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Evaluation of Antimicrobial Activity of Indigenous Wine Yeasts

Master in molecular and Microbial Biology

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Authorship Statement

I hereby declare to be the author of this work, which is original and unpublished. Authors and papers consulted are duly cited in the text and are listed in the included references.

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Abstract

Killer yeasts represent a very interesting part of microbiology, they are yeasts that can produce toxins that kill sensitive strains. They have various mechanisms of action such as: damaging the cell wall, permeabilising the cell membrane, disrupting the cell cycle and fragmenting RNA.

This phenotype was first described in Saccharomyces cerevisiae, but various studies have shown that it extends to several other yeast species, and they can be present in various ecosystems such as soil, plants, animals or vineyards.

Since *killer* yeasts are present in wine, indigenous yeasts from must have been studied for their antimicrobial capabilities, which could have various applications in terms of biocontrol, potential substitutes for commonly used antimicrobial agents.

Moreover, in the winemaking, these yeasts could be substitutes for sulphites, which are commonly used to inhibit spoilage yeasts like *Pichia guilliermondii* that can negatively affect the wine's aroma, flavor, and mouthfeel.

This study focused on screening and identifying *killer* yeasts from wine must isolates. *Mestchnikowia pulcherrima*, a yeast with a *killer* phenotype, stood out among the isolates under study. It exhibits a *killer* phenotype not through conventional toxins but via a pigment known as pulcherrimin, which targets strains reliant on iron for survival.

It was possible to identify two proteins that are involved in the *killer* phenotype, the Ski3 and Ski8 proteins., that are part of a protein complex that promotes the degradation of mRNA, thus interfering with the cell cycle of sensitive microorganisms.

The results of this study highlight the potential of *killer* yeasts as new, natural antimicrobial agents that can be used to improve wine fermentation and preservation as well as protect wine from spoilage organisms. Furthermore, this research highlights their potential to treat pathogenic microorganisms, resistant to antimicrobial agents, suggesting a more natural, healthy, and sustainable strategy in the context of basic medicine.

Keywords: *Killer* yeasts; *Killer* toxins; *Saccharomyces cerevisiae; Pichia guilliermondii; Mestchnikowia pulcherrima;* Super*killer* proteins.

Resumo

As leveduras *killer* são uma parte muito cativante da microbiologia, estas leveduras têm a capacidade de produzir toxinas que têm como competências inibir ou matar estirpes de microrganismos sensíveis, não demonstrando citotoxicidade para as células animais.

As toxinas *killer* apresentam vários mecanismos de ação, tais como: a) danificar a parede celular através da hidrólise de glucanos da parede celular ou inibição de β-1,3-glucano sintase; b) permeabilizar a membrana celular resultando na libertação de K+, H+, ATP, e outros metabolitos; c) perturbar o ciclo celular bloqueando a progressão do ciclo celular na fase G1/S ou a conclusão da fase G1; d) fragmentar o RNA em termos das subunidades 18S e 25S rRNA, ou tRNA.

O fenótipo *killer* foi descrito pela primeira vez em *Saccharomyces cerevisiae* onde foram identificadas as toxinas *killer* K1, K2 e K28, posteriormente muitos estudos mostraram que este fenótipo se estende a várias outras espécies de leveduras tais como *Candida, Cryptococcus, Debaryomyces, Kluyveromyces, Hanseniaspora, Pichia, Torulaspora, Ustilago, Williopsis e Metschnikowia*. Assim, essas leveduras podem ajudar a manter o equilíbrio em vários ecossistemas, como no solo, nas plantas, nos animais ou na vinha.

Uma vez que as leveduras *killer* também estão presentes no vinho, as leveduras indígenas têm vindo a ser estudadas quanto às suas aplicações, como potenciais substitutos de agentes antimicrobianos ou biocontrolo natural.

Estas leveduras apresentam potencial para substituir os agentes antimicrobianos sintéticos, e por isso podem ser utilizadas de forma a reduzir a resistência, que é um dos maiores problemas ao nível da saúde mundial.

O biocontrolo natural está ligado com a inibição de crescimento de microrganismos deteriorantes em comida ou bebidas, tais como o vinho, de forma a reduzir o uso de conservantes químicos.

Na indústria do vinho microrganismos como leveduras, fungos filamentosos e bactérias constituem um papel fundamental pois influenciam a saúde da vinha, na fermentação, sabor, cheiro e na qualidade final do vinho. Assim de forma a obter vinhos de alta qualidade tem sido cada vez mais proposta a utilização de leveduras indígenas, na substituição dos sulfitos.

Os sulfitos são utilizados no tratamento do vinho para evitar a proliferação de leveduras de deterioração presentes no vinho, como a *Pichia guilliermondii*, que alteram as caraterísticas do vinho, assim as leveduras *killer* podem ser usadas para substituir estes conservantes químicos.

A *P. guilliermondii* é uma levedura sensível a toxinas *killer*, frequentemente identificada como uma levedura de deterioração no contexto vinícola, notória por comprometer a qualidade do vinho. Esta levedura pode introduzir vários problemas durante a fermentação e o armazenamento do vinho, tais como gerar aromas estranhos e a alterar a cor e o sabor do vinho.

Neste trabalho foram utilizadas leveduras isoladas, em estudos anteriores, a partir do mosto do vinho. Assim este estudo incidiu sobre o rastreio e na identificação de leveduras *killer* entre vários isolados de leveduras do mosto de vinho.

Com esse objetivo foi necessário selecionar os isolados utilizando o meio YMA-MB, visto que este meio contém azul de metileno que penetra para o interior das células mortas, sendo possível a visualização de halos de inibição quando existe atividade *killer.* Apenas nove entre os sessenta isolados analisados apresentaram o fenótipo *killer*, sendo ainda possível separar os isolados em dois grupos: aqueles que provocaram halo de inibição e aqueles que provocaram halo de células mortas. Como controlo positivo foi utilizada a levedura *Saccharomyces cerevisiae* PYCC 4620, uma vez que esta levedura apresenta potencial *killer* contra a levedura *Pichia guilliermondii.*

Depois da seleção dos isolados com perfil *killer,* as proteínas foram extraídas para análise por SDS-PAGE. A extração foi realizada através de um método mecânico que permitiu a separação das proteínas responsáveis pelo fenótipo *killer* dos restantes componentes celulares. Assim foi possível verificar que entre os isolados, as toxinas *killer* encontram-se em diferentes localizações celulares: no citoplasma, em que as leveduras apresentavam o fenótipo *killer* quando se testava o sobrenadante resultante do processo de extração proteico e no núcleo, em que este fenótipo se encontrava apenas quando se testava o extrato celular. Após a avaliação da atividade *killer* procedeu-se à identificação das leveduras *killer* por sequenciação do gene *18S rRNA*. As leveduras com atividade *killer* no extrato celular foram identificadas como *Mestchnikowia pulcherrima*.

Esta levedura destacou-se entre os isolados por apresentar os halos de inibição mais significativos. A levedura *Mestchnikowia pulcherrima* é considerada uma levedura com atividade antimicrobiana que não possui as toxinas *killer*, mas sim um pigmento de coloração vermelha que lhe confere esse fenótipo, o pulcherrimina.

O pigmento pulcherrimina é responsável pela precipitação do ferro, tornando-o indisponível no meio, assim os microrganismos que dependem de ferro acabam por não sobreviver.

