are most favourable for noble rot [16]. The soil in Sauternes varies between calcareous and clayey and the vineyards are planted on gravel soil on a subsoil of clay [18].

These wines have a strong *Botrytis cinerea* character, with slight taste of apricots, honey, and peaches. Due to the relatively high sulphur dioxide content, they can be preserved for a very long time in bottle [16].

2.2. Production of Botrytized Wines

Noble rot involves fungal development on ripe grapes allowing the production of botrytized wines, which can only be made in particular conditions. Indeed, the production of botrytized wines is a great challenge for winemakers due to the uncertain nature of *B. cinerea* development, the low grape production and low juice yield, the technological difficulties and the high risk of grey rot. Therefore, the production of these type of wines is limited, being one of most expensive of the world due to volume loss and high production costs.

2.2.1. Harvest

In botrytized winemaking, various strategies and techniques are applied, but all start with the late harvest of the grapes – for this reason they are also known as Late Harvest Wines. Therefore, it bears a high risk of losing the harvest due to bunch rot, other infections or adverse climate conditions. Indeed, the late harvest is a rather demanding process that oblige to go through the vineyard periodically and to collect perfectly noble-rotted berries, leaving the rest until they reach the optimal shrivelled state. Thus, the grape clusters are left on the vine until November [19].

2.2.2. Grape Processing and Juice Extraction

The noble-rotten grapes should be handled according with the general rules of white winemaking, and especially during transport it is of extreme importance to avoid spontaneous physical damage to the grape skin. Once at the winery, they should be handled with particular care to avoid all the events that cause the wines to be more difficult to clarify [20].

In many regions, the noble-rotten grapes gently crushed and macerated are left in their own juice overnight to release sugar and aromatic substances. The oxidation is avoided by the use of closed vessels or a layer of CO_2 gas [16]. On the other hand, often the grapes are not destemmed to favour the circulation and the drainage of juice during

pressing. Indeed, in winemaking, gravity methods should be used since that mechanical operations (crushing, pumping and pressing) are not as soft as required in the production of these type of wines. Nonetheless, due to its increased viscosity, the juice extraction is extremely difficult and its slow pressing over many repeated cycles is necessary [20]. While the standard pneumatic pressure is not strong enough, continuous pressing should not be used because grapes are ripped and transfers suspended solids and glucan into the juice [16]. Despite being essential, crushing and pressing are the most difficult operations in botrytized winemaking, compromising the quality of produced wines. Importantly, and unlike healthy grapes, the juice obtained from the latter pressing cycles contains more sugar, iron and tannin concentrations and, therefore, is of higher quality [20].

A light sulfiting at 3-5 g/hl of the must protects against oxidation, restricts the proliferation of some spoilage microorganisms, and favours the development of alcoholic fermentation [16, 20].

Must clarification before fermentation is typically applied in white wines technologies but has particular difficulties in botrytized winemaking, due to the presence of *Botrytis*-derived polysaccharides, as well as large amounts of suspended solids, still, excessive clarification is undesirable. Sometimes this step improves flavour quality, but it also may accentuate any nutrient deficiencies already present in the must. Indeed, the most effective clarification at the moment takes place at between 18 and 24 hours of decantation at low temperature (0ºC) and it settles during 2-3 days [4, 16, 20].

2.2.3. Fermentation

The fermentation process of botrytized musts starts with a large population of multiple yeasts species. However, for most of them the botrytized musts represents an adverse environmental condition [21]. The high sugar concentration is the principal limitation factor, but the growth of *Botrytis cinerea* creates a nutritional deficiency [20, 22]. Indeed, the fermentation of noble-rotten musts is difficult and can take 1 to 6 months, or even a year [16].

Previous studies have showed the dynamics of yeasts population during spontaneous fermentation, wherein botrytized wines revealed a higher level of biodiversity than normal wines [16]. However, the principal yeasts of botrytized wines, namely *Saccharomyces cerevisiae*, *S. uvarum* and *C. zemplinina*, can survive until the

end of fermentation. These are thought to be able to coexist because their sugar preferences and carbon source are different [21].

2.2.3.1. Fermentation Techniques and Chemical Composition

In Hungary (Tokaji) and France (Sauternes) wooden barrels have been widely used as fermenters, but in most of regions stainless steel tanks are used. It is recommended that the addition of thiamin (0.6 mg/l), diammonium phosphate (300 mg/l), and active dry yeast (10–15 g/hl) to achieve an optimal fermentation rate, as the faster yeasts propagation, and lower the amounts of requires SO_2. [16].

In respect to the control of temperature there are no general rules for botrytized wine fermentations. Nonetheless, a special aspect of botrytized winemaking is the cessation of fermentation at desired residual sugar content. Traditionally, fermentation stops spontaneously at various ethanol levels, although sometimes the levels of ethanol were higher than desirable, which led to insufficient residual sugar concentration [16].

In the case of Tokaji Aszú, due to very high sugar content of the juice (occasionally more than 50%), fermentation occurs slowly and often reaches little more than 5–7% alcohol before termination. Fermentation continues slowly and may take several weeks or months to finish and the alcohol content may reach as high as 14%. When fermentation ceases, the fermentation barrels are usually left half open, as the oxygen uptake is restricted by the growth of a common cellar mould on the wine's surface [4]. Typically, for the BA and TBA, most sugar in the juice is not converted during alcoholic fermentation. The wines are consequently sweet and low in alcoholic strength, commonly 6–8% [4]. Conversely, the sweet wines produced in Sauternes contrasts with the gradation of botrytized styles produced in Germany, where the major difference is in the alcohol levels. French styles commonly exceed 11–13% alcohol, whereas German versions occasionally exceed 10% [4].

Briefly, the unique chemical composition of botrytized grape juice has a great influence in the products and by-products of alcoholic fermentation. Due to the high sugar content of the juice, the growth, the fermentation rate and the survival of yeasts are dramatically reduced. On the other hand, during fermentation, glycerol content increases proportionally less in botrytized than in non-botrytized wines and the stimulating effect of high sugar contents on volatile acidity is particularly marked in botrytized wines by

the heteropolysaccharides of *Botrytis*. The volatile acidity in botrytized wines may reach the 2 g/l (a level permitted by law in some situations) [16].

Moreover, due to their high sugar contents, fermentation can be prevented through chemically induced termination. The most frequent technique is by the addition of sulphur dioxide [15]. However, such form of promoting the end of the fermentation is one of the technical problems in botrytized wine production that needs further research and development [16].

2.2.4. Aging and Stabilization

A unique feature of botrytized wines, comparatively to normal still wines, is the formation and precipitation of calciummucate crystals, a salt of galactaric or mucic acid. Thus, before bottling, the wines are normally stabilized by removing protein and avoiding tartrate salt crystallization, through the reduction of calcium content in wine with DL-tartaric acid addition [16].

Due to its high sugar content, botrytized wines are microbial unstable and there is a risk of in-bottle re-fermentation. Thus, the bottling process needs to be extremely careful and sterile to avoid microbial spoilage. Sterile filtration of the wine into sterile bottles, sealed with sterile corks, is a frequent procedure [4, 16]. For example, Tokaji Aszú wines may be pasteurized before bottling to prevent subsequent fermentation and sulphur dioxide is also required to prevent undesirable microbial activity during maturation and after bottling [4].

2.3. Biochemical characterization of Botrytized Wines

In *Botrytis* spp. infected grapes, apart from the concentration of sugars, their physicochemical characteristics are also changed, which contribute to the unique profile of the resulting sweet wines (Table 1). The principal causes of the sensorial effects of noble rotting are berry dehydration, combined with the metabolic action of *Botrytis cinerea*. Some of the impacts of drying can simply be related to concentration, such as the increase in citric acid and sugar content. Other type of impacts can be related to the presence and/or absence of metabolites, as the fungal metabolism is sufficiently active to

decrease the concentration, despite concentrating effect of water loss. This is particularly noticeable with tartaric acid [4, 8].

Table 1 | Chemical and physical parameters of grape and juice by noble rot [16].

	Sauternes		Germany			Tokaj	
Constituent	**Healthy berry**	**Noble-rotted berry**	**Healthy berry**	**Noble-rotted berry (BA)**	**Noble-rotted berry (TBA)**	**Noble-Rotted berry (Eszencia)**	**Noble-rotted berry (Eszencia)**
Weight per 100 berries (g)	202	98	209	85	36	–	–
Sugar (g/l)	247	317	182	295	500	685	708
Glucose/fructose ratio	–	–	0.98	0.80	0.94	0.98	0.88
Glycerol (g/l)	–	–	0.09	8.00	20.67	24.3	30.7
Tartaric acid (g/l)	5.33	2.48	7.3	2.6	2.4	4.81	4.44
Malic acid (g/l)	5.43	7.84	4.2	8.0	10.1	5.82	7.42
Citric acid (g/l)	0.17	0.22	0.19	0.20	0.24	0.11	0.99
Acetic acid (g/l)	0.32	0.41	0.00	0.45	0.13	–	0.49
Gluconic acid (g/l)	0	2.08	0.02	1.5	2.17	3.20	3.88

2.3.1. Organoleptic Characterization of Botrytized Wines

During noble rot, the metabolic activity of *B. cinerea* leads changes in chemical composition and physical properties in the grapes. Both factors play an important role to the quality of noble-rotted grapes. Whereas *B. cinerea* activity alone leads to rotten and inferior quality grapes, dehydrates concentrating results in over matured and shrivelled berries. The latter are appropriate for making high quality sweet wines, with a lower content of alcohol and distinctive aroma compounds produced by *Botrytis* [16]. In fact, the aroma of botrytized wines have been widely investigated and the main aroma compounds identified as specific botrytized odorants are indicated in Table 2.

Table 2 | Volatile compounds associated with botrytized wines as characteristic aroma substances [15, 16].

Compound	Sensory descriptor	Wine type
Phenylacetaldehyde	Honey	Sauternes
2-Phenyletanol	Rose, floral	Sauternes
3-Mercaptohexan-1-ol	Grapefruit	Sauternes
Ethylhexaonate	Pineapple, green apple, banana	Sauternes
β-Damascenone	Fruity, quince, canned apple	Sauternes
γ-Nonalactone	Peach, apricot	Sauternes Tokaji Aszú
δ-Decalactone	Coconut, peach, apricot	Sauternes Tokaji Aszú
Sotolon	Caramel, curry, nut	Sauternes
3-Sulfanylpentan-1-ol	Grapefruit	Sauternes
3-Sulfanylheptan-1-ol	Citrus	Sauternes

Therefore, botrytized wines are sweet white wines and they are famous for their distinctive and exceptional range of aromas, such as citrus and dried fruit in young wines, orange peel in older wines, and honey nuances in wines subjected to oxidative ageing [15]. As fortification is not allowed, their alcohol content arise only from the fermentation of the original sugar content of the juice [16].

2.3.2. Botrytized Wines and Health

The composition of botrytized wines includes a number of physiologically beneficial substances in significantly higher concentrations than found in other type of wines. They contain much higher fructose than glucose and also contain high organic acids and a large amount of minerals [16].

In general, wines contain a large variety of antioxidants. They include resveratrol, catechin, epicatechin, and proanthocyanidins and they are considered free radical terminators, which eliminate reactive oxygen species from the human body. However, the most significant health-related compounds in wines are polyphenols. In comparison with normal white wines, botrytized wines generally contain much higher quantities of polyphenols, enriching their antioxidant capacity. One possible explanation is related with dehydration of the grape and with the berry skins maceration before pressing. In

addition, Tokaji Aszú has higher values for these parameters, due to the much longer maceration time and the alcoholic environment during maceration [16].

3. General Microbiology of Wine Grapes

Grapes are inhabited by a complex population of microorganisms, including yeasts, filamentous fungi and bacteria. They have different physiological characteristics which can change in response to factors such as the climate, grape variety and geographical region. Therefore, they play a critical role in wine production, namely in fermentation process and organoleptic properties, compromising the quality of produced wines [23, 24]. While some species, such as fungi and environmental bacteria are only found in grapes, others like yeasts, lactic acid bacteria (LAC) and acetic acid bacteria (AAB) have the ability to survive and grow in wines [25, 26].

Grapevine is also colonised by other ubiquitous microorganisms, known as endophytes and epiphytes, which may have a beneficial or neutral effect on plants. Even though they do not cause disease symptoms, some of them have the ability to protect plants against pathogenic agents through an antibiosis mechanism [24, 27]. While epiphytes microbes live on surface of the epidermis of the plant, endophytes microbes have the ability to penetrate and survive within the internal tissues of the epidermis of the plant without causing any deleterious effect on the host plant [28, 29, 30]. However, grapes are also susceptible to several phytopathogens attacks that have negative impact, resulting in a decrease of fruit yield and quality, thus reducing the economic return of the vineyard. In addition, these microorganisms can be transferred to the winery, compromising the quality of wine produced [24, 25].

After *véraison*[2] microorganism population of grape berries comprised *basidiomycetous* yeasts (for example, *Cryptococcus* spp., *Rhodotorula* spp. *Sporobolomyces* spp.) and the yeast-like fungus *Aureobasidium pullulans*. When the berries begin to fissure, the nutrient availability is increased and the fruit sugars are converted into alcohol (ethanol) by the yeast flora, likely dominance by the oxidative *ascomycetous* populations (e.g. *Candida* spp., *Metschnikowia* spp. and *Pichia* spp.).

[2] Change of colour of the grape berries.

Additionally, the high sugar concentrations availability on berry surface favours the growth of ascomycetes with higher fermentative activity, such as *Pichia* spp. *Hanseniaspora/Kloeckera* spp. and *Zygoascus hellenicus* and also includes wine spoilage yeasts (e.g. *Zygosaccharomyces* spp., *Torulaspora* spp.), as well as acetic acid bacteria (e.g. *Gluconobacter* spp., *Acetobacter* spp.) [4, 23, 25].

Therefore, wine is an alcoholic beverage obtained from the fermentation of sugars in the grapes and their juice by the yeasts. The principal fermentative yeast in wine production is *Saccharomyces cerevisiae*, which converts glucose and fructose from grapes to alcohol, with the release of carbon dioxide and energy (Figure 4). Fruit juices contain water and nutrients that create an ideal environment for *S. cerevisiae* to start a sustained fermentation, and to completely metabolize fermentable sugars and produce an alcoholic beverage [5]. In the absence of *S. cerevisiae*, the fermentation usually ceases before all the sugars are converted to alcohol, resulting in a stuck fermentation [4].

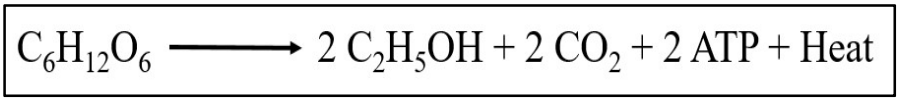

Figure 4 | Alcoholic fermentation.

3.1. *Botrytis cinerea*: Grey Rot and Noble Rot

Microorganisms can be grouped according to their effect on grape and quality of wine, depending on their technological significance in grape and wine production [25, 31]. *Botrytis cinerea* is a haploid Euascomycete fungus that belongs to the class of Leotiomycetes, with a genome size of 30 Mb. It is one of the principal pathogenic fungi of grapevines responsible for major viticulture losses in Europe. Indeed, *B. cinerea* is a phytopathogenic fungus with necrotrophic lifestyle that causes infections, which are characterized by the rapid destruction of the tissues of host plant [32, 33].