Após a identificação dos isolados foi realizada a técnica de SDS-PAGE com o objetivo de separar as proteínas obtidas no extrato celular e realizar a técnica de zimografia. A zimografia permitiu a visualização do efeito *killer* em algumas frações proteicas. As frações proteicas que provocaram a inibição de crescimento de *P. guilliermondii* foram selecionadas e identificadas

por MALDI-TOF/TOF. Os resultados foram inesperados, já que as frações correspondiam as enzimas fosfotransferase e transaldolase, que não têm qualquer ligação com a atividade *killer*, dado que a fosfotransferase é uma enzima responsável pela catalisação da transferência do grupo fosfato para um substrato específico, e, por outro lado, a enzima transaldolase está integrada na via metabólica das pentoses fosfato, responsável pela biossíntese de nucleótidos e ácidos nucleicos. Deste modo foi realizada a análise do proteoma completo, em que foi possível identificar duas proteínas que estão envolvidas no fenótipo *killer,* as proteínas Ski3 e Ski8. Estas proteínas *superkiller* fazem parte de um complexo proteico constituído por as proteínas Ski2, Ski3 e Ski8. O complexo Ski é responsável pela degradação do mRNA, interferindo assim no ciclo celular de microrganismos sensíveis. No entanto, estão reportadas evidências que mesmo na ausência de Ski2, o complexo formado pelas proteínas Ski3 e Ski8 é capaz de provocar o mesmo efeito.

Na análise do proteoma não foram encontradas as proteínas responsáveis pela produção do pigmento pulcherrimina, o que pode significar que estas proteínas podem estar codificadas no citoplasma e assim estariam no sobrenadante e não no extrato celular.

Uma vez que o sobrenadante da levedura *Mestchnikowia pulcherrima* não inibiu a levedura alvo *Pichia guilliermondii,* é possível que não haja a produção do pigmento pulcherrimina nas condições testadas e que apenas foi possível observar a ação das toxinas *superkiller.*

Os resultados deste estudo realçam o potencial das leveduras *killer* como novos agentes antimicrobianos naturais que podem ser utilizados para melhorar a fermentação e a preservação do vinho, bem como para o proteger de organismos de deterioração. Além disso, esta investigação destaca o seu potencial para tratar microrganismos patogénicos, resistentes a agentes antimicrobianos, sugerindo uma estratégia mais natural, saudável e sustentável no contexto da medicina básica.

Palavras-chave: Leveduras *Killer*; Toxinas *Killer*; *Saccharomyces cerevisiae; Pichia guilliermondii; Mestchnikowia pulcherrima;* Proteínas Super*killer*.

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Abbreviations

BSA: Bovine Serum Albumine

C+: Positive Control

CBB: Comassie Brilliant Blue

ITS: Internal Transcribed Spacer

K1: *Killer* Toxin 1

K2: *Killer* Toxin 2

K28: *Killer* Toxin 28

KHR: *Killer* Toxin Heat Resistance

KHS: *Killer* Toxin Heat Susceptible

Klus: *Killer* Toxin encoded in Mlus dsRNA

KTP: *Killer* Toxin Production

L: Ladder

LC: Liquid Chromatography

MALDI-TOF/TOF: Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight/Time-of-Flight

MS: Mass Spectrometry

PCR: Polymerase Chain Reaction

PYCC: Portuguese Yeast Culture Collection

SDS-PAGE: Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis

SKi: Super*Killer*

WHO: World Health Organization

WLN: Wallerstein Laboratory Nutrient

YM: Yeast Malt

YMA: Yeast Malt Agar

YMA-MB: Yeast Malt Agar with Methylene Blue

YMB: Yeast Malt Broth

1. Introduction

1.1.Fungi Kingdom

The Fungi kingdom contains a vast number of species, with approximately 144,000 species known to date. In addition, this number is tending to increase, as around 2,000 new species are found and identified every year (Tullio, 2022).

However, it is important to emphasise that the definition of "being a fungus" is constantly being updated, as new discoveries often make it necessary to re-evaluate this concept (Blackwell, 2011). As we learn more about fungi, we realise that this kingdom is the basis of all life on Earth (Tullio, 2022), since fungi are ubiquitous organisms that can thrive in a variety of natural environments, including soil, plants, animals and even the human body (R. Brown *et al.*, 2021).

The characteristics of fungi and their ecology, including associations with other organisms, have allowed them to develop a fundamental role as agents of ecosystems and evolutionary processes (Bahram & Netherway, 2022), so we can consider that fungi are not only essential for decomposing matter and recycling nutrients (Tullio, 2022), but that these organisms are unique in terms of their morphological and ecophysiological properties, as they act as mutualists, commensalists and antagonists in interaction with plants, animals, bacteria or even other fungi (Figure 1.1), mediating the health, performance, population dynamics and biogeography of these organisms (Bahram & Netherway, 2022).

Although fungi are often invisible to the human eye, their varied ecological activities have definite and notable impacts on the functioning of ecosystems and global societies (Horianopoulos *et al.*, 2021).

Currently, fungi are used in the food and pharmaceutical industry, mainly in the production of bread and beer, through yeasts, cheeses, through filamentous fungi, antibiotics, enzymes, and organic acids. This potential, as well as the ease of management, cultivation, reproduction, and safety, has led to an increase in studies into yeasts to evaluate more natural and sustainable applications (Tullio, 2022).

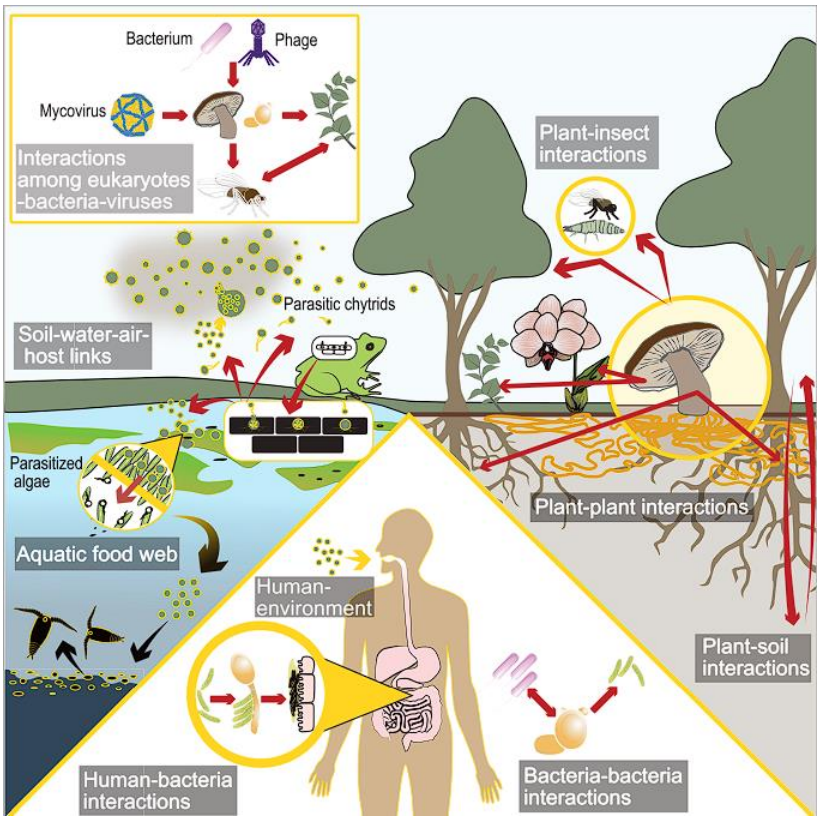


Figure 1.1 Illustration of the mediation of fungi in different ecosystems. The organisms of this kingdom are capable of functioning as mutualists, commensalists and antagonists in contact with plants, animals, bacteria and other fungi (Bahram & Netherway, 2022).

1.2. Yeasts

The word yeast is based on words meaning "foam" and "to rise", direct references to the fermentation processes involved in many applied fields, such as brewing, baking, wine making and distilling (Boekhout *et al.*, 2022; Kurtzman *et al.*, 2011).