Botrytis cinerea is considered a generalist pathogen because it has the ability to infect a wide variety of plant hosts, or even just some organs (more than 200 plant species [34]) and also has a great capacity to adapt to different environmental conditions causing pre- and postharvest losses worldwide [35, 36]. The conidial form is ubiquitous and their reproduction is performed by asexual spores (conidia), which are produced on specially modified filaments, called conidiophores (Figure 5) [16].

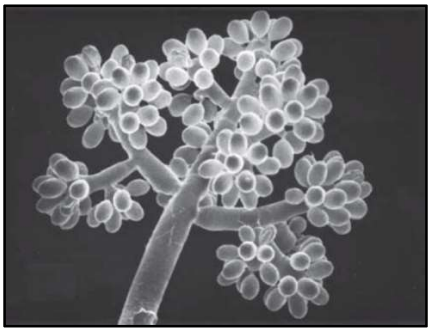

Figure 5 | Conidiophore (filament) and conidia (spores) of Botrytis cinerea [4].

During infections, *B. cinerea* releases diverse proteins, including enzymes, such as hydrolases, oxidases, endo- and exopectinases, cellulases, proteases, phospholipases, amongst others. These enzymes may modify the host cell walls, thus compromising the integrity of host tissues and inducing cell death. These enzymes also catalyse drastic changes in juice composition due chemical degradation of the epidermis that diffuse into berry flesh. Therefore, mycelia and conidiophores contribute to destroy berry skin and enable the berry dehydration via evaporation under dry conditions [4, 35, 16].

B. cinerea has great importance in viticulture, causing frequently grey rot (bunch rot), and occasionally noble rot, wherein the loss of moisture is a crucial factor for the direction of infection [16]. Under moist conditions it may cause grey rot or *Botrytis* disease, creating favourable conditions for the growth of other spoilage organisms [4]. Then, *B. cinerea* and a mix of other microorganisms, including yeast and bacteria, are involved in diverse crop species rot worldwide. However, under certain microclimatic conditions – humidity in the morning followed by warm, dry and sunny conditions in the afternoon – the fungus has a beneficial role in the production of sweet white wines and is also known "Noble Rot" [37, 38]. Therefore, depending on the conditions of development the same fungus (*Botrytis cinerea*), can causes grey rot or noble rot [4] and it is important to realize the difference between them.

3.1.1. Grey Rot

B. cinerea contaminates the grapevine's tissues causing grey rot if conditions are favourable to the development of this disease [39]. One of the main characteristics of this fungus is their saprophyte behaviour, which allows the existence in the environment either spores or mycelium on living or dead plant material. It is capable of causing loss

in the amount as well in the quality of wines if the climate and host physiological conditions are suitable [40].

B. cinerea overwinters as sclerotia or mycelium, which allows it to survive in the soil for many years and contributes to their persistent and widespread in nature (Figure 6) [36]. In the spring, conidiophores formed from sclerotia and mycelium release conidia [36, 39], which are dispersed by wind and rain, and directly penetrate plant tissues [41].

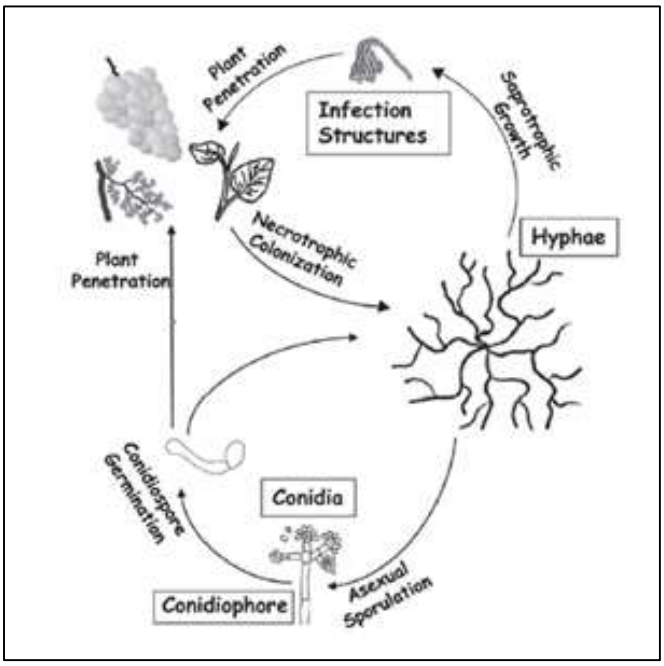

Figure 6 | Lifecycle of *B. cinerea*, with different stages of sexual and asexual development [36].

Although all the organs of the vine may be affected by the pathogen, ripe bunches are the most affected [42] and their action is more effective if there are wounds in the plant [40]. The determinant climatic factors for *Botrytis* bunch-rot development are temperatures between 20-25°C and relative humidity of 90-100% [41]. The main sign of *Botrytis* bunch-rot is the growth of grey sporulation of fungus on the surface of infected berries, with a rapid contamination of neighbouring berries (Figure 7) [43].

Figure 7 | Grapes infected with *Botrytis* bunch rot showing characteristic symptoms of grey fungal growth [44].

Therefore, *B. cinerea* associated with saprophytic fungi, including *Penicillium* spp., *Aspergillus* spp., *Rhizopus* spp. and *Mucor* spp., frequently causes the development of organoleptic defects in grapes and wines, like mouldy off-odours. Indeed, the transformation of terpenes into less odorous compounds and the hydrolysis of ethyl esters of fatty acids results from deterioration of phenolic compounds (anthocyanins, hydroxycinnamic acids, flavonoids), caused by the strong oxidase and esterase activities of fungi [4, 25]. Moreover, these fungi develop less mycelial mass than *B. cinerea* which affects the accumulation of glycerol, gluconic acid and sugars [8, 45].

Also, grapes are inhabited by yeasts and bacteria, whose negative impact is much lower than the unpleasant effects of *B. cinerea* [25]. Nisiotou and Nychas [46] assessed the yeast flora associated with healthy and *Botrytis*-infected grapes. The dominant species found in healthy grapes are *Hanseniaspora uvarum*, *Candida zemplinina*, *H. opuntiae and Aureobasidium pullulans* while damage *Botrytis*-infected grapes were inhabited by *H. uvarum*, *C. zemplinina*, *Issatchenkia occidentalis*, *I. terricola* and *H. opuntiae*. As regarding to bacteria, a previous study [47] suggests that the microbial ecology between *Botrytis*-infected and healthy grapes may be different, wherein population of acetic acid bacteria, in particular *Glucanobacter* spp. were increased in comparison with what was observed on healthy samples.

3.1.1.1. Effects of grey rot on wine quality

Grey rot has adverse consequences because the grape composition is modified. Inside of the grape berry, the fungus mycelium develops and consume large amounts of

organic acids, nitrogen and sugars. The sugar consumed by the fungus causes glycerol and gluconic acid accumulation. However, the reduction of sugar is not always compensated for the concentration effect that occurs by evaporation of water in the berries where grey rot develops. Indeed, the main chemical indicators of grey rot are the presence and concentration of ethanol, glycerol, gluconic acid, galacturonic acid, citric acid, acetic acid and laccase [40].

3.1.1.2. Measures of protection

As grey rot depends on the climate and sensitivity of the grape varieties, prophylactic measures to limit the significance of the damage on production are still under study. The measures of protection in vineyard should follow these recommendations:

- Avoid the varieties or clones in very sensitive areas or parcels considered favourable to the disease;
- Limit the excessive use of nitrogenous fertilizers, which cause great force and increase the sensitivity of the vine;
- The rate of the disease is much lower if the deep rooting allows modular water stress;
- To promote the pruning systems which allow good aeration of foliage and clusters and preventing injuries in the berries;
- Proceed to pare back bunches to reduce the volume and production and limit their compression;
- Exposing bunches (leaf thinning) in order to promote their precocity;
- Ensure good protection from mildew and moths.

When these preventive measures are properly applied, the *B. cinerea* development is decreased and should contribute to better limit the use of fungicides [48].

3.1.2. Noble Rot

Noble rot or late-harvest wines are typical in certain wine regions and it is a result of grapes infection by *B. cinerea* (Figure 8) under particular climate (temperature, humidity) conditions. In contrast to bunch rot, noble rot promotes favourable changes in grape berries by desirable *B. cinerea* activities that enhance the quality of the wines produced [25, 49].

Figure 8 | Grapes infected with *Botrytis cinerea* presenting noble rot [49].

To date, the molecular and biochemical processes that lead *Botrytis*-grape berry interactions to noble rot are unknown. However, the type of rot might depend on the combination of diverse factors, such as effects of the environment, winemaking practices, intrinsic characteristics of the grape cultivar and the berry microbiome [50]. The desirable conditions for noble rot to take place are temperature at around 20-25°C and a relative humidity of 85-95% for a maximum of 24 hours. Afterwards, the relative humidity should fall below 60%, which plays an important role in the dehydration of the infected berries [38].

3.1.2.1. Development of noble rot

In both cases grey and noble rot, epidermal penetration by germinating conidia seems to be essentially the same. Infection occurs through micro-fissures during berry growth, which provides nutrients for conidial germination and produce additional sites for fungal penetration. The colonized berry skin changes colour to pink and subsequently to chocolate-brown, become permeable and then gradually withers [16, 49, 50].

During infection, *B. cinerea* is not able to metabolize all the sugars present in the grapes. The result is the increase of sugar concentration and the loss of water, which together promote the accumulation of characteristic fruity aromas, as well as the concentration of sugars, being one of the most significant changes during noble rotting [4, 49, 50]. In contrast, during grey rot the infection progresses to complete degradation of the berry [16].

In addition, activity of *B. cinerea* is associated with physicochemical changes that include reduced glucose/fructose ratio, higher concentration of malic acid and lower concentration of tartaric acid and formation of glycerol and gluconic acid when compared with uninfected grapes [38, 50]. Although *B. cinerea*, *Aspergillus* spp. and *Penicillium* spp. produce gluconic acid by directly glucose oxidation into gluconic acid [8, 51], the presence of acid bacterium such as *Gluconobacter oxydans* which frequently colonize grapes infected by *Botrytis cinerea*, is probably responsible for most of the gluconic acid synthetized found in infected grapes [52].

3.1.2.2. Grape microbiota of noble rotten grapes

The skin injuries on grapes generated by *Botrytis cinerea* allows secondary colonization by additional microorganisms of filamentous fungi, yeasts and bacteria. They became a tiny ecosystem with interaction between them [21].

The development of saprophytic fungi such as *Penicillium*, *Aspergillus*, *Mucor* and *Cladosporium* species are commonly found on botrytized grapes [53]. However, the presence of *Aureobasidium pullulans* was also reported [16] and previous studies have reported a higher constant microbial numbers of yeast during pre-harvest colonization on/in *Botrytis*-infected grapes and grape musts (Table 3).

Table 3 | Occurrence of yeasts identified on/in *Botrytis*-infected grapes and grape musts [21, 46].

Country, Region	Specie
Attica, Greece	*Hanseniaspora uvarum, Metschnikowia pulcherrima, Hanseniaspora opuntia, Hanseniaspora guilliermondii, Zygosaccharomyces bailii, Candida zemplinina, Issatchenkia terricola*
South Africa	*Klockera apiculate (Hanseniaspora uvarum), Torulopsis stellata (Candida zemplinina/stellata)*
France, Sauternes	*Kloeckera apiculata (Hanseniaspora uvarum), Torulopsis stellata (Candida zemplinina/stellata), Metschnikowia pulcherrima, Candida krusei, Saccharomyces cerevisiae*
Hungary, Tokaj	*Aureobasidium pullulans, Hanseniaspora uvarum, Metschnikowia fructicola, Metschnikowia pulcherrima, Rhodotorula kratochvilovae, Rhodotorula nothofangi, Kluyveromyces thermotolerance, Cryptococcus magnus var. magnus, Candida zemplinina/stellate, Saccharomyces cerevisiae, Saccharomyces uvarum*
USA, California	*Saccharomyces cerevisiae, Hanseniaspora uvarum, Pichia kluyveri, Metschnikowia pulcherrima, Candida zemplinina*

Although grapes are the main source of bacteria in wine production, providing must with both beneficial and potentially damage species, affecting the fermentation course and consequently the quality of the final product, the bacterial diversity on *Botrytis*-infected grapes has not been well studied. Nevertheless, the population of acetic acid bacteria significantly increases on the botrytized grapes, similar to what happens in grey rot [16].

3.1.2.3. Effects of noble rot on juice composition

B. cinerea is a complex species that causes noble rot and gives a characteristic flavour to sweet wines [54]. Therefore, botrytized wines are recognize as a distinct category of natural dessert wines. The residual sugar content of these wines derives from the fermentation of grape juice affected by *Botrytis cinerea* under particular environmental conditions. Noble rot increases grape quality and makes it possible to produce aromatic and sweet wines. The main characteristic of botrytized wines is the extreme variety and abundance of the aroma compounds produced by *Botrytis cinerea*. The descriptors most often applied to these wines, feature the wines by peach, apricot, pear, quince, raisin, and honey flavours. They are also features by their high acid contents,

which prevent them from appearing cloying, even if the sugar content is usually more than 200 g/l. In addition, these wines may present various types marked by differences in style, depending on the grape variety, the vinification technology, and the length and method of aging [16].

3.1.2.4. Induction and control of noble rot

The production of sweet wines from botrytized grapes depends on the suitable combination of diverse factors such as climatic conditions, vine management and grape harvest. Left grapes on the vine after ripening become more susceptible to damage by animals, other rot fungi and meteorological adverse conditions. Indeed, global climatic change drives uncertainty on the regular development of the noble rot even in regions considered heretofore climatically ideal. On the other hand, the selection of noble-rotted grapes requires time and experienced manpower [49].

The inoculation of *Botrytis* spores and mycelia directly into juice was performed. However, field inoculation is closer to the natural noble-rot process and therefore, is more acceptable to consumers [16, 49].

Therefore, the artificial induction of noble rot where conditions are unfavourable for their natural development, were also performed. However, weather conditions still play a key role in the process and some experiments resulted in inferior wine quality in comparison to postharvest inoculation of grape under controlled conditions [16]. Thus, the possibility of obtaining controlled botrytization in postharvest conditions represents an option for producing sweet botrytized wines and could be recommended to standardize the quality of these wines as well [45, 49].

In later studies, harvested grapes were inoculated with spore suspensions of *B. cinerea* and stored under severely controlled environmental conditions, such as incubation at 90–100% relative humidity at 20–25ºC for 24 h, followed by longer storage under cool, dry conditions to limit the growth of the *Botrytis* and facilitate berry dehydration. Even though the method seems promising, has not been adopted due to the high costs [16, 49].

Lately, two international patents were registered an Italian enterprise which are Sordato Kinensis™ and Sordato *Botrytis* control™. While the first is an automatic drying

patented process to obtain the highest sugar concentration in a predetermined time, the second allows the development and control of the noble form of *B. cinerea* in larval form in drying rooms [55]. These technologies represent an alternative to development of noble rot and production of sweet botrytized wines from grapes cultivated in areas that are not suitable for noble rot development. Therefore, the common yield losses in traditional noble rot areas during climatically adverse seasons, can be compensated for [49].