Yeasts can be defined as those fungi whose asexual growth predominantly results from budding or fission (Kurtzman *et al.*, 2011). Under the right conditions, these microorganisms can reproduce indefinitely through mitotic divisions, resulting in huge clonal populations of haploid, diploid or even polyploid stages. Yeasts are distinguished from other fungi by their capacity for infinite clonal development in unicellular form and by the fact that their sexual states do not create fruiting bodies (Liti & Schacherer, 2011). Yeasts can have various structures for asexual and sexual reproduction, as can be seen in Figure 1.2.

Previously in some areas of molecular biology it was common to consider "yeast" and "Saccharomyces" as synonyms (Kurtzman *et al.*, 2011), but this was considered wrong, because a greater part of yeasts are in the phylum Ascomycota, subdivided in Saccharomycotina and Taphrinomycotina (Buzzini *et al.*, 2017), and a couple of them are in the phylum Basidiomycota (Aaron, 2021).

Since the beginning of human history, there are records that yeasts have been associated with various activities. It is difficult to establish when these microorganisms began to be used, but there is evidence that a Neolithic village in China used yeast to produce bread and fermented beverages (Hernández *et al.*, 2018). However, only in 1860 that was discovered that yeasts can convert sugar into ethanol and carbon dioxide, which is designated fermentation (Sicard & Legras, 2011).

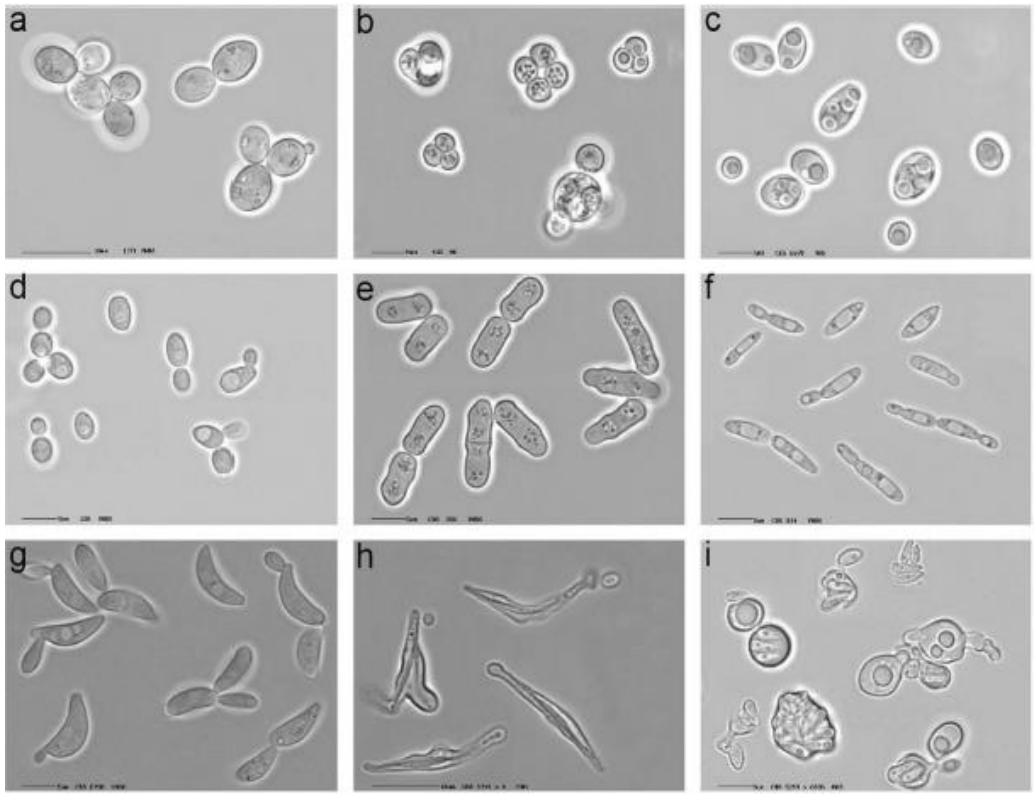


Figure 1.2 Forms of asexual and sexual reproduction in some yeasts. a) multipolar budding yeast cells of *Saccharomyces cerevisiae*; b) asci with globular ascospores of *Saccharomyces paradox*; c) asci with saturn-shaped ascospores of *Pichia scutulata*; d) buding yeast cells of *Candida glabrata*; e) fission of yeasts cells of *Schizosaccharomyces pombe*; f) bipolar budding yeast cells of *Hanseniaspora uvarum*; g) lunate yeasts cells of *Metschnikowia lunata*; h) asci with liniform ascospore of *Metschnikowia agaves*; i) asci with free clavate ascospores of *Clavispora lusitaniae*. (Boekhout *et al.*, 2022)

1.3.*Killer* Yeasts

The first description of the *Killer* yeast phenomenon occurred more than 60 years ago, through the isolation of a strain of *Saccharomyces cerevisiae* that had the ability to inhibit the growth of other strains of the same species (Bevan & Makower, 1963).

Bevan and Makower classified the strains into three phenotypes: *killer*, sensitive and neutral. *Killer* yeasts produce toxins that are lethal to sensitive strains, but have no effect on neutral strains, which are immune to these *killer* effects (Figure 1.3). On the other hand, a sensitive yeast may have receptors for various toxins (Billerbeck *et al.*, 2024; Mannazzu *et al.*, 2019).

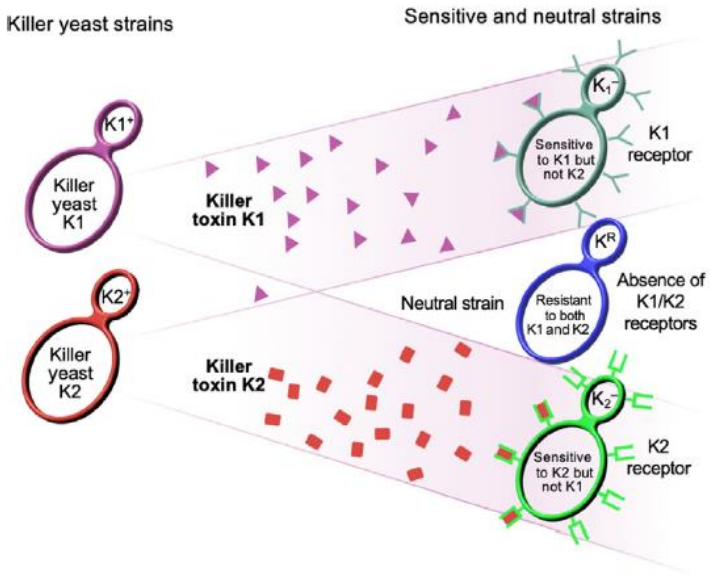


Figure 1.3. Scheme representing the interactions between *killer*, neutral and sensitive yeasts.
The toxins produced by *killer* yeasts require a specific receptor in sensitive yeasts, so if the yeast has no receptors, it is considered neutral and resistant to a given toxin (Billerbeck *et al.*, 2024).

Killer toxins, which can also be called mycocins, zymocins and zymocides, are generally proteins or glycoproteins that interact with receptors present on sensitive cells (Boynton, 2019).

So, there are different interactions between *killer* toxins and sensitive cells. The action of *killer* toxins occurs in two steps, the first of which is binding to the primary receptor, followed

by binding to the sensitive cell's secondary receptor. After binding to the secondary receptors, different mechanisms of action can occur (Figure 1.4): a) cell wall damage due to hydrolysis of cell-wall glucans or inhibition of β-1,3-glucan synthase; b) cell membrane permeabilization resulting in release of K+, H+, ATP, and other metabolites; c) cell-cycle perturbation that blocks progression of the cell cycle in the G1/S phase or completion of the G1 phase; d) fragmentation of RNA in terms of 18S and 25S rRNA, or tRNA (Mannazzu *et al.*, 2019).