3.1.2.5. Noble rot in Portugal

In Portugal, the wine regions can be divided in two groups: the group of regions which undergo a moderated influence of sea winds (Vinhos Verdes, Bairrada, Estremadura and Terras do Sado) and the group of regions, generally more hot, which are under the Atlantic influence (Douro, Trás-os-Montes, Dão, Beira Interior, Ribatejo, Alentejo and Algarve). Although the climate is classified as temperate, the environmental conditions are highly favorable to the development of diseases, including grey rot [56], which is particularly important in production of vine grapes [57]. Indeed, this disease may be responsible for significant losses in the amount of wine production and, ultimately can be transferred into the winery, compromising its quality [48]. For this reason, several treatments in vineyard, including chemical treatments have been applied [58] to prevent this disease.

Currently, there are many producers from Douro to Alentejo that have produced botrytized wines created from overripe grapes [59, 60]. In Quinta do Casal da Granja (Douro appellation), in Herdade do Esporão and in Herdade da Mingorra (both Alentejo appellation) have been produced late harvest wines with *Botrytis*- infected grapes, by the method of the sweet wines of Sauternes region, from Semillon grapevine variety, that reached the overripening and dehydration in vineyard [61, 62, 63]. However, other grapevine varieties have also been used to produce late harvest wines in Portugal. For instance, Muscat grapes produced in Portugal, which are typically used for fortified wines and brandy, have also been used to produce late-harvest wines [64]. The Rozès noble late harvest (Douro appellation) is produced exclusively from Malvasia Fina grapevine variety [65]. In Dão appellation, while in Quinta dos Carvalhais are used 70% Encruzado and 30% Semillon from grapevine varieties to produce late harvest wines [66] in Casa de Santar the late harvest wine is produced from 100% of Encruzado grapevine variety [67].

4. OBJECTIVES OF THE WORK

It is well known that the wine sector is affected by biotic and abiotic factors and also geographical and climatic conditions influencing the equilibrium of the grapevine microecosystem and affecting the quality of grape production [25]. One of the causes is the development of diseases such as grey rot due to infection by the fungus *B. cinerea*. In fact, the great interest in this mould is related to the fact that it is the main agent responsible for grey rot that causes great losses in the production of wine. Nevertheless, in certain regions of the world and under specific climatic conditions, this fungus causes noble rot, allowing the production of highly prized and expensive special sweet white wines.

The main objectives of this study were to query for the presence of *Botrytis cinerea* in grape samples, to determine and to compare the microorganism's quantity present in *Botrytis*-infected grapes from two types of rot, and then to analyse the microbial ecology in both rotten grapes. Therefore, the present study aims at giving a contribution to the development of scientific knowledge of the microbial nature of late harvest wines.

CHAPTER II – MATERIALS AND METHODS

This chapter describes the materials and experimental techniques used to characterize the microbial communities of *Botrytis*-infected grapes. The used methods involve molecular approaches and statistical analysis.

1. SAMPLING

The grape samples used in this study were collected on 20th of October 2014, in the Dão Appellation (Figure 9), Viseu district. The Dão region is located in the Beira Alta region, in center north Portugal. The geographical conditions are excellent for wine production: the mountains of Caramulo, Montemuro, Buçaco and Estrela protect the vineyards both from direct influence of the continental climate, and from the chill and rains from the ocean. The region is extremely mountainous, but the altitude in the south is lower. The 20,000 hectares of vineyards are located mostly between 400 and 700 meters and develop on low depth schist (in the southern area) or granite soils [68].

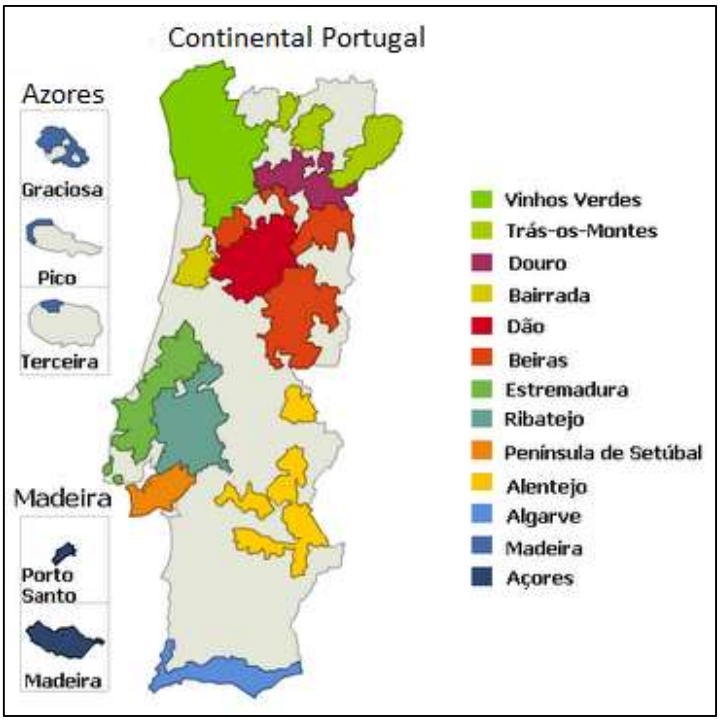

Figure 9 | Wine regions of Portugal [69].

In October, the territory was affected by a heat wave and the average of temperature was 18.95º C. In Viseu district, the average of minimum temperature was 13.7ºC and the average of maximum temperature was 21.9ºC. The first days were affected by some cloudiness and between 18th and 20th of October the passing of cold front surfaces caused weakly rainfall; during the month the total precipitation, in this region, was 194.0 mm [70].

In this study, we have collected two grapevine varieties commonly used to produce botrytized wines, which, due to confidentiality reasons, will herein be denominated Variety A and Variety B. For each grapevine variety, three representative grey and noble rotten bunches (Figure 10) visually identified by trained farmers, were randomly and aseptically collected throughout the vineyard.

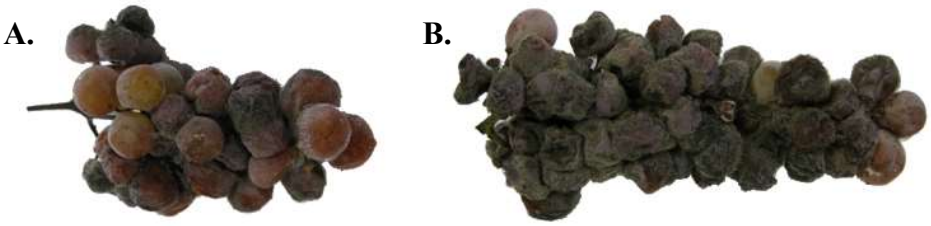

Figure 10 | Grapes infected with *Botrytis cinerea*. A) Noble rot; B) Grey rot.

In total, 12 grape samples were collected and maintained chilled until further processing. Microorganisms were isolated from grape berries and musts, through classic microbiology techniques.

2. MICROORGANISMS ISOLATION

For the isolation of microorganisms two strategies were carried out. The first consisted of plating portions of grape berries directly on two culture medium: yeast extract peptone dextrose (YEPD agar: 1% yeast extract, 2% peptone, 2% glucose, 2% agar) and Potato Dextrose Agar (PDA, 42 g/L), both supplemented biphenyl (150 mg/L) or cycloheximide (0.01%). Bacteria and yeasts were grown at 28°C for ~2 days and filamentous fungi at 28°C for 7–15 days. A total of 187 isolates were stored in duplicate at -80°C, in correspondent YEPD (1% yeast extract, 2% peptone, 2% glucose) or potato dextrose broth (PDB, 27 g/L) and glycerol 20% (for fungi) or 80% (for bacteria or yeast) until further processing by molecular analysis. In the second approach, the harvested

grapes were crushed for 20 minutes, filtered into a falcon of 50 mL and centrifuged at 4000 rpm for 10 minutes at 4ºC. The pellet was washed with 50 mL of NaCl 0.9%, vortexed and centrifuged at 4000 rpm for 10 minutes at 4ºC (the supernatant was discarded). The pellet was resuspended in 1 mL of TE 1X and 1 mL of glycerol 80% and stored at -80ºC in duplicate until further processing. For enumeration of microorganisms, serial dilutions (10-2 to 10-6) were prepared and spread in triplicate on the following culture medium: (i) YEPD agar (1% yeast extract, 2% peptone, 2% glucose, 2% agar) (Annex I), (ii) PDA (42 g/L) and (iii) WL (Wallerstein Laboratory Agar) (77 g/L), and incubated for 24/72h at 28ºC. Isolates were counted from plates with 30-300 colonies and colony-forming units (CFU/mL) were calculated using the following formula:

$$\mathbf{CFU/mL} = \frac{(\text{no. of colonies x dilution factor})}{\text{volume of culture plate x } 25}$$

Afterwards, three to six isolates, with the same morphologic characteristics were randomly selected for molecular identification, as described in the following sections.

3. MOLECULAR IDENTIFICATION

3.1. DNA Extraction

For both bacteria and yeasts, isolates with approximately 48h of growth in culture medium plates were used. The cells were removed from the plate and added to 500 µl of sterile H_2O miliQ. Pellets cells was obtained by centrifuge for 2 minutes at 10.000 rpm and the supernatant was discarded (the pellet was stored at -20ºC until DNA extraction).

3.1.1. Bacterial DNA Extraction

The bacterial DNA extraction was performed using the kit Promega Genomic DNA (Promega, USA). The pellet was resuspended with 480 µl of EDTA (50 mM) and 120 µl of lysozyme (10 mg/ml), and incubated at 37ºC for 30-60 minutes. Samples were centrifuged for 2 minutes at 13000 – 16000 x g, the supernatant was discarded and 600 µl of Nuclei Lysis solution was added to the pellet and incubated at 80°C for 5 minutes. Samples were then left to cool at room temperature and 3 µl of RNase solution was added. The solution was homogenized by inverting the tubes 2-5 times and incubated at 37°C during 60 minutes. Samples were kept at room temperature, and after cooling, 200 µl of

Protein Precipitation Solution was added and tubes were vigorously vortexed for 20s. Subsequently, samples were placed on ice for 5 minutes and centrifuged at 13000 – 16000 x g for 3 minutes, the supernatant was transferred to a new 1.5 ml tube, which already contained 600 µl of isopropanol at room temperature. This was carefully mixed by inversion and allowed to precipitate the DNA. Samples were centrifuged at 13000 – 16000 x g for 2 minutes and the supernatant was discarded. Then a wash was carried out with 600 µl of 70% ethanol and inverting gently several times and centrifuged at 13000 – 16000 x g for 2 minutes. The supernatant was discarded and the pellet was dried in the speed vacuum (DNA 120 Speedvac concentrator, USA) for 5 – 10 minutes. The DNA pellet was resuspended in 50 – 100 µl of DNA rehydration solution and incubated overnight at 4°C.

3.1.2. Yeasts DNA Extraction

For yeast DNA extraction, a laboratory implemented protocol was used [71]. The pellet cell was resuspended with 100 µl of solution I (1 M sorbitol, 0.1 EDTA-Na_2, pH 7.5), vortexed until resuspension and 5 µl of Lyticase solution (1U/µl) was added and vortexed 1s. Samples were incubated 1 hour at 37ºC (spheroblasts formation). After this, 100 µl of solution II (50 mM Tris-HCl, 20 mM EDTA-Na_2, pH 7.4) and 5 µl of sodium dodecyl sulphate – SDS 10% was added (spheroblasts lysis), vortexed and incubated at 65ºC, at least for 10 minutes. The precipitation and removal of proteins was achieved by adding 100 µl of potassium acetate and incubation for at least 5 minutes and centrifuged at 16000 rpm, at 4ºC, during 25 minutes. The supernatant (330 – 350 µl) was transferred to new tubes and centrifuged at 16000 rpm during 15 minutes. The supernatant (~ 300 µl) was transferred to new tubes already containing 300 µl of isopropanol (precipitation and concentration of nucleic acids). Tubes were carefully mixed by inversion 2 – 3 times, and incubated at least 10 minutes at room temperature, and centrifuged 5 minutes at maximum velocity. The supernatant was discarded and the DNA pellet was washed with ethanol 70%, incubated 5 minutes at room temperature and centrifuged 3 minutes at maximum velocity. The supernatant was discarded and the pellet was dried in the speed vacuum (DNA 120 Speedvac concentrator, USA) for 2 – 6 minutes. The DNA pellet was ressuspended in 50 – 60 µl of 1X TE buffer and incubated overnight at 4°C.

3.1.3. DNA Extraction of Filamentous Fungi

For filamentous fungi DNA extraction, cultures with 7-15 days of growth were used and the protocol was based on [72]. Briefly, approximately 200 µl of glass beads were previously placed in tubes and then a portion of sliced fungi mycelium was added. Then 400 µl of preheated 2x Cetyl Trimethylammonium Bromide (CTAB: 2% CTAB (p/v), 100 mM Tris-HCl (pH8), 20 mM EDTA (pH8), 1.4 M NaCl) buffer at 65ºC was added and the tubes were vortexed. To allow the mechanical breaking of fungi cells, 2 cycles of 10s in Mini Bead Beater was applied, and between cycles, samples were allowed to rest on ice for 2 minutes. Afterwards, samples were centrifuged at 5000 g for 10 minutes at 4°C and the supernatant was transferred to a new tube. This step was repeated with 300 µl of 2x CTAB buffer by macerating with a glass rod (or a plastic tip) and a cycle of 60 seconds in Mini Bead Beater. The samples were centrifuged at 5000 g for 10 minutes at 4°C and the supernatant was added to the previous tube. The samples were incubated at 65°C for 1 hour and then centrifuged at 13000 rpm for 5 minutes. The supernatant (~300 µl) was transferred to another tube and 600 µl of chloroform were added. The mixture was homogenized by inversion until a colloidal suspension is formed, and centrifuged at 13000 rpm for 5 minutes. The supernatant was carefully transferred to a new tube and 750 µl of cold isopropanol (-20ºC) were added, and the mixture was gently homogenized by inversion. Then, samples were placed at -20°C overnight to allow the precipitation of DNA. After this, samples were centrifuged at 13000 rpm for 30 minutes at 4°C and the supernatant was discarded. The pellet was washed with 200 µl of 70% ethanol (-20°C) and vortexed to release the pellet, and then the tubes were centrifuged at 7000 rpm for 5 minutes at 4ºC. The supernatant was discarded and the pellet dried using the speed vacuum (DNA 120 Speedvac concentrator, USA) for 5-10 minutes. The DNA was the ressuspended in 50-100 µl of 1X TE buffer and incubated overnight at 4°C.

3.1.4. DNA Extraction of Grape Musts

Musts DNA extraction was performed using the kit DNeasy® Plant Mini 250 (Quiagen, Germany). Approximately 200 µl of glass beads were previously placed in cryopreservation tubes and 400 µl of preheated AP1 buffer were added. Then, 200 µl of grape must and 40 µl of PVP 10% were added. To allow the mechanical breaking of cells, 2 cycles of 5 minutes in Tissue Lyser (30Hz/s) was applied, and between cycles, samples were allowed to rest on ice for 2 minutes. The tubes were centrifuged at 5000 g for 10 minutes at 4°C and the supernatant was transferred to a new tube. Glass beads were washed with 300 µl of AP1 buffer and a pulse of 10 seconds in Mini Bead Beater was applied. The samples were

centrifuged at 5000 g for 10 minutes at 4°C and the supernatant was added to the previous tube. Then, 4 µl of RNase A 100mg/ml were added and the samples were incubated at 65°C for 10 minutes. After this, 130 µl of P3 buffer were added and the mixture was vortexed and incubated for 5 minutes on ice. The samples were centrifuged at 20000 g for 5 minutes and the supernatant was transferred to another tube and centrifuged over again at 20000 g for 5 minutes. The supernatant was transferred to QIAshredder Mini spin column, previously placed on 2 mL collector tubes, and centrifuged at 20000 g for 2 minutes. The flow-through was transferred to new 2 mL tube and 1.5 of volume (~1200 µl) of AW1 buffer was added, quickly mixed and transferred to DNeasy Mini spin columns in fractions of 650 µl. After centrifugation at 6000 g for 1 minute, the flow-through was discarded. The columns were placed in new collector tubes and washed with 500 µl of AW2 buffer and centrifuged at 6000 g for 1 minute. The columns were washed once again with 500 µl of AW2 buffer, centrifuged at 20000 g for 2 minutes and transferred to new 1.5 mL tubes. Finally, 50 µl of AE buffer were added and centrifuged at 10000 g for 1 minute after 5 minutes of incubation at room temperature.