Several yeast species show the *killer* phenotype, which is normally manifested at acidic pHs between 4 and 6 and temperatures below 30ºC (Crabtree *et al.*, 2023; Woods & Bevan, 1968). The presence of the *killer* phenotype is not linear at the species level, which means that it can be present in different strains of the same species(Travers-Cook *et al.*, 2023).

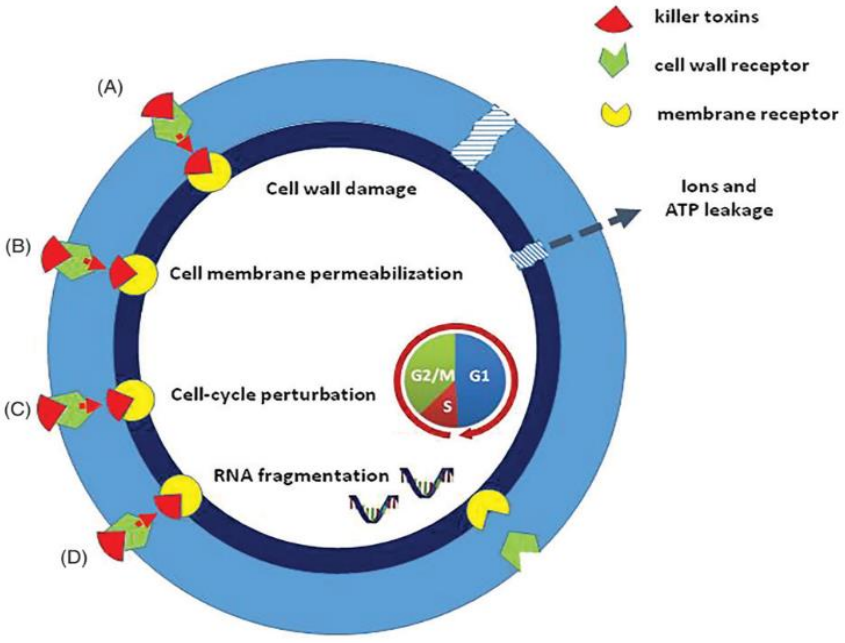


Figure 1.4 Visual representation of the effects of *killer* toxins on sensitive yeasts. The *killer* toxin first binds to the receptors on the cell wall and then enters the cell via the cell membrane receptor. The type of toxin can lead to different mechanisms in the sensitive yeast: A) cell wall damage; B) Cell membrane permeabilization; C) Cell-cycle perturbation; D) RNA fragmentation. (Mannazzu *et al.*, 2019).

1.3.1. *Saccharomyces cerevisiae Killer* Yeast

This work used the yeast *Saccharomyces cerevisiae* PYCC 4620, known for its *killer* potential against the spoilage yeast *Pichia guilliermondii* (da Silva *et al.*, 2008).

To date, six types of *killer* toxins present in *Saccharomyces cerevisiae* have been described: K1, K2, K28, Klus, KHR and KHS (Sambuk *et al.*, 2019).

The internal toxins K1, K2, K28, and Klus are induced by dsRNA, while the exotoxins KHR and KHS are encoded by chromosomal DNA (Magliani *et al.*, 1997a).

On the other hand, there is also the Super*Killer* complex (SKi complex), responsible for mRNA degradation (Vodkin *et al.*, 1974; Widnert & Wickner, 1993).

1.3.1.1.K1 and K2 *Killer*s Toxins

Despite having distinct proteins, toxins K1 and K2 have a very similar method of action. *Saccharomyces cerevisiae* produces these toxins, which is an α-β dimer linked by a disulfide. Hydrophobic amino acids and a relatively high charge are present in both subunits. The β subunit is the source of the toxin's excess charge. From a basic dimer, the toxin may generate several dimers, and the creation of these dimers is essential to the toxin's mechanism of action. *Killer* toxins can have many mechanisms of action based on their dimeric structure (Marquina *et al.*, 2002).

The most widely recognized hypothesis to date (Figure 1.5), proposes that the channel domain is attributed to the α subunit, which has two hydrophobic domains that may be membrane expanders, causing membrane damage.

The proton-binding sites for the cation channels that toxins produced in the target cells' membranes are displayed by this subunit. According to this concept, binding to the cell wall receptor depends on the β subunit (Martinac *et al.*, 1990).

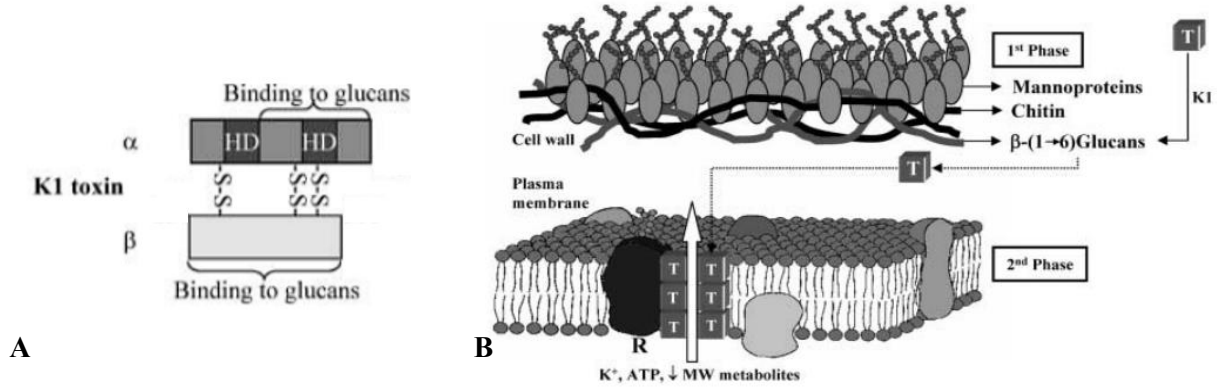


Figure 1.5 Mode of action of K1 toxin. A) Representation of K1 toxin. K1 toxin is formed by an α-β dimer. The α subunit contains two hydrophilic domains (HD) and is linked by disulfides (S) to the β subunit. Both subunits have a relatively high charge, which allows the β subunit to bind to the glucan layer present in the cell wall of the sensitive yeast.
B) In the first phase, the K1 toxin (T) binds to the layer of glucans present in the cell wall through the β subunit, thus accessing the cell membrane. In the second phase, the α subunit binds to receptors (R), allowing its hydrophobic domains to function as expanders, opening channels and allowing essential metabolites to leave the cell (Marquina *et al.*, 2002).

1.3.1.2.K28 *Killer* Toxin

The identification of K28 has been facilitated by the fact that the *S. cerevisiae* K28 *killer* toxin is attached to the mannoprotein portion of the yeast cell wall, setting it apart from K1 and K2 *killer* toxins. K28, on the other hand, suppresses nuclear DNA synthesis rather than having any ionophoric effects (Marquina *et al.*, 2002; Schmitt *et al.*, 1996).

The cells treated with K28 come to a stop in the budded phase of the cell cycle (Figure 1.6), containing a single nucleus in the mother cell that contains unreplicated (G1) DNA. This toxin's action is not immediate, and it causes sensitive yeasts to stop proliferating as unbudded cells, indicating that it prevents the G1 phase of the cell cycle from being completed (Butler *et al.*, 1991).