3.2. DNA Quantification

Total DNA concentration and its purity was quantified by measurement of sample absorbance at 260 nm and 280 nm with a NanoDrop ND-1000 UV-Vis spectrophotometer (ThermoScientific). Samples were stored at -20°C until further use.

3.3. Amplification: Polymerase Chain Reaction (PCR)

The internal transcribed spacer (ITS) region and the gene which encodes for the subunit 16S ribosomal RNA (Figure 11) were used to identify the microorganism. DNA concentrations were standardized to 100 ng/ µl before PCR amplification by dilution with TE buffer.

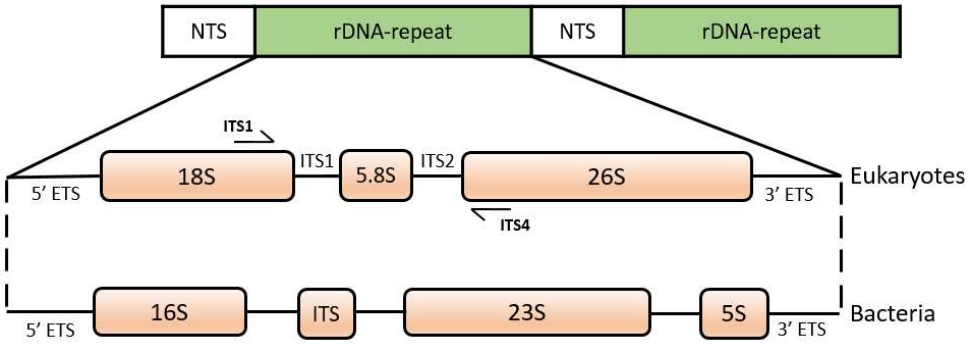

Figure 11 | Conserved organization of the pre-rRNA, adapted from Lafontaine *et al* [73].

3.3.1. ITS Region Amplification

The ITS region was amplified by PCR using the following primers: ITS1: 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4: 5'-TCCTCCGCTTATTGATATGC-3' [74].

The PCR reaction was carried out in a total volume of 25 µl containing: 2.5 µl of 10X PCR buffer Incomplete (Bioron, Germany), 2 µl of $MgCl_2$ 25 mM, 0.5 µl of dNTP's 10 mM, 1 µl of Primer ITS1 10 mM, 1 µl of Primer ITS4 10 mM, 0.5 µl of Taq polymerase 5U (Bioron, Germany), H_2O until 24 µl and 1 µl of DNA 100 ng/µl. H_2O or *S. cerevisiae* 5288C were used as negative and positive control, respectively. Dimethyl sulfoxide (DMSO) an enhancing agent, frequently included as part of a standard optimization of PCR amplifications can also be used when the ITS region not amplifies. In these cases, 2.5 µl of DMSO 100% was included in the PCR reaction (25 µl). The PCR amplification was performed in Eppendorf thermocycler (Eppendorf, USA) with an initial 6 min. denaturation at 94°C, followed by 35 cycles that consisted of 40 s at 94°C, 40 s at 53°C, and 1 minutes at 72°C, and a final 5 min. extension at 72°C [74].

3.3.2. 16S Region Amplification

The 16S rRNA gene region was amplified by PCR using the 16S_F2: 5'-AGAGTTTGATCCTGGCTCAG-3' and 16S_R2: 5'-GGTTACCTTGTTACGACTT-3' primers [75].

The PCR was carried out in a total volume of 25 µl containing: 2.5 µl of 10X PCR buffer Incomplete (Bioron, Germany), 1.7 µl of $MgCl_2$ 25 mM, 0.5 µl of dNTP's 10 mM, 0.5 µl of Primer 16S_F2 10 mM, 0.5 µl of Primer 16S_R2 10 mM, 0.5 µl of Taq

polymerase 5U (Bioron, Germany), H_2O until 24 µl and 1 µl of DNA 100 ng/µl. H_2O or *Lactobacillus curvatus* were used as negative and positive control, respectively. The PCR amplification was performed in Eppendorf thermocycler (Eppendorf, USA) with an initial 4 min. denaturation at 94°C, followed by 25 cycles that consisted of 30 s at 94°C, 30 s at 50°C, and 45 s at 72°C, and a final 5 min. extension at 72°C. Alternatively, the number of cycles was increased to 28 when the 16S region does not amplify [74].

3.3.3. Detection of *Botrytis cinerea* using specific primers

The PCR was carried out using the 21_F: 5'- CTTTGTTGCTTTGGCGAGC-3' and 22_R: 5'- GCAGAAGCACACCGAGAAC-3' primers, developed and tested by Genomic Unit Laboratory (Biocant, Portugal).

The reaction was carried out in a total volume of 15 µl containing: 1.5 µl of 10X PCR buffer Incomplete (Bioron, Germany) 1.2 µl of $MgCl_2$ 25 mM, 0.3 µl of dNTP's 10 mM, 0.6 µl of Primer 21_F 10 mM, 0.6 µl of Primer 22_R 10 mM, 0.3 µl of Taq polymerase 5U (Bioron, Germany), H_2O until 13 µl and 2 µl of DNA 100 ng/µl. H_2O was used as negative control.

The PCR amplification was performed in Eppendorf thermocycler (Eppendorf, USA) with an initial 2 min. denaturation at 94°C, followed by 25 cycles that consisted of 18 s at 94°C, 24 s at 65°C, and 1 minutes at 72°C, and a final 5 min. extension at 72°C [74].

3.4. Detection of PCR Products: Electrophoresis

The amplification profiles were separated by electrophoresis in agarose gel 1% (w/v) in TAE 1X, using Safe-Green (1.2 µl/40 ml). For each sample, 8µl of PCR product was runned with 2 µl of loading buffer. Sizes of fragments were determined by comparison with 1.5–2 µl of a standard molecular weight marker NZYDNA Ladder I (NZYTech). The electrophoresis was run at 90V for ~30 minutes. Then, gels were photographed under UV light using the Gel Doc™ Ez Imager (Bio-Rad, USA).

3.5. Purification of PCR Products

PCR products were purified using Exostar kit (GE HealthCare, USA), according to the manufacturer's instructions. This method was specifically developed to be used in PCR clean-up reaction, and involves two proprietary enzymes: Alkaline Phosphatase and Exonuclease 1 which are formulated to work together to remove unincorporated primers and dNTPs from amplification reactions [76].

In these reactions was added 0.7 µl of each enzyme to 3.5 µl of the PCR product into new 0.2 ml PCR tubes. Subsequently, the tubes were put in Eppendorf thermocycler (Eppendorf, USA) at 37ºC for 15 minutes and then at 80°C for 15 minutes. After this purification step, the fragments were sequenced.

3.6. Sequencing: Sanger Method

3.6.1. Sequencing Reaction

The sequencing was carried out in a 10 µl reaction mix containing: 2 µl of BigDye Terminator v3.1 (Applied Biosystems, USA), 2 µl 5X Sequencing Buffer, 0.5 µl of 16S_R2 Primer (for bacteria) or ITS4 Primer (for yeasts and filamentous fungi), 0.6 µl of H_2O and PCR product until 10 µl.

The sequencing reaction was performed in Eppendorf thermocycler (Eppendorf, USA) with an initial 3 min. denaturation at 96°C, followed by 25 cycles that consisted of 10 s at 96°C, 5 s at 50°C (for bacteria) or 53°C (for yeasts and filamentous fungi) and 4 minutes at 60°C.

The products were then purified with BigDye® XTerminator™ Purification Kit (Applied Biosystems, USA), according to the manufacturer's instructions, and subsequent sequencing in the Sequencer Hitachi 3500 Genetic Analyzer (Applied Biosystems, USA).

3.6.2. DNA Sequences Alignment

The DNA sequences were visualized through Chromas Lite (Technelysium Pty Ltd) software; and identification was performed by comparison with known sequences in databases. Then, the nucleotide blast (BLASTn) network service on NCBI/GenBank database (http://blast.ncbi.nlm.nih.gov) was used to find the closest match for each

sequence in order to identify the microorganisms isolated, based on the maximum percentage of the identity and query cover, and an e-value below 10^{-5} [24].

4. STATISTICAL ANALYSIS

To determine the statistical significance ($p < 0.05$) of quantitative microbial composition on the surface of grape samples, a *t*-student pairwise test was performed using SPSS Statistics 23 (IBM, US).

For the analyse of the structure of microbial communities, it was carried out a square root transformation of the microbial abundance data matrixes, to compute a Bray – Curtis resemblance matrixes. The data obtained were explored by (i) principal coordinate analysis (PCO), (ii) one-way analysis of similarities (ANOSIM) for significance difference and (iii) SIMPER to identify the taxa responsible for similarity and dissimilarities between samples within each group, and between groups. These analyses were performed using Primer E version 6 & Permanova + software [77].

CHAPTER III – RESULTS AND DISCUSSION

The objective of this work was to characterize the microbial community from grape samples displaying noble and grey rot. The grape microbiota can be influenced by several factors, including rainfall, temperature, grape variety, berry maturity, and the application of agrichemicals, which impact on their sanitary status. Indeed, microbial diversity in grapes berries is described as divergent and most probably associated with the vineyard [78]. The following diagram (Figure 12) represents the experimental strategy carried out to characterize the microbial community from *Botrytis*-infected grapes.

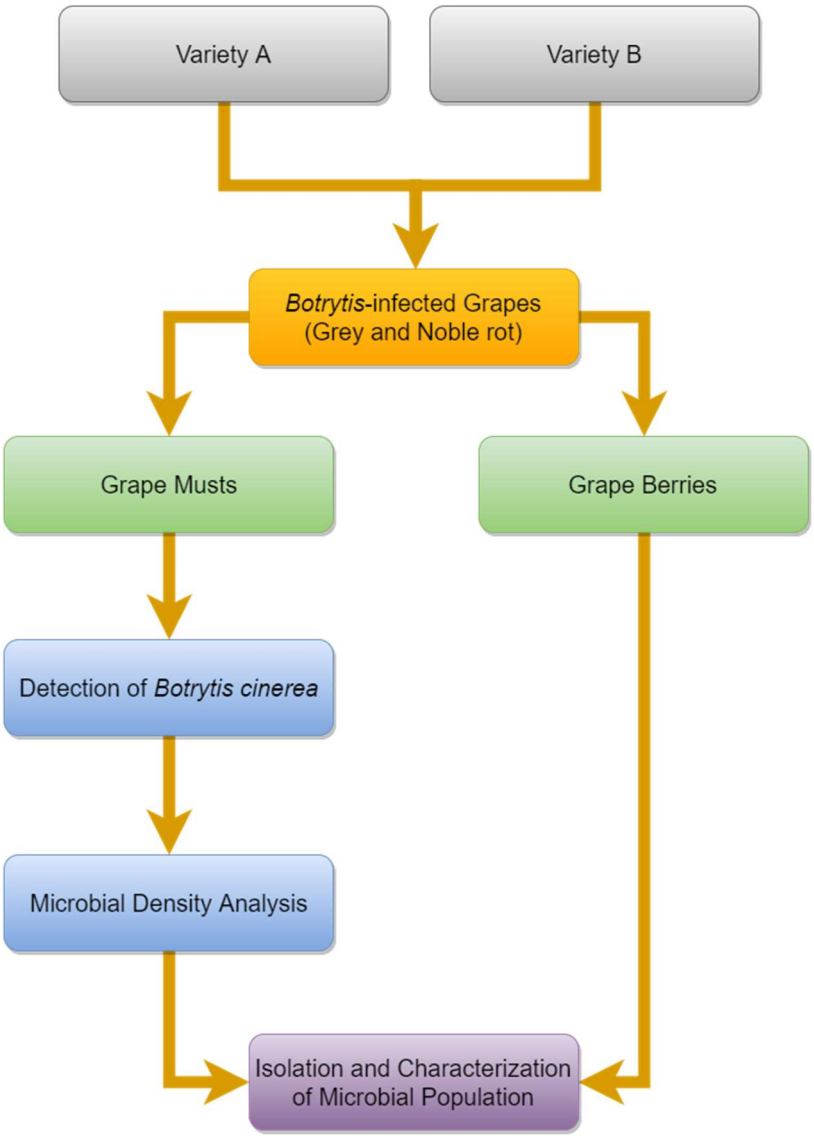

Figure 12 | Experimental strategy carried out for the molecular characterization of *Botrytis*-infected grapes. The molecular characterization was carried out for grape musts samples and grape berries.

This study was built upon three parts: 1) to query the collected grape samples for the presence of *Botrytis cinerea;* 2) to determine and to compare the microbial density of the collected samples, by means of colony-forming units (CFU's) count; and only then, 3) to characterize the microbial populations of the collected grapes, isolated both from the grape musts and from the grape skin.

1. DETECTION OF *BOTRYTIS CINEREA* ON GRAPE MUSTS

According with literature, *Botrytis cinerea* is responsible for both grey and noble rot [25]. Thus, the first step of this work was to detect its presence (Figure 13) in grape musts, by PCR amplification with specific primers.

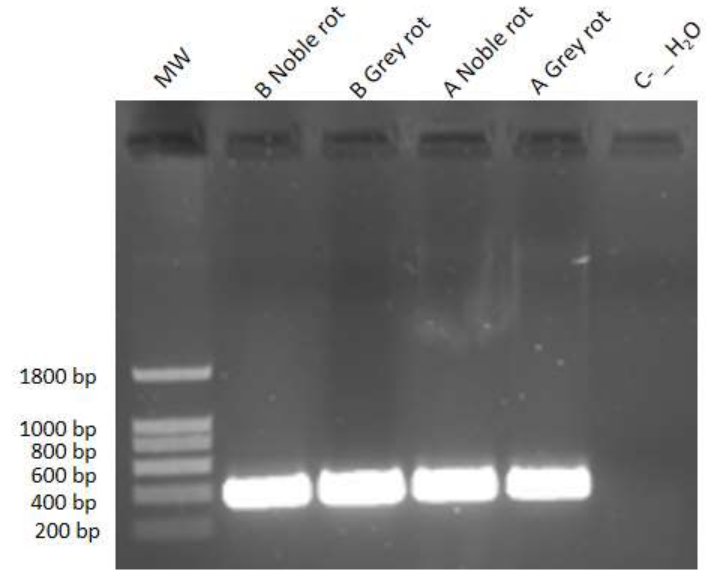

Figure 13 | Detection of *Botrytis cinerea* in grape musts samples. Electrophoretic separation of PCR products from *B. cinerea* amplification in 1% agarose gel. MW – Molecular weight.