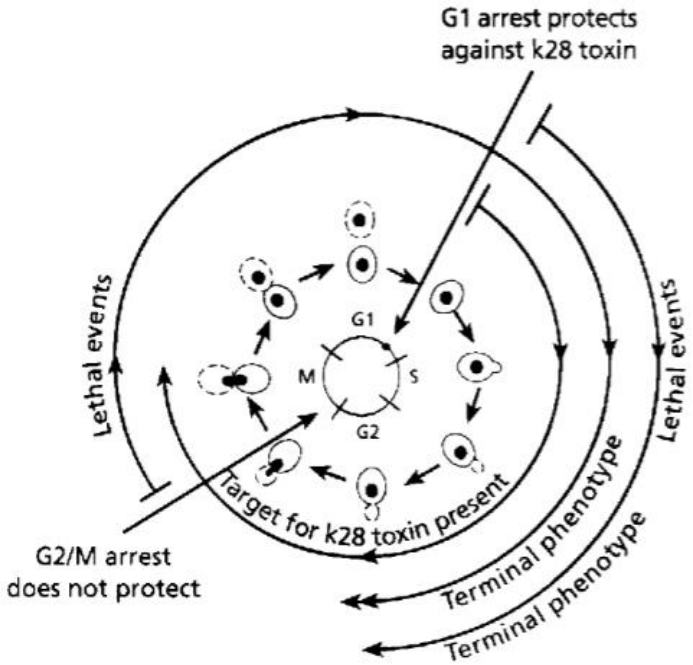


Figure 1.6 Mode of action of K28 toxin. The K28 toxin induces the arrest of medium-sized buds or a single nucleus in the mother cell. These events can derive from cells blocked in the G1/S phase after going through mitosis or in cells blocked in the G2/M phase. The cells thus reach the terminal phenotype that leads to lethal events (Schmitt *et al.*, 1996).

1.3.1.3.Klus *Killer* Toxin

Rodríguez-Cousiño *et al.* (2011) discovered a new *killer* toxin (Klus) in wine yeast strains from a region of Spain that was encoded in Mlus dsRNA.

Klus strains carry one of four Mlus isotypes (Klus-1 to Klus-4), with 2.1 to 2.3 kb sizes. The ORF for the preprotoxin is ancestrally related to the host chromosomal gene YFR020W, offering for the first time a hint as to the origin of a dsRNA-encoded toxin. Klus toxin is active over a wide range of yeasts other than *S. cerevisiae* (Rodríguez-Cousiño *et al.*, 2013).

1.3.1.4.KHR and KHS *Killer* Toxins

As previously mentioned, additional chromosomally encoded *killer* toxins have been identified in *S. cerevisiae*. These toxins have been identified as *killer* of heat susceptible (KHS) and *killer* of heat resistant (KHR) due to differences in their optimal pH and thermostability (Magliani *et al.*, 1997).

The mature KHR and KHS toxins are single proteins that are encoded on the left arm of chromosome IX and the right arm of chromosome V, respectively. It is believed that some protein processing takes place during maturation because the mature toxins have molecular masses that are lower than those of their precursors. The KHR and KHS genes are distinct from other *killer* genes. (Goto *et al.*, 1990, 1991; Magliani *et al.*, 1997).

KHR encodes a 33-kDa preprotoxin with a possible hydrophobic signal sequence in the N-terminal site region, four competent glycosylation sites, and five cleavage sites that could be cut by kex2 protease. Since the mature KHR toxin lacks regions that are obviously hydrophobic, its mechanism of action is most likely distinct from K1 (Goto *et al.*, 1990).

KHS encodes a hydrophobic N-terminal sequence that results in a 79-kDa precursor, which is likely spliced to produce the mature toxin. The mature KHS toxin contains three clusters of hydrophobic amino acid sequences that may have an ionophore function similar of K1 or K2 toxins (Goto *et al.*, 1991).

1.3.1.5.SKi Complex

The SKi complex is encoded in the nuclear genome, and it was initially thought that this complex caused the overexpression of *killer* toxins, however this complex represses the translation of poly(A) minus RNAs (J. T. Brown *et al.*, 2000).

This complex is made up of 3 subunits: Super*Killer* proteins 2, 3 and 8 (J. T. Brown *et al.*, 2000; Synowsky & Heck, 2008).

The SKi2 protein has 146 kDa and is a putative RNA helicase, SKi 3 is the largest subunit of this complex with 163 kDa and contains a tetratricopeptide motif, finally Ski 8 with 44 kDa and contains a WD repeat domain (Halbach *et al.*, 2013).

Synowsky & Heck (2008) proposed that the complex has a stoichiometry 1:1:2 with two copies of SKi8. They presented three possible models shown in Figure 1.7.

Halbach *et al.* (2013) later described that the SKi3 subunit would be linked to the SKi2 subunit, which in turn binds to the two SKi8 proteins, suggesting that model II (Figure 1.7), presented by Synowsky & Heck (2008), was the most correct.

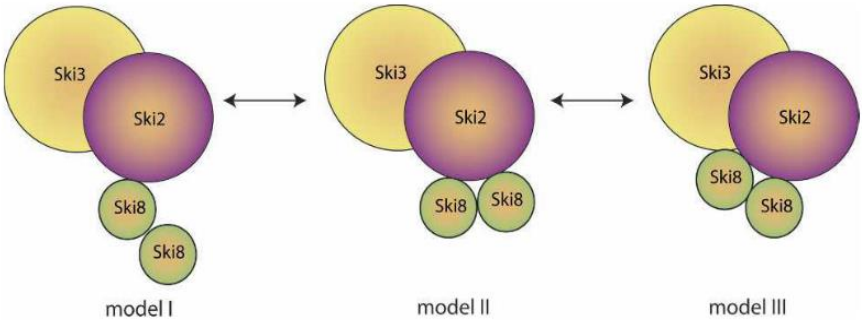


Figure 1.7 Models for structuring the SKi complex. (Synowsky & Heck, 2008)

Model I: The Ski3 protein is bound to the Ski2 protein, which in turn binds to SKi8, which binds to the second SKi8.
Model II: The Ski3 protein is bound to the Ski2 protein, which is bound to the two SKi8 proteins.
Model III: All the proteins in the complex are interconnected.

1.3.2. Other *Killer* Yeasts

Many mycocins have been isolated from yeasts of over 100 species from 20 different genera since they were first described. These species include *Saccharomyces* and also *Candida, Cryptococcus, Debaryomyces, Kluyveromyces, Hanseniaspora, Pichia, Torulaspora, Ustilago, Williopsis,* and *Metschnikowia* (Table 1.1) (Gil-Rodríguez & Garcia-Gutierrez, 2021).

Mestchinokowia pulcherrima has been shown to be an important antimicrobial agent because it contains pulcherrimin, a red pigment that has natural antifungal properties. So far, this yeast is considered a *killer* yeast, but the production of *killer* toxins has not been verified. Its mechanism of action starts with the medium becoming less iron-rich due to the precipitation of iron (III) ions brought on by the interaction with pulcherriminic acid. This makes the environment hostile to other microorgisms that depend on iron for growth (Morata *et al.*, 2019).

Table 1.1 Species in which the *killer* phenotype has been found. Information adapted from Boynton (2019).