The result shows that *Botrytis cinerea* is present in both grapevine varieties and in noble and grey rotten grapes. Indeed, this mould is responsible by the digestion and the mechanical disruption of the berry skin, enabling the growth of the other saprophytic moulds, such as *Penicillium* and *Aspergillus* [79], typically associated with grape surfaces [80] that are responsible for grape rot. Moreover, some species produce mycotoxins, which are toxic for both yeasts and human [81]. For this reason, we have also tested for their presence in this study, although these genera were not detected.

2. Microbial Density of Noble and Grey Rotten Grape Musts

In this study, one of the main goals was to assess whether or not, grapes with noble rot have different microbial population both in quantity and quality. As such, the very first addressed question was if there was any significant difference in their microbial density, between the two types of rot. Then, the quantitative composition of microorganisms in the grape musts samples was measured (Figure 14) by the colony-forming unit (CFU's).

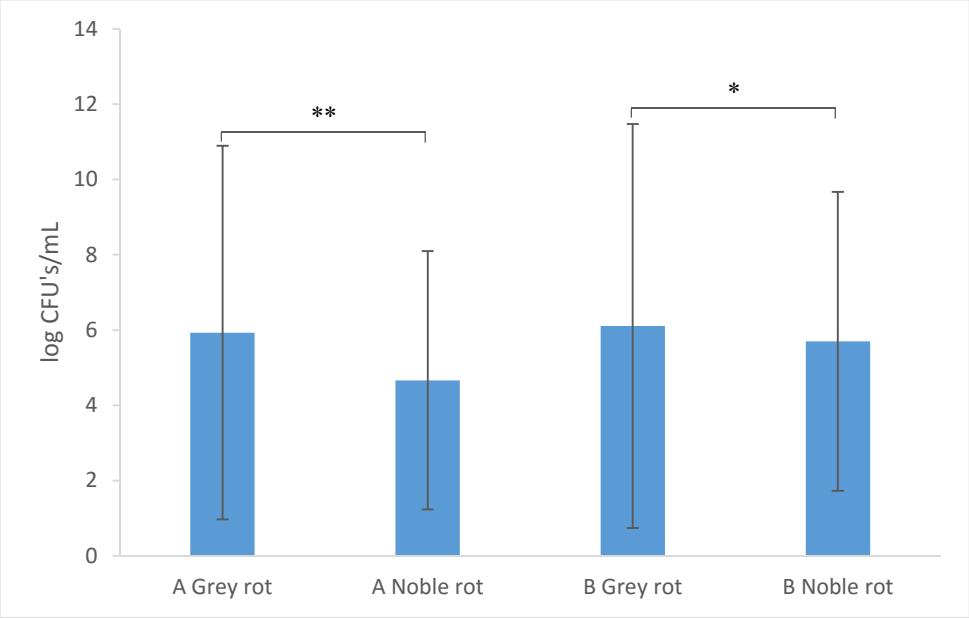

Figure 14 | Microorganisms counted in *Botrytis*-infected grapes musts. The means of log CFU's ± SD are represented in the graph. Significance was assessed with *t*-student analysis and differences between the mean values are considered significant if $p < 0.05$ (*), highly significant if $p < 0.01$ (**) and extremely significant if $P < 0.001$ (***).

These results clearly indicate that within the same variety, the quantitative differences between grey rot and noble rot were statistical significant. The differences reveal a relationship between the microbial community density and the grape health status, namely the grapes with noble rot had a lower microbial density, when compared to the grapes with grey rot. This result may be explained by the fact that damaged berry skin provides a more favourable environment to microbial growth, such as nutrient availability to the growth of the associated microbiota [79]. As the collected grapes samples were under same environmental conditions, the damages on grape skins may

have been caused by diverse factors, including the presence of bacteria, yeasts, as well as microbial vectors such as *Drosophila* spp., birds, moth, ants, wasps and nematodes [40].

3. MOLECULAR CHARACTERIZATION OF THE MICROBIAL POPULATIONS WITHIN NOBLE- AND GREY- ROT GRAPES

To fully characterize the structure of the microbial communities, it was carried out the characterization of the isolated microorganisms from both grape musts and the skin of grape berries. To achieve this, the DNA of isolated microorganisms was extracted and a PCR amplification was carried out to identify both bacterial and eukaryotic microorganisms.

3.1. Microbial diversity from grape musts samples

As above referred, to understand which microorganisms were present in grapes musts, molecular analysis methods were employed for both eukaryotic and bacterial communities.

Regarding the eukaryotic community, it was identified a higher quantity of the *Hanseniaspora uvarum* (Figure 15A) than *Metschnikowia pulcherrima* (Figure 15B) species.

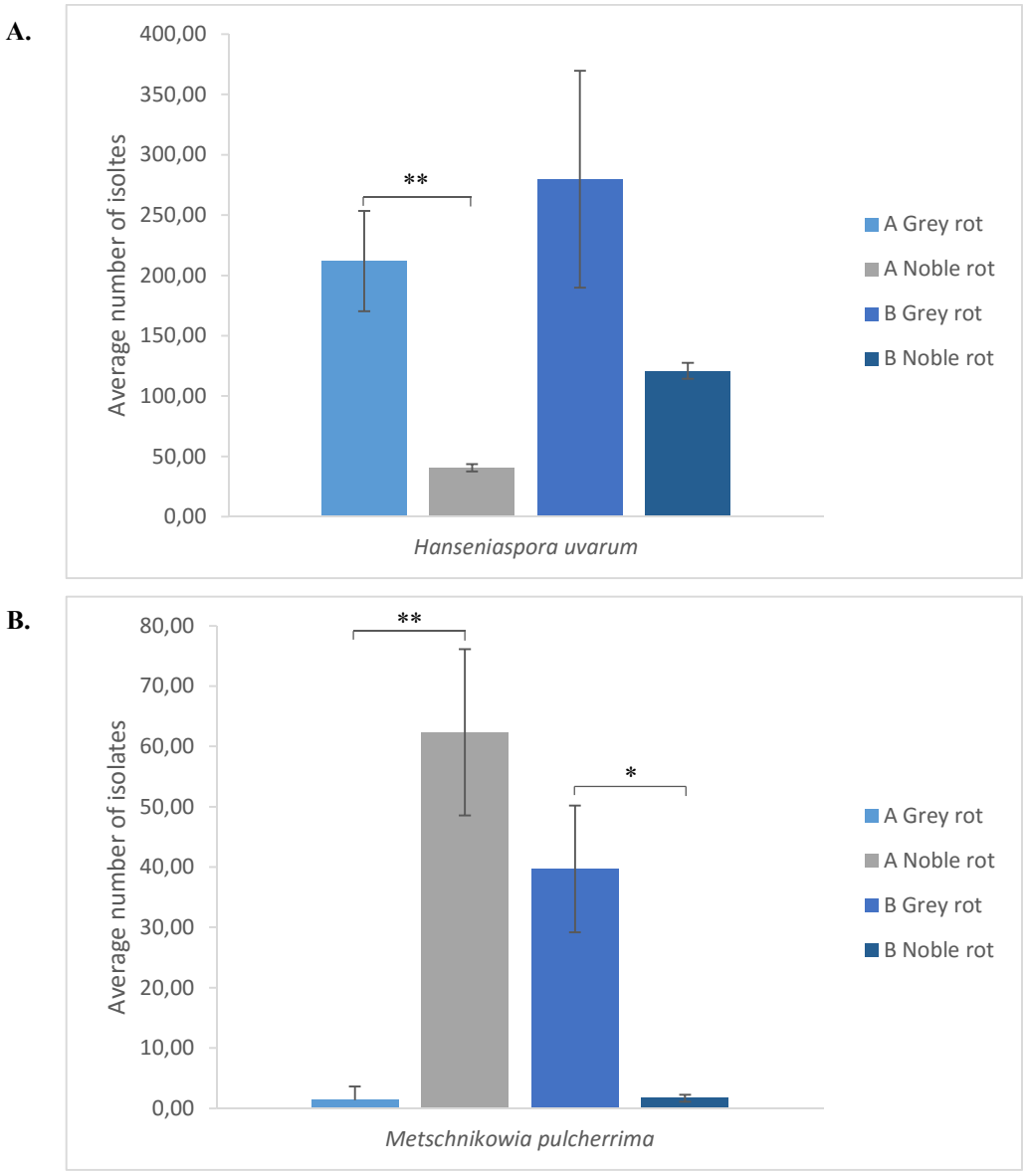

Figure 15 | Microbial diversity and abundance of eukaryotic community in *Botrytis*-infected grapes musts. Average abundance from *H. uvarum* **(A)** and *M. pulcherrima* **(B)** species. The average number of colonies ± SD are represented in the graph. Significance was assessed with *t*-student analysis and differences between the mean values are considered significant if $p < 0.05$ (*), highly significant if $p < 0.01$ (**) and extremely significant if $P < 0.001$ (***).

The comparison among eukaryotic community revealed differences ($p < 0.05$) between the *H. uvarum* and *M. pulcherrima* quantity in Variety A and between the *M. pulcherrima* quantity in Variety B. Indeed, the fermentative ascomycetous *Hanseniaspora uvarum* has been reported as a standard component of the natural colonising of pre-harvest yeast flora [21, 77]. Regarding the *M. pulcherrima* specie, it has been found at low concentrations on undamaged berries, whose incidence on damaged grapes increases at the harvesting time appearing in areas of the grape surface where some juice might leak [23, 82]. Indeed, this oxidative ascomycetous yeast commonly colonizes

grapes during maturation. However, it has also been reported that *M. pulcherrima* might positively influence the onset of noble rot and its development is more frequently on noble-rotted grapes [49].

Concerning the prokaryotic community, it was only found acetic acid bacteria from the *Glucanobacter* genus in noble rotten grape musts (Figure 16). This result highlighted the fact that the population of acetic acid bacteria is significantly increased on the botrytized grape [16]. The result is the gluconic acid formation and its presence is one of the typical indicator of *Botrytis* infection [4, 45].

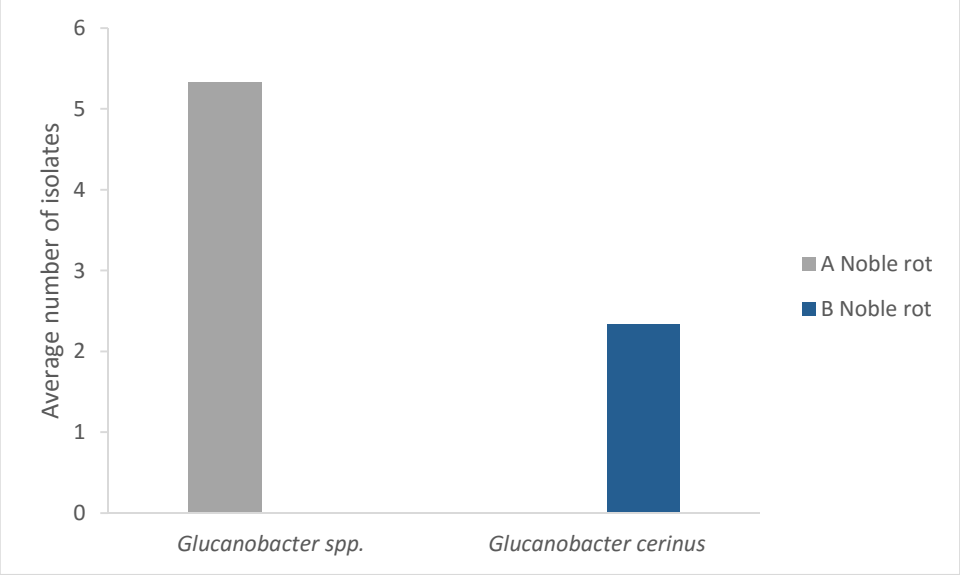

Figure 16 | Microbial diversity and abundance of prokaryotic community in *Botrytis*-infected grapes musts. The average abundance from *Glucanobacter* genus identified in musts samples is represented in the graph.

However, acetic acid bacteria also produce acetic acid and ethyl acetate. Therefore, they are probably the main source of the high concentrations of these compounds in some botrytized wines [4, 16].

3.2. Microbial diversity within grape berries

To fully understand the distribution of the microorganism according the variety and type of rot, they were also isolated from grape berries. On the Variety A and Variety B *Botrytis*-infected (noble rot and grey rot) grape samples, a total of 187 microorganisms were isolated (Figure 17). Of these, 176 formed circular colonies, which could be bacteria or yeast, while 11 were identified as filamentous fungi.

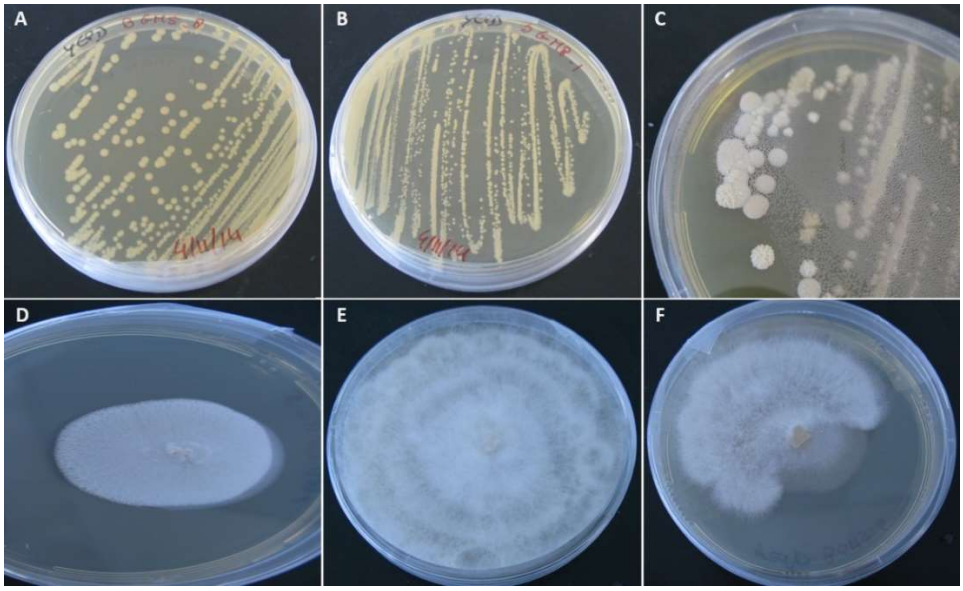

Figure 17 | Examples of isolates. A), B), C) Bacteria or Yeast; D), E), F) Filamentous fungi.

Moreover, on grapes from Variety A noble rot samples, the growth of filamentous fungi was not detected (Figure 18). Indeed, a previous study reported that fungal mycelium and conidia can be totally lacking on noble rotten grapes [16].