Phylum	**Family**	**Example species**	**Citation(s)**
Ascomycota	Debaryomycetaceae	*Meyerozyma guilliermondii*	(de Lima *et al.*, 2013)
Ascomycota	Dipodascaceae	*Dipodascus geotrichum*	(Vadkertiova & Slavikova, 1995)
Ascomycota	Lipomycetaceae	*Lipomyces lipofer*	(Mushtaq *et al.*, 2013)
Ascomycota	Metschnikowiaceae	*Metschnikowia saccharicola*	(Tan *et al.*, 2018)
Ascomycota	Pichiaceae	*Pichia punctispora*	(Golubev & Blagodatskaya, 1994)
Ascomycota	Saccharomycetaceae	*Debaryomyces hansenii*	(Gunge *et al.*, 1993)
Ascomycota	Saccharomycetales Incertae sedis	*Candida maltosa*	(Buzzini & Martini, 2000; Mehta *et al.*, 1982)
Ascomycota	Saccharomycodaceae	*Hanseniaspora uvarum*	(Radler *et al.*, 1985)
Ascomycota	Schizosaccharomycetaceae	*Schizosaccharomyces pombe*	(Bonilla-Salinas *et al.*, 1995; López Hernández & Zanders, 2018)
Ascomycota	Wickerhamomycetaceae	*Wickerhamomyces anomalous*	(Golubev, 2015)
Basidiomycota	Agaricostilbaceae	*Sterigmatomyces halophilus*	(Antunes & Aguiar, 2012)
Basidiomycota	Bulleraceae	*Bullera hannae*	(Golubev *et al.*, 1996)
Basidiomycota	Cystofilobasidiaceae	*Cystofilobasidium infirmominiatum*	(Golubev *et al.*, 2003)
Basidiomycota	Kondoaceae	*Kondoa miscanthi*	(Mushtaq *et al.*, 2010)
Basidiomycota	Malasseziaceae	*Malassezia furfur*	(Arzumanian *et al.*, 2009)
Basidiomycota	Mrakiaceae	*Tausonia pullulans*	(Golubev *et al.*, 2002; Vadkertiova & Slavikova, 1995)
Basidiomycota	Piskurozymaceae	*Piskurozyma capsuligena*	(Keszthelyi *et al.*, 2006)
Basidiomycota	Tremellales Incertae sedis	*Hannaella sinensis*	(Golubev & Nakase, 1997)
Basidiomycota	Tremellaceae	*Naganishia albida*	(Vadkertiova & Slavikova, 1995)
Basidiomycota	Trichosporonaceae	*Vanrija humicola*	(Golubev & Shabalin, 1994)
Basidiomycota	Robbaueraceae	*Robbauera albescens*	(Golubev, 1998)
Basidiomycota	Sporidiobolales Incertae sedis	*Rhodotorula mucilaginosa*	(Libkind *et al.*, 2004)
Basidiomycota	Sporobolomycetaceae	*Sporobolomyces salmonicolor*	(Vadkertiova & Slavikova, 1995)
Basidiomycota	Ustilaginaceae	*Pseudozyma tsukubaensis*	(Golubev *et al.*, 2006)

1.4.*Killer* yeast applications

The demand for *killer* yeasts is continuously growing across various sectors such as environmental biotechnology for the biological control of plant pathogens, medicine for antifungal immunotherapy and treating infections in animals and humans, and the food industry for pest control in cheese and wine production (Servienė & Serva, 2023).

There are numerous possible uses for *killer* yeast and their toxins in different industrial processes (Bajaj & Singh, 2017).

Currently, *killer* yeast application as biocontrol agents in pre-harvest and post-harvest control of phytopathogenic fungi (Figure 1.8) has been intensively used, since fungal diseases of crops cause significant losses in food production worldwide (Mannazzu *et al.*, 2019). For this reason, the biological control of fungal diseases using antagonistic microorganisms represents a possible alternative to the use of fungicides (Maria Rosa-Magri *et al.*, 2011).
Some yeasts with this capability have been granted Qualified Presumption of Safety status by the European Food Safety Authority (Andreoletti *et al.*, 2013), which authorize their use in food preservation (BIOHAZ, 2012).

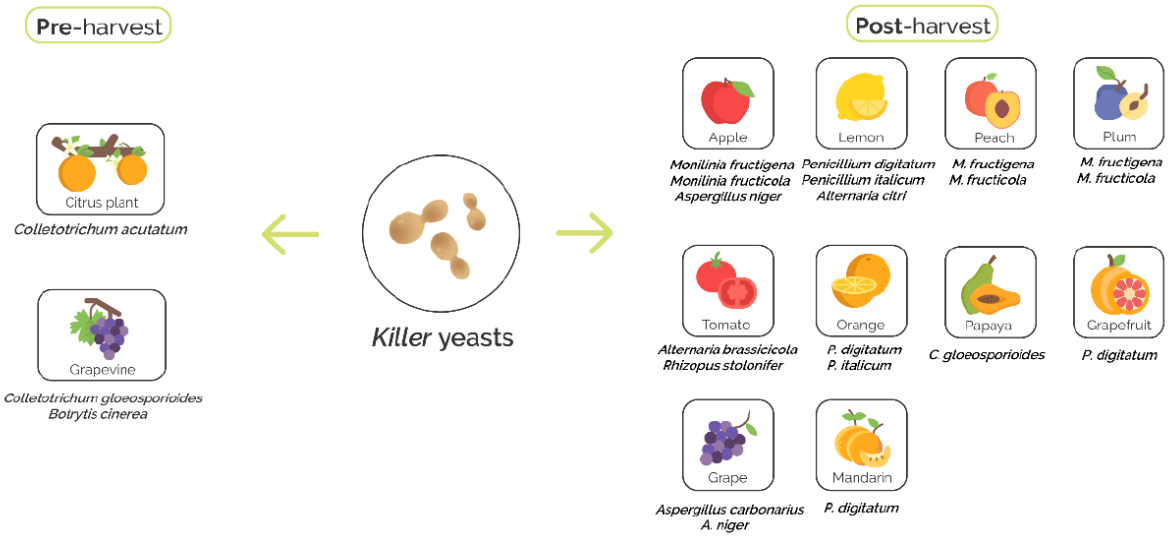


Figure 1.8 Schematisation of the application of *killer* yeasts as a biocontrol for fungal infections, before and after harvesting (Díaz *et al.*, 2020).

1.4.1. Winemaking

At first, wine was the result of a coincidental combination of biology and chemistry, with microbes playing a key role. Wine makers have been adapting their methods in accordance with scientific knowledge and advancements from these ancient fermentations to the modern monitored industrial processes (Belda *et al.*, 2017).

Since at least the eighteenth century, sulfur dioxide has been a common preservative in wine. Because of its antibacterial, antioxidant, and antioxidasic qualities, its use has even become crucial in the production of fine wines (Divol *et al.*, 2012).

In wine making microorganisms, including yeasts, filamentous fungi and bacteria constitute an important role in wine production, by their impact on grapevine health, wine fermentation, and the flavor, aroma, and quality of finished wines (Liu *et al.*, 2020). To obtain premium quality wines it has been increasingly proposed the use of indigenous yeast starters, selected among yeast biota of specific environments (Comitini *et al.*, 2017). The controlled inoculation of musts with *Saccharomyces* and non-*Saccharomyces* yeasts in winemaking is an interesting biocontrol tool in helping the improvement of wine quality by removing undesirable spoilage yeasts and reducing the amount of applied chemistry preservatives (Sidari *et al.*, 2021; Villalba *et al.*, 2020).

Although it is thought that using *killer* toxins as biocontrol agents in winemaking is a better and an alternative method for traditional synthetic antimicrobials like sulfur dioxide, not much progress has been made in this area, mostly because of toxin instability in winemaking conditions (Escott *et al.*, 2017).

1.4.1.1.Grape varieties

The biotic and abiotic environmental factors that influence grape-associated yeast communities are far more varied, including the berry's hygienic state, the weather and climate, the farming practices used, and also the grape varieties (Belessi *et al.*, 2022).