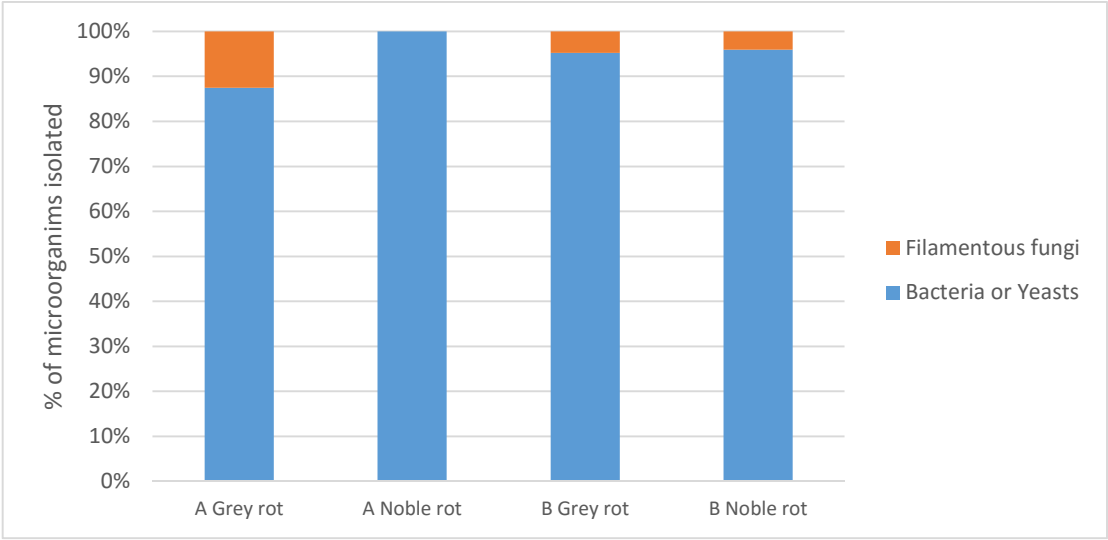

Figure 18 | Diversity of the microbial community found on *Botrytis*-infected grapes samples. Microbial community distribution in the different samples.

This observation highlighted the fact that grape berries had higher percentage of bacteria and yeasts than of filamentous fungi, which is in line with a previous study. The acidic pH as well as the high sugar content of sugar and alcohol can be the main cause that explains the strong presence of yeasts and bacteria on grapes [25].

Independently of the species, 16S rRNA gene primers amplify DNA fragments with ~1500 bp (Figure 19A). Otherwise, depending on the specie, ITS primers amplify DNA fragments with different sizes (Figure 19B).

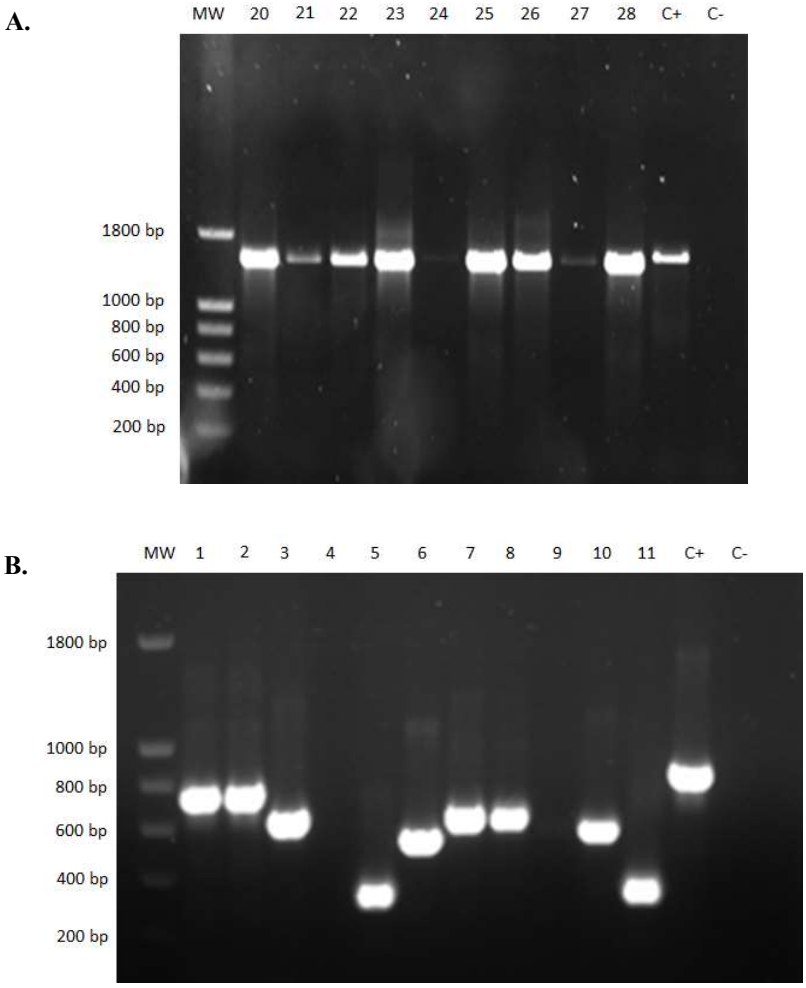

Figure 19 | PCR amplification. Electrophoretic separation of PCR products of 16S rRNA **(A)** and ITS **(B)** amplification in 1% agarose gel. MW – Molecular weight.

From 187 isolates analysed, 124 were eukaryotic (66%), 48 were prokaryotic (26%), and only 15 were not possible to identify (8%) (Figure 20) due to technical constrains.

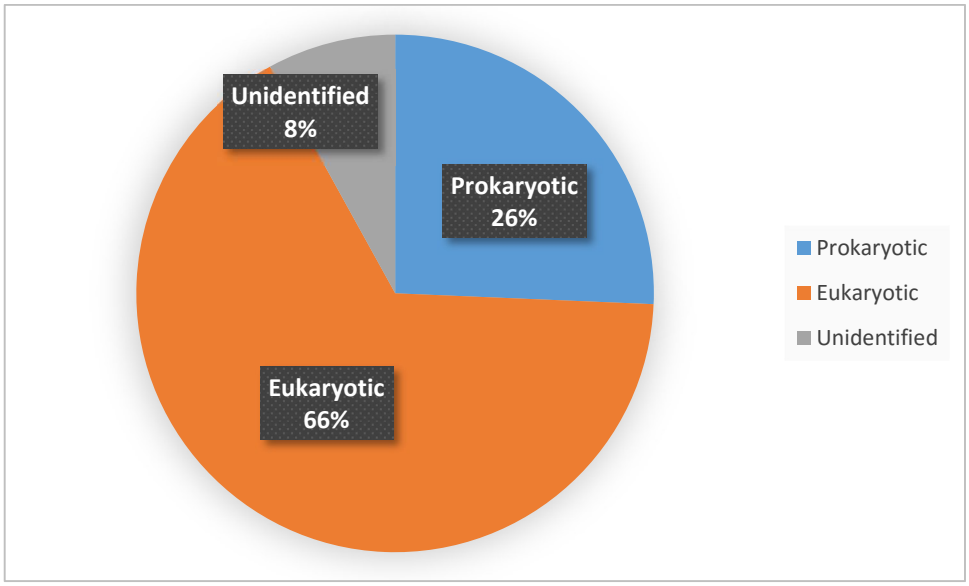

Figure 20 | Diversity of isolated microorganisms. Percentage of prokaryotic and eukaryotic communities identified.

The prokaryotic community was mostly dominated by bacteria of the *Glucanobacter* genus (Figure 21). As referred above, the presence of these bacteria is a typical indicator that grapes are infected with *Botrytis cinerea* [45]. Indeed, it was identified *G. cerinus* (9 isolates) and *G. oxydans* (2 isolates). However, *Acetobacter* spp. (3 isolates), *Gluconacetobacter saccharivorans* (3 isolates), *Gluconobacter oxydans* (2 isolates) and *Asaia* spp. (1 isolate) were also detected through this approach.

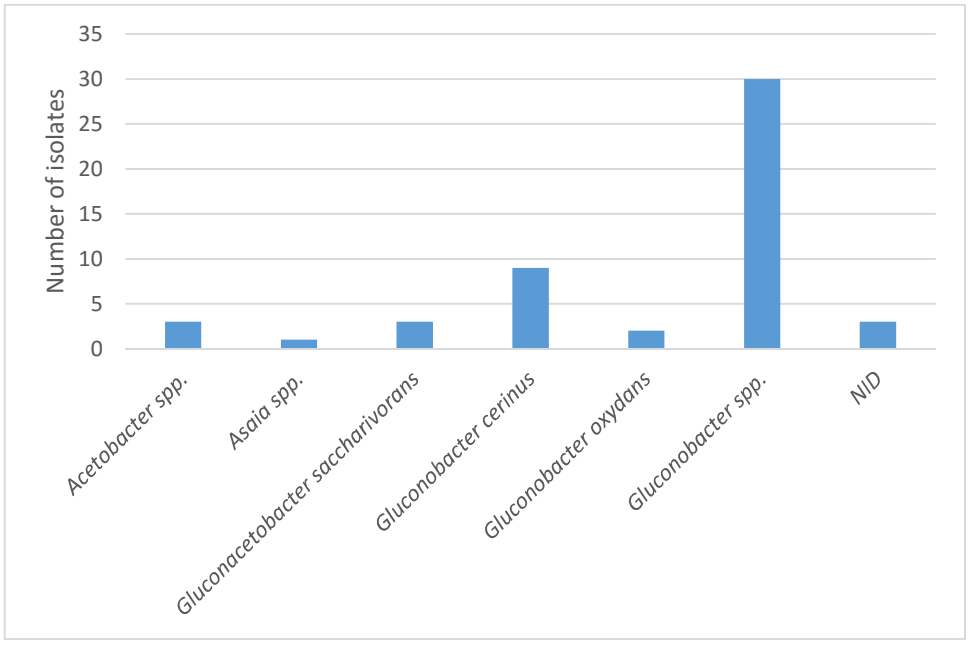

Figure 21 | Microbial diversity and abundance of the bacterial species. NID: Not identified.

Concerning the yeasts community, it was mostly dominated by microorganisms from the *Hanseniaspora uvarum* specie (54 isolates) and from the *Pichia* genus (Figure 22). The species belonging to the *Pichia* genus were *Pichia fermentans* (14 isolates), *Pichia kluyveri* (3 isolates) *Pichia membranifaciens* (4 isolates) and *Pichia* spp. (23 isolates). According to the literature, *Hanseniaspora uvarum*, an apiculate yeast, it has only been detected after *véraison* [83] and has been widely identified on *Botrytis*-infected grapes and grape musts [21]; *Pichia* spp., which are fermentative species, were mainly described to be present on mature berries [83]. The *Metschnikowia* genus, herein represented by the *Metschnikowia pulcherrima* specie, was also isolated and identified from grape samples.

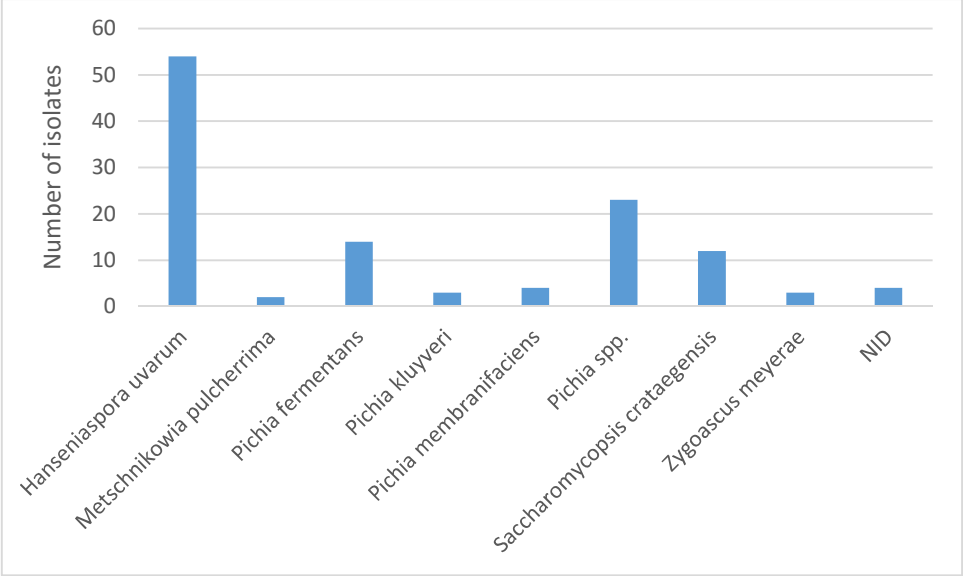

Figure 22 | Microbial diversity and abundance of the yeasts species. NID: Not identified.

These results are in accordance with previous results obtained from grape musts samples. However, through this approach it was possible isolate and identify a higher number of *Pichia* species.

Regarding the filamentous fungi herein identified, they were present at lower frequencies (Figure 23). In this study were identified *Cunninghamella* (2 isolates) and *Mucor* (2 isolates) genera. These fungi can attack the grapes, which influence their quality and the taste of the resulting wines [81].

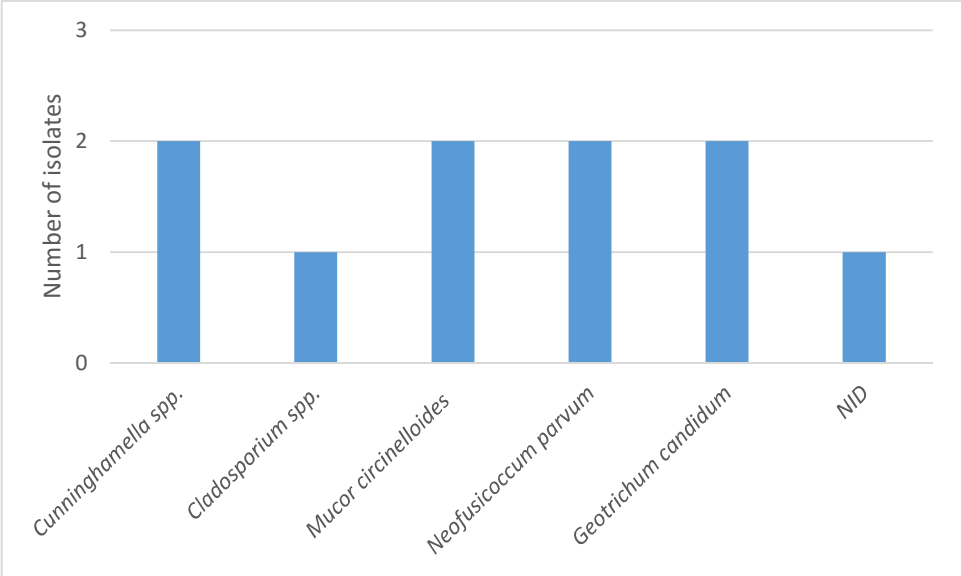

Figure 23 | Microbial diversity and abundance of the filamentous fungi species. NID: Not identified.

The *Cladosporium* genus is a ubiquitous filamentous fungus in vineyard that was also detected in this study (1 isolate). The *C. herbarum* is the relevant specie whose technological significance is *Cladosporium* rot, characterized as a post-harvest disease on fruits and crops. Although no relevant mycotoxins are produced, volatile organic compounds are accumulated attributing organoleptic defects to the affected grapes [25, 81].

The *Neofusicoccum* genus, herein represented by the *N. parvum* specie was also identified. This is a genus of fungi from the Botryosphaeriaceae family mainly associated with diseases of the woody tissues, canker and dieback, but they can also affect grape berries causing bunch rot [84, 85].

3.2.1. The structure of microbial communities of the Noble- and Grey- Rotten grapes

In order to understand the microbial population of each type of rot on grapes of each variety, the composition of microbial communities was assessed. Therefore, the microbial communities were assessed separately into bacterial (Figure 24) and eukaryotic communities (Figure 25). On Variety A grapes grey rot, it was detected the presence of *Gluconobacter* spp. (87.5%) and *G. cerinus* (7.1%). On noble rotten grapes these species were also detected, but *G. cerinus* revealed to have a greater impact on the population structure (30.8%). However, *Asaia* spp. was also identified (7.7%). This genus is

characterized by reduced production of acetic acid from ethanol and by the absence of growth when the acetic acid concentrations are higher than 0.35% (v/v) [86].

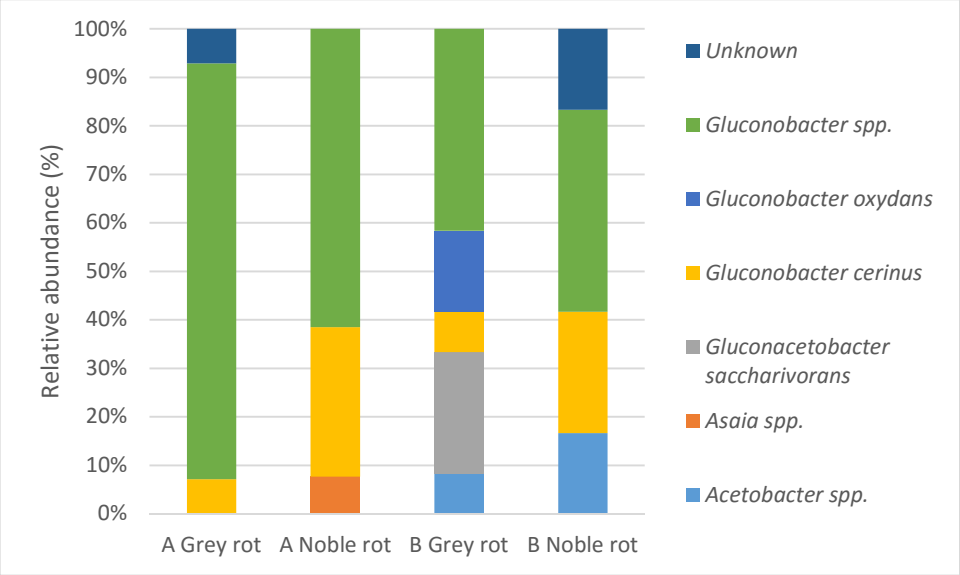

Figure 24 | Distribution of the bacteria according to their isolation source. Relative abundance of the prokaryotic community.

Regarding the Variety B grey rot, the bacterial community on grapes was richer, and the species present were *Acetobacter* spp. (8.3%), *Gluconacetobacter saccharivorans* (25%), *Gluconobacter cerinus* (8.3%), *Gluconobacter oxydans* (16.7%) and *Gluconobacter* spp. (41.7%). On surface of grapes affected by noble rot, the community is composed by *Acetobacter* spp. (16.7%), *Gluconobacter cerinus* (25%) and *Gluconobacter* spp. (41.7%).

The previous figure also shows that *Acetobacter* spp., *Gluconacetobacter saccharivorans* and *Gluconobacter oxydans* are only present on Variety B and the last one was present on grey rot grape samples. Although *Gluconobacter* genus is related with wine spoilage and vinegar production, grapes and wine are the main source of these bacteria [25]. It has also been reported that grapes are the primordial source of Acetic Acid Bacteria (AAB) and Lactic Acid Bacteria (LAB) that may influence the fermentation course and, consequently the quality of the final product [78]. However, little is known about the presence and importance of LAB on noble-rotted berries [16]. In fact, no LAB growth was detected in this study, probably due to the culture medium used, and whose detection may involve the use of a specific enrichment medium [87].

Regarding the eukaryotic community, based on their isolation source (Figure 25), the dominant specie present on Variety A grapes, was *Hanseniaspora uvarum*, representing 47.2% and 59.3% of the eukaryotic population in grey and noble rot, respectively. This finding is in accordance with the literature, in which *H. uvarum* was found on healthy and on *Botrytis*-infected grapes (noble and grey rot) [46]. This fermentative ascomycetous has been reported as a standard component of the natural colonising of pre-harvest yeast flora. However, the yeast community can be diverse due to environmental conditions, grape variety and *terroir* [21, 77]. Indeed, although the *H. uvarum* specie has had impact on the structure of grapes of Variety B (31% and 32.4% in grey and noble rot, respectively), the genus dominant was *Pichia*, representing 48.3% and 37.8% in grey and noble rot, respectively.

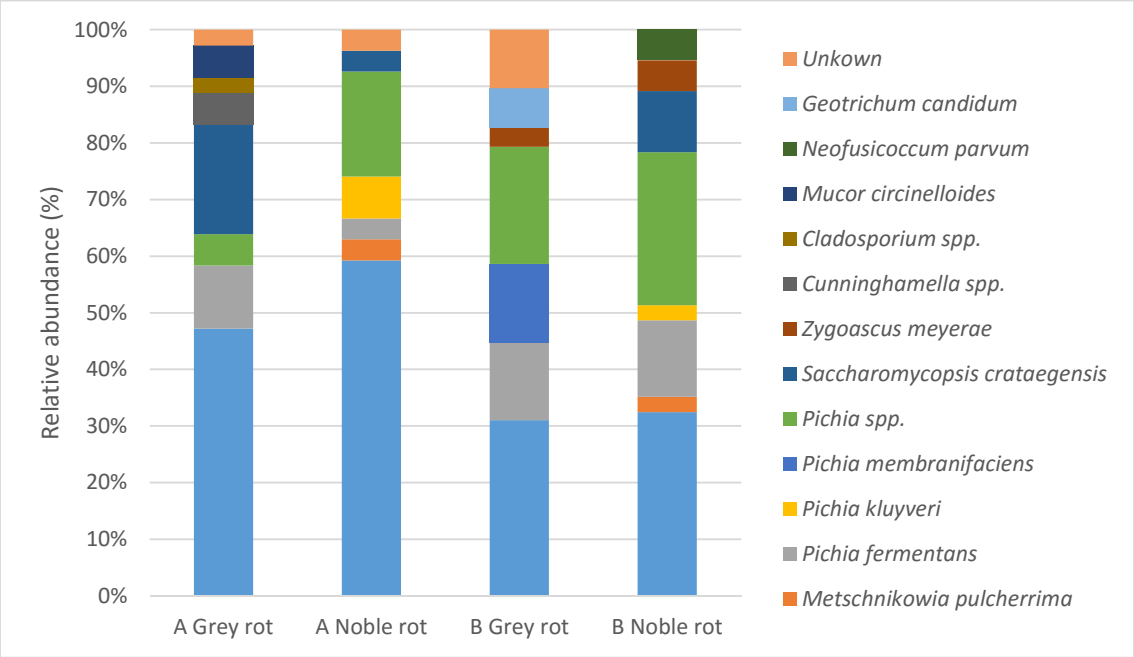

Figure 25 | Distribution of eukaryotic community according to their isolation source. Relative abundance of the eukaryotic community.

Metschnikowia pulcherrima and *Pichia kluyveri* were present on noble rot grapes. Although these yeasts may be associated with wine fermentations [77, 88], the development of ascomycetous populations on grapes can be explained by the increase of nutrients availability due to microfissures in grape skins [25]. On the other hand, the presence of *M. pulcherrima* can also be justified by their ability to survive in noble rot condition, which is characterized by water evaporation and consequent increase of the sugar concentration [89]. Although their antagonist effect has not been characterized in this study, growth of *M. pulcherrima* can strongly antagonize the growth of various

filamentous fungi, yeasts, and bacteria by depletion of the iron in the environment [89]. Therefore, as referred above, its presence might positively influence the onset of noble rot grapes [49].

The *Pichia* genus, a film-forming yeasts, in particular *P. membranifaciens* found on Variety B grey rot grapes samples. This specie is a common contaminant found in grapes, fermentation and wines and it has the ability to produce off-flavours [25].

The presence of the *Zygoascus* genus, represented by the specie *Zygoascus meyerae*, on Variety B grapes, can be explained by larger injuries of grapes skin which favours the growth of higher fermentative yeasts [25]. Nonetheless, dangerous wine spoilage yeasts (e.g. *Zygosaccharomyces* spp., *Torulaspora* spp.) were not detected in this study.

The *N. parvum* specie was isolated from Variety B noble rot grapes, probably because of their ability to survive and spread in dried conditions [90]. Nevertheless, Lorenzini *et al* [90] also suggests that this microorganism plays an important role to change the aroma of passito wines.

Overall, previous studies have shown that grapes are colonised by a wide range of yeasts without a linear correlation. According with Barata *et al* [25], the yeast communities can be grouped based on their similar physiological characteristics and, consequently in their behavior in grape berries. In fact, based on the observed results, the yeast community can be inserted in the copiothrophic[3] group, which includes weakly fermentative (*Hanseniaspora* spp.), film-forming (*Pichia* spp.) and fermentative (*Metschnikowia* spp.) yeasts. Moreover, the balance among yeast community predominantly depends on the availability of nutrients on the grape berry surface.

[3] It is an organism that tends to be found in environments which are rich in nutrients, particularly carbon.

4. COMPARISON OF MICROBIAL COMMUNITIES

The microbial community was analysed to query for possible population variations between groups of samples. Thus, to examine whether there are "proven" community differences between groups of samples identified *a priori*, a multivariate statistical analysis was carried out to compare which of these samples share particular species, at comparable abundance levels, enabling the clustering of samples into groups which are mutually similar. In this sense, cluster analysis aims to find groups of samples which are more similar to each other and the distances between samples reflects their relative dissimilarity of species composition [91]. To the abundance data matrix (Table 4), a square rot transformation was applied in order to reduce the heterogeneity of variance.

Table 4 | Raw data matrix.

	Isolated microorganism	Variety A Grey rot			Variety A Noble rot			Variety B Grey rot			Variety B Noble rot		
		AG1	AG2	AG3	AN1	AN2	AN3	BG1	BG2	BG3	BN1	BN2	BN3
Bacteria	*Acetobacter* spp.	0	0	0	0	0	0	0	1	0	0	0	2
	Asaia spp.	0	0	0	0	1	0	0	0	0	0	0	0
	Gluconacetobacter saccharivorans	0	0	0	0	0	0	0	2	1	0	0	0
	Gluconobacter cerinus	1	0	0	2	2	0	1	0	0	1	0	2
	Gluconobacter oxydans	0	0	0	0	0	0	0	0	2	0	0	0
	Gluconobacter spp.	4	0	8	2	3	3	2	1	2	1	2	2
Yeasts	*Hanseniaspora uvarum*	4	7	6	4	6	6	4	2	3	6	4	2
	Metschnikowia pulcherrima	0	0	0	0	0	1	0	0	0	1	0	0
	Pichia fermentans	4	0	0	1	0	0	2	0	2	2	0	3
	Pichia kluyveri	0	0	0	2	0	0	0	0	0	0	1	0
	Pichia membranifaciens	0	0	0	0	0	0	0	3	1	0	0	0
	Pichia spp.	1	1	0	1	0	4	4	1	1	2	2	6
	Saccharomycopsis crataegensis	7	0	0	1	0	0	0	0	0	0	0	4
	Zygoascus meyerae	0	0	0	0	0	0	0	1	0	0	0	2
Filamentous fungi	*Cunninghamella* spp.	0	2	0	0	0	0	0	0	0	0	0	0
	Cladosporium spp.	0	1	0	0	0	0	0	0	0	0	0	0
	Mucor circinelloides	0	1	1	0	0	0	0	0	0	0	0	0
	Neofusicoccum parvum	0	0	0	0	0	0	0	0	0	2	0	0
	Geotrichum candidum	0	0	0	0	0	0	2	0	0	0	0	0

Then, a Bray-Curtis resemblance matrix was computed for eukaryotic (Table 5) and prokaryotic (Table 6) communities.

Table 5 | Bray-Curtis similarity calculation: resemblance matrix for eukaryotic community.

AG1												
AG2	40.80											
AG3	36.05	65.65										
AN1	71.12	44.53	40.55									
AN2	39.62	51.52	83.05	45.13								
AN3	45.82	55.15	55.05	50.57	62.02							
BG1	60.99	43.20	38.92	60.41	43.11	65.16						
BG2	37.75	39.56	32.90	41.77	37.24	45.57	40.32					
BG3	64.83	44.76	40.30	64.57	45.61	51.57	69.25	66.34				
FN1	57.56	46.77	43.97	56.71	48.31	74.02	66.50	37.61	64.59			
BN2	49.75	52.29	50.87	73.88	58.28	69.23	60.74	50.50	57.15	56.40		
BN3	73.80	30.05	22.70	57.24	24.68	47.22	60.97	48.24	54.09	50.80	42.14	
	AG1	**AG2**	**AG3**	**AN1**	**AN2**	**AN3**	**BG1**	**BG2**	**BG3**	**BN1**	**BN2**	**BN3**

Table 6 | Bray-Curtis similarity calculation: resemblance matrix for prokaryotic community.

AG1											
AG3	68.63										
AN1	82.84	50.00									
AN2	76.46	49.67	81.11								
AN3	73.21	75.96	62.02	58.93							
BG1	89.18	53.95	92.10	73.60	68.22						
BG2	31.18	32.04	32.04	26.45	38.86	34.31					
BG3	41.42	42.49	42.49	35.47	50.87	45.31	55.23				
BN1	80.00	41.42	82.84	65.08	53.59	90.62	36.94	34.31			
BN2	64.08	66.67	66.67	50.87	89.90	73.88	41.42	53.95	58.58		
BN3	66.67	40.00	80.00	67.43	47.34	72.53	52.24	35.04	64.08	50.00	
	AG1	**AG3**	**AN1**	**AN2**	**AN3**	**BG1**	**BG2**	**BG3**	**BN1**	**BN2**	**BN3**

Therefore, to investigate whether there are differences on *Botrytis*-infected grapes microbiome, the structure of microbial communities was explored by principal coordinates analysis (PCO; Figure 26). For both eukaryotic (Figure 26A) and bacterial (Figure 26B) communities, samples were grouped according with their health status at different similarity levels, where the first axis explains 43.6% and 48.6% of the total variation, respectively.