In previous work, indigenous yeasts from the Alentejo region have been isolated and identified from the musts of Touriga Nacional and Aragonez grapes varieties (Figure 1.9), through classical microbiology under the research Project MicroBioWines. This work was supported by Fundo Europeu de Desenvolvimento Regional (FEDER) funds through

COMPETE2020 – Programa Operacional Competitividade e Internacionalização (POCI), (POCIALG-01-0247-FEDER-017987)). According to the Instituto da Vinha e do Vinho (2018), Touriga Nacional is a portuguese indigenous grape variety, with a significant increasing popularity worldwide. The Aragonez variety is an Iberian red grape that is grown in several regions, and very well adapted in the Alentejo region.

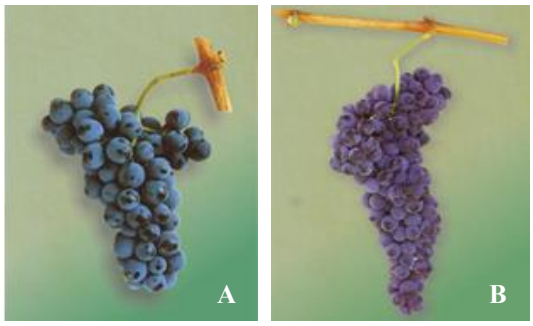


Figure 1.9 Touriga Nacional (A) and Aragonez (B) red grape varieties. (Instituto da Vinha e do Vinho, 2018).

1.4.1.2.*Killer* non-Saccharomyces yeasts in wine

Non-Saccharomyces *killer* yeasts are becoming increasingly popular since some of them have *killer* toxins. In winemaking, these yeasts can inhibit spoilage organisms, contributing to improved wine quality. Often these yeasts are also used at the level of wine aroma, flavor complexity, and mouthfeel, particularly when used in combination with *Saccharomyces cerevisiae* (Oro *et al.*, 2014).

Some of the non-*Saccharomyces killer* yeasts include species from genera such as *Metschnikowia*, *Pichia*, *Torulaspora*, *Hanseniaspora*, and *Candida* (Morata *et al.*, 2019, 2022).

1.4.1.3.Spoilage yeasts in wine

Particularly in fermented foods and beverages, where the metabolites produced contribute to the flavour, aroma, and taste of the finished items, microbial spoilage is frequently difficult

to detect. In actuality, there isn't much of a distinction between what is viewed as a positive activity and spoiling for cultural or ethnic reasons.

Wines that have been stored may develop a film due to spoiling, bottled wines may develop sediments and gas production, and all phases of wine manufacturing may result in off-tastes and off-odours (Loureiro & Malfeito-Ferreira, 2003).

The most discussed spoilage yeast species include *Pichia guilliermondii* and *Brettanomyces bruxellensis*. *P. guilliermondii* is frequently identified as a spoilage yeast within the winemaking context, notorious for compromising wine quality. This yeast can introduce various issues during wine fermentation and storage, such as producing off-flavors and possibly altering the wine's color and taste (Escott *et al.*, 2017). Consequently, it is critical to monitor and control its presence to preserve the desired quality and characteristics of the wine. *Pichia guilliermondii* is known to be vulnerable to *killer* toxins secreted by several *killer* yeast species, including *Hanseniaspora uvarum*, *Metschnikowia pulcherrima*, *Wickerhamomyces anomala* (previously known as *Pichia anomala*), *Pichia kluyveri*, *Debaryomyces hansenii*, *Kluyveromyces lactis*, *Zygosaccharomyces bailii*, and some *Saccharomyces spp, among others* (Lopes *et al.*, 2009). These *killer* yeasts produce toxins that can inhibit or eliminate sensitive strains like *Pichia guilliermondii*, which is notorious for its adverse impact on wine quality, potentially leading to spoilage (Hernández *et al.*, 2018).

1.4.2. Using *Killer* Yeasts as Antimicrobial Agents

Recent research has demonstrated that certain yeast strains possess the ability to inhibit the growth of a variety of microorganisms, suggesting their potential as effective antimicrobial agents (Acuña-Fontecilla *et al.*, 2017). Furthermore, studies such as those by Abu-Mejdad *et al.*, (2020) have evaluated the cytotoxicity of some *killer* toxins on human cells and found them to be non-toxic, thereby enhancing their appeal as alternatives to conventional antimicrobials.

Given the escalating issue of antimicrobial resistance, it is crucial to explore innovative approaches to address this global health concern. *Killer* toxins represent a promising avenue worth considering for their potential to offer a new method of microbial control (Junges *et al.*, 2020). This could lead the way for breakthroughs in both medical, food and environmental applications of microbial management (Abu-Mejdad *et al.*, 2020; Acuña-Fontecilla *et al.*, 2017; Junges *et al.*, 2020).

1.5.Antimicrobial resistance as global challenge

The convergence of political factors, escalating populations, climate change, and rising antibiotic resistance poses significant global challenges that impact food security and health. Research into *killer* yeasts as new antimicrobial agents offers a promising alternative, potentially reducing dependence on chemicals and addressing antimicrobial resistance more sustainable and healthier.

1.5.1. Bacterial pathogens

According to WHO antimicrobial resistance is one of the ten most important threats to global health. Effective antimicrobial medications are essential to modern medicine, yet all WHO regions have high documented rates of resistance infections from a wide variety of bacteria (World Health Organization, 2024).

In 2019, it is estimated that out of 4.95 million deaths caused by antibiotic-resistant bacteria, 1.27 million are associated with multi resistant bacteria (Murray *et al.*, 2022).

Due to the problem caused by antibiotic resistance, there was a need to categorise bacteria into priority groups. Figure 1.10 shows which bacteria are included in the three groups (critical, high and medium).

The critical group represents antibiotic-resistant bacterial pathogens that pose the greatest threat to health due to limited treatment options, high mortality, morbidity and transmissibility and difficult to prevent. These bacteria may have global resistance mechanisms. The high group, on the other hand, presents bacteria with significant difficulty in treatment, substantial mortality and morbidity and a tendency towards increased resistance. These bacteria are difficult to prevent and highly transmissible, but they are not globally critical.

Finally, the medium group contains pathogenic bacteria with moderate treatment difficulty, moderate mortality, morbidity and resistance. They are not critical at a global level (World Health Organization, 2024).

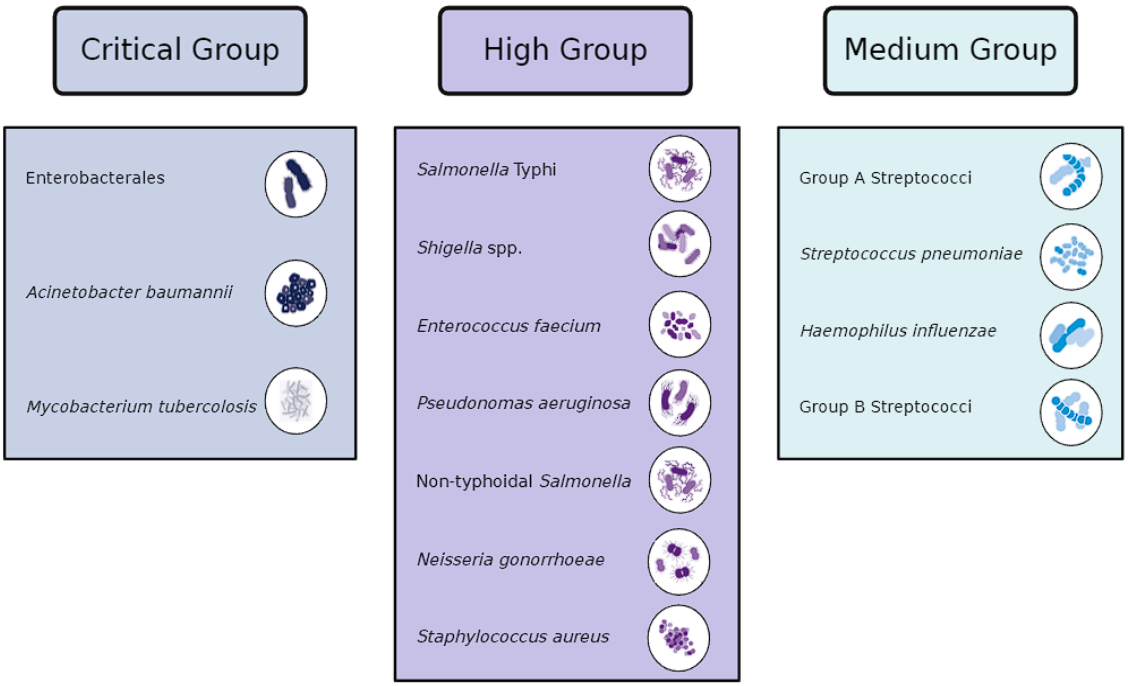


Figure 1.10 Bacteria of greatest concern for human health in 2024 (World Health Organization, 2024).