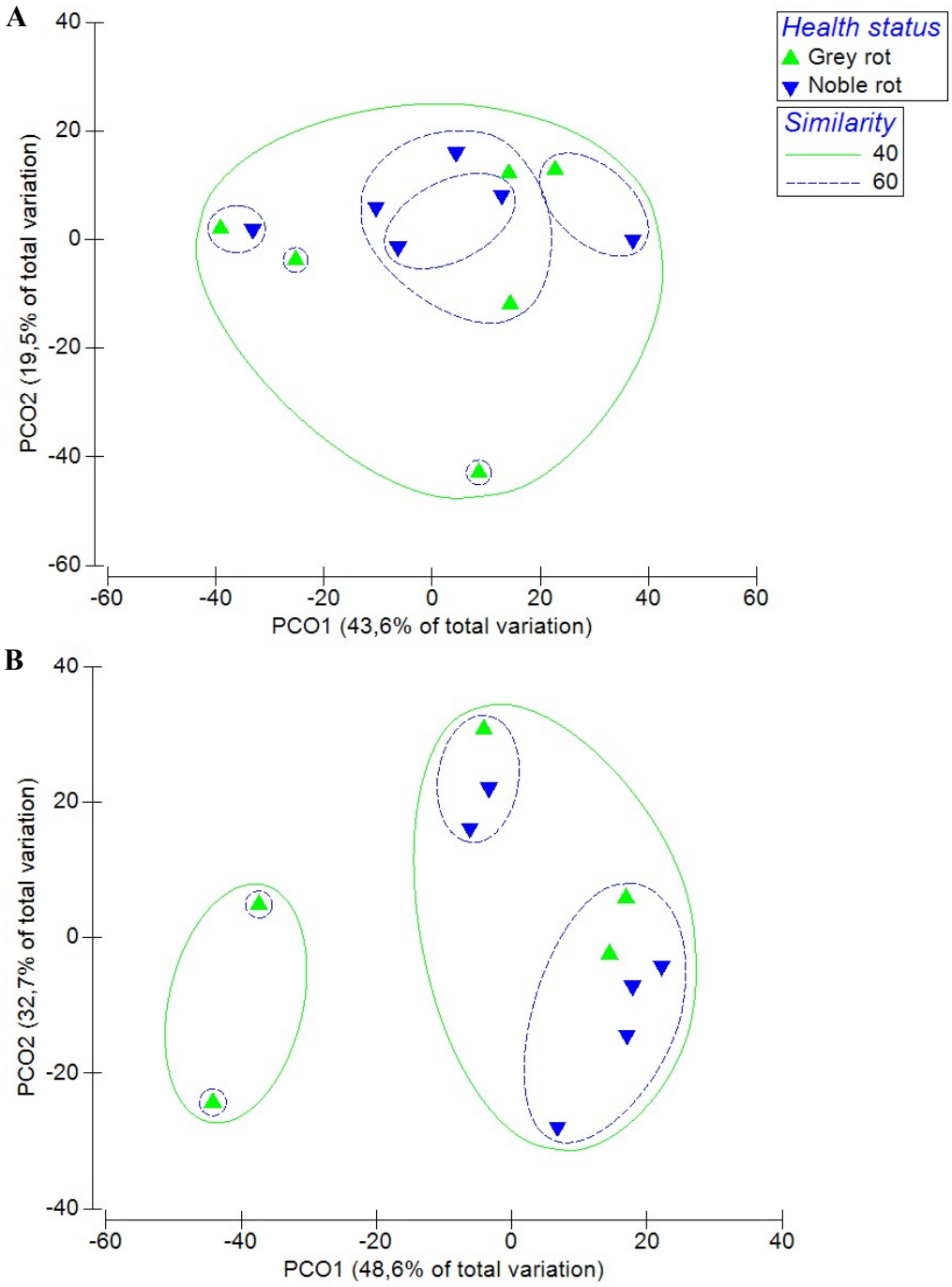

Figure 26 | Principal coordinate analyses (PCO) diagram of microbial community on *Botrytis*-infected grapes. Principal coordinates analysis based on health status of eukaryotic **(A)** and bacterial **(B)** communities.

The statistical analysis was carried out through an analysis of similarities (ANOSIM; Table 7), a nonparametric test for significant difference between two or more groups, based on any distance measured which compares the ranks of distances between groups with ranks of distances within groups. The most important information given by ANOSIM is not so much the significance level, but mainly the R statistic value, since it is an accurate measure for the dissimilarity between the defined groups. Thus, the R

statistical allow us to know how the groups are separated, and varies in a range (-1, 1) wherein: R<0 is unlikely since it would represent that similarities within groups would be smaller than those in different groups; R=0 means that similarities between and within groups will be the same on average; R=1 represents that the similarities of all replicates within groups are higher than any similarity between groups [91]. *R* values >0.75 are commonly interpreted as well separated, *R*>0.5 as separated, but overlapping, and *R*<0.25 as hardly separable [92]. Therefore, this analysis (type one-way ANOSIM) was based on 2 groups defined *a priori* based on their health status (grey rot and noble rot).

Table 7 | ANOSIM of microbial diversity based on health status.

Factor	Community	R	P
Heath status	Eukaryotic	-0.039	0.589
	Prokaryotic	0.052	0.266

The results show that were not found significant differences between grey and noble rot in terms of identified species ($R_{ANOSIM} \sim 0$; $p > 0.05$) for both eukaryotic and bacterial communities. Therefore, the results highlighted the fact that the development of noble rot does not depend on the diversity of the involved species, as referred Fournier *et al* [93]. Probably, the noble rot depends on the quantity of microorganisms, which depends on the grape skins damage and, consequently of the nutrients availability.

In order to better understand the major dissimilarities in the composition of eukaryotic (Table 8) and bacterial (Table 9) community between the different health status, a SIMPER analysis (similarity percentage breakdown) was also performed. This analysis aims to determine the individual contribution of each species to the dissimilarities found between the various groups and the similarity within each group. The SIMPER is an exploratory analysis that determines the average dissimilarity between all pairs of samples between groups and then unfold this average the isolated contribution of each species to the average dissimilarity. The statistical tests are not involved; the SIMPER analysis essentially indicates the most responsible species for grouping the samples or the difference between the groups samples defined *a priori* [91]. It was considered the 90% threshold as cutoff value, to list only the species with a greater contribution.

Table 8 | Analysis of the dissimilarity between the different health status for the eukaryotic community.

Grey rot vs Noble rot (Average dissimilarity = 48.07%)		
Species	**Contribution (%)**	**Cumulative Contribution (%)**
Pichia spp.	15.68	15.68
Pichia fermentans	14.89	30.57
Saccharomycopsis crataegensis	12.31	42.88
Pichia membranifaciens	8.93	51.81
Hanseniaspora uvarum	8.27	60.08
Pichia kluyveri	7.49	67.56
Mucor circinelloides	6.69	74.25
Zygoascus meyerae	5.78	80.03
Metschnikowia pulcherrima	5.70	85.73
Geotrichum candidum	3.97	89.70
Cunninghamella spp.	3.89	93.59

Through the comparison between grey rot and noble rot groups of eukaryotic communities, the dissimilarity value obtained was 48.07%. Microorganisms belonging to the *Pichia* spp., *Saccharomycopsis crataegensis* and *Hanseniaspora uvarum* were those that mostly contributed for the dissimilarity of groups, which contributed with 67.56% for the dissimilarity between groups.

Table 9 | Analysis of the dissimilarity between the different health status for the bacterial community.

Grey rot & Noble rot (Average dissimilarity = 42.73%)		
Species	**Contribution (%)**	**Cumulative Contribution (%)**
Gluconobacter cerinus	28.94	28.94
Gluconobacter spp.	23.40	52.34
Gluconacetobacter saccharivorans	18.48	70.82
Acetobacter spp.	13.38	84.20
Gluconobacter oxydans	10.39	94.59

The dissimilarity value obtained for bacterial community, between grey rot and noble rot groups, was 42.73%, where the genus *Glucanobacter*, including the *G. cerinus* and *G. oxydans* contributed with 62.73% for the dissimilarity between groups.

Regarding the average of similarity within each group, it was higher on noble rot than grey rot, for eukaryotic (noble rot: 54.44%; grey rot: 48.11%; Table 10) community.

Table 10 | Analysis of the similaritiy within each group for eukaryotic community.

	Noble rot Average similarity: 54.44%		Grey rot Average similarity: 48.11%	
Species	**Contribution (%)**	**Cumulative Contribution (%)**	**Contribution (%)**	**Cumulative Contribution (%)**
Hanseniaspora uvarum	64.19	64.19	63.63	63.63
Pichia spp.	24.70	88.89	21.99	85.62
Pichia fermentans	5.40	94.29	9.05	94.67

Microorganisms belonging to the genus *Pichia,* including the *P. fermantans*, and the *H. uvarum* are the main responsible for the similarity for both noble and grey rot (Figure 27). In fact, this was expectable, because these microorganisms represent the main structure of eukaryotic population of rotten grapes in this study.

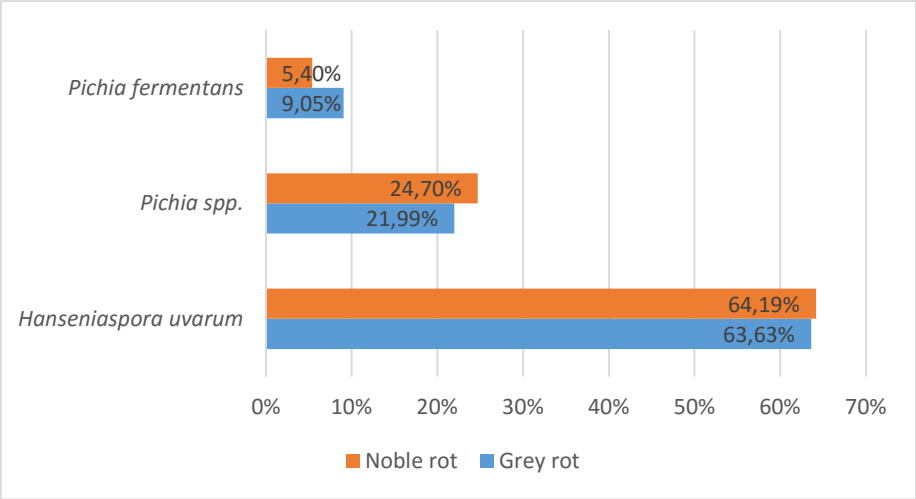

Figure 27 | SIMPER analysis for the eukaryotic community. The main species responsible for the similarities within each group (Grey rot or Noble rot).

For bacterial community, the average of similarity within each group, it was also higher on noble rot than grey rot (noble rot: 65.23%; grey rot: 49.37%; Table 11).

Table 11 | Analysis of the similaritiy within each group for prokaryotic community.

	Group Noble rot Average similarity: 65.23%		Group Grey rot Average similarity: 49.37%	
Species	**Contribution (%)**	**Cumulative Contribution (%)**	**Contribution (%)**	**Cumulative Contribution (%)**
Gluconobacter spp.	77.49	77.49	86.93	86.93
Gluconobacter cerinus	22.51	100.00	7.48	94.41

These results also revealed the same behaviour, where the microorganism responsible for the similarity within each groups are the same for both noble and grey rotten grapes, namely the *Glucanobacter* genus, including *Gluconobacter cerinus* specie (Figure 28). This is also expectable, since these microorganisms have a great impact on the structure of bacterial community. Indeed, these results suggest that noble rot development tends to be more similar and less diverse than grey rot development.

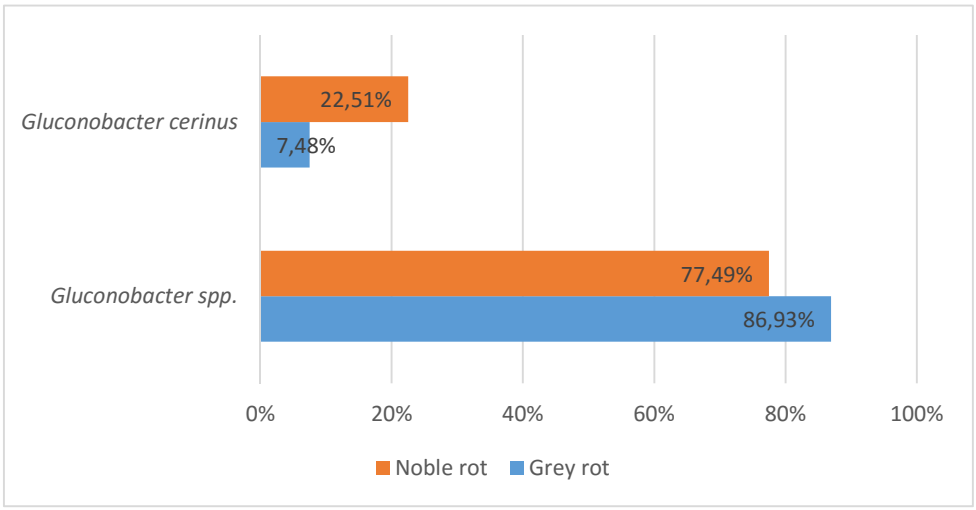

Figure 28 | SIMPER analysis for the prokaryotic community. The main species responsible for the similarities within each group (Grey rot or Noble rot).

The collected data contribute to characterization of the biodiversity of two grapevines varieties from Dão Appellation displaying grey and noble rot symptoms. Altogether, this work reinforces that grey and noble rot symptoms on grapevine are both caused by *B. cinerea* and there is no differentiation between microbial consortia diversity.

Despite the unveiled biodiversity from grapes displaying grey and noble rot symptoms, there is a large gap in the knowledge of the functional diversity and

significance of microbial community interactions. Therefore, this study highlighted the need of a more detailed research in the microbial interaction on grapes, once there is differences on microbial density. In fact, the co-existence of different microbial population generates competition for the nutrients and produces enzymatic compounds, which could have a toxic effect on other species [24].

CHAPTER IV – CONCLUSION AND FUTURE PERSPECTIVES

1. CONCLUSION

The objective of this work was to acquire knowledge on the microbial population on *Botrytis*-infected grapes, either involved in grey or noble rot. Although *Botrytis cinerea* is the main responsible for the two types of rot, the direction of disease is supported by the special climatic conditions, soil characteristics, grape varieties and viticulture practices [79]. This study was addressed to understand whether there were or not differences in microbial community between grey and noble rot. Such understanding could contribute for the development of environmental friendly strategies for noble rot grapes production.

Although grapes are the main source of bacteria involved in wine production, the range of bacterial diversity on botrytized has been poorly addressed [78]. In this study, the prokaryotic community was characterized by the presence of the *Glucanobacter* and the *Acetobacter* genera. Indeed, the presence of these acetic acid bacteria produce gluconic acid, which is the chemical indicator of infection by *Botrytis cinerea*, and they also responsible by the changes in the volatile acidity in wines [18].

Regarding the eukaryotic community, it was found the predominance of fermentative yeasts such as *Hanseniaspora uvarum*, *Metschnikowia pulcherrima* and *Pichia* spp. Although it was not found differences between the two types of rot concerning the species diversity, it was found differences ($p < 0.05$) between grey and noble rot at microbial population density level. This difference was notable by the *H. uvarum* and *M. pulcherrima* species. Since that the collected grapes samples were exposure under same environmental conditions, these differences could be due to damages on grape skins, which may have been caused by vectors associated with the grapevine [40].

2. FUTURE PERSPECTIVES

This study should be further continued and new lines of research should be developed in order to contribute to a more sustainable wine sector, both in terms of late

harvest wines production and in reduction of pesticide used to control development of grey rot.

Wine production, quantity and quality, depends on the climatic conditions of each year. For instance, in 2014 wine production was 7% lower than 2013 due to weather conditions [7]. Furthermore, the direction of this disease depends largely on climatic conditions. Thus, I consider that this study should be made comparison with grapes harvested in different years, as did Bene and Magyar [79] to confirm whether high quality vintages provide low yeast populations with low mould conidia count.

Regarding to the methodology, isolate and cultivate microorganisms in pure culture is typically the first step to investigate them, but in many environments uncultured microorganisms comprise about 99% of the microbial population. Therefore, culture-independent methods have emerged as a powerful tool to answer fundamental questions in microbial ecology, namely to understand the genetic diversity, population structure and ecological roles of the majority of microorganisms. Indeed, metagenomics or the culture-independent genomic analysis can be used to analyse microbial communities through genetic material recovered directly from environmental samples, providing significant information about microbial community from a mixed population of microbes [94, 95]. Indeed, the next generation sequencing (NGS) technologies has revolutionized the field of genomics, enabling fast and cost-effective generation of genome sequence data [95, 96, 97]. Moreover, further molecular approaches such as metaproteomics, metatranscriptomics, and proteogenomics are required to explore the vast microbial diversity and to understand their interactions with biotic and abiotic environmental factors [98].

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