Critical group: antifungal resistance and of global concern.

High group: Difficult to treat and quite transmissible, but not critical at a global level.

Medium group: Moderately difficult to treat, not a global concern.

1.5.2. Fungal pathogens

The emergence of drug-resistant fungal infections is a significant clinical issue. This is primarily because more patients are experiencing immune suppression or compromised immunological function, as well as invasive fungal infections as a result of intricate surgical procedures. When invasive fungal infections occur alongside other severe medical problems, the mortality rate of affected people is often higher than that of uninfected patients (Berman & Krysan, 2020).

As with bacterial pathogens, the WHO has also categorised fungal pathogens into three groups: critical, high and medium (Figure 1.11).

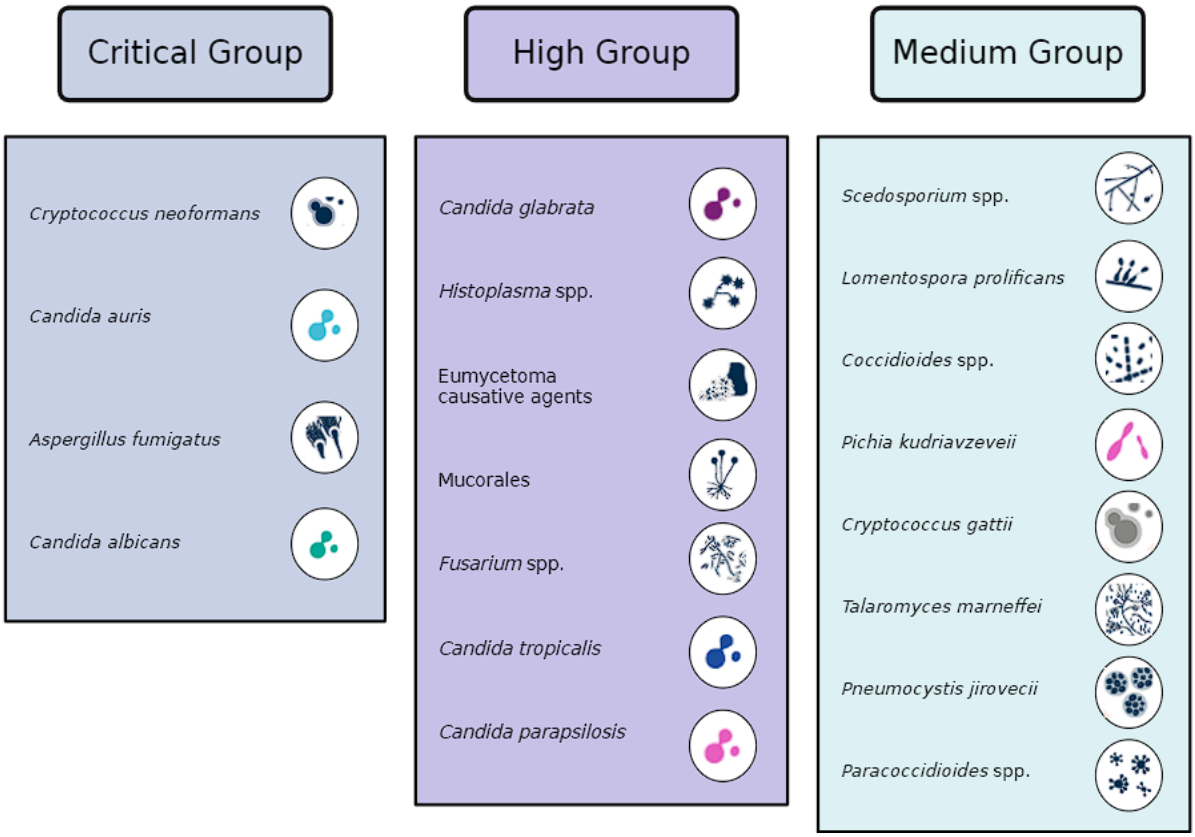


Figure 1.11 Fungi of greatest concern for human health in 2022 (World Health Organization, 2022).

Critical group: antifungal resistance and of global concern.

High group: Difficult to treat and quite transmissible, but not critical at a global level.

Medium group: Moderately difficult to treat, not a global concern.

1.6.Methodologies for detecting and identifying *killer* activity

Until now, the most widely used techniques for identifying the *killer* phenotype were tests in which the *killer* yeast was in contact with the sensitive yeast (Bevan & Makower, 1963). These tests are carried out using the YMA-MB medium in essentially two ways: the inoculum is incorporated into the medium before it is solid and then wells are made where the *killer* yeast inoculum is deposited (Wójcik & Kordowska-Wiater, 2015); the second way is by spreading the sensitive yeast inoculum along the plate and an amount of *killer* yeast biomass is applied to the plate (Bevan & Makower, 1963).

Pasqual *et al.* (1990) have shown that a differential medium can be used at industrial level to select yeasts with a *killer* phenotype, the WLN medium (Wallerstein Laboratory Nutrient).

2. Objectives

Considering the potential applications of *killer* yeasts, the objectives of the current study are as follows:

i) To screen yeast isolates from wine must for possible *killer* activity against sensitive yeast strains and common wine-contaminating species. This involved isolating and characterizing yeast strains found in wine must, followed by evaluating their ability to inhibit or kill competing microorganisms, particularly those that negatively impact the quality of the wine. In addition to identifying strains capable of enhancing microbial stability and fermentation efficiency during the winemaking process, the screening approach aimed to investigate the potential uses of *killer* yeasts in practical winemaking applications.

ii) To identify the proteins responsible for the observed *killer* activity. This included analysing the mechanisms underlying the *killer* phenotype, focusing on identifying and characterizing the specific proteins or toxins that contribute to the yeast's antimicrobial properties. Studying the genetic and metabolic elements that control *killer* yeast activity as well as the ways in which environmental factors (pH, temperature, and nutrient availability) affect their effectiveness were also necessary to comprehend these mechanisms. These findings may further enhance the use of *killer* yeasts in wine fermentation and preservation processes, potentially improving both wine quality and protection against spoilage organisms.

These findings could improve the employment of killer yeasts in wine fermentation and preservation procedures, which could lead to an increase in wine quality and protection against spoilage organisms.

On the other hand, this study can also be seen as a way of finding alternatives to conventional antimicrobial agents, as they are a natural and healthier option with the potential to contribute to reducing the problem of antimicrobial resistance.

3. Materials and methods

3.1.Culture media

3.1.1. Yeast Malt Medium

Yeast malt (YM) medium was used to grow the yeasts isolated from the wine and the yeast strains that were used throughout this work.
For growth in liquid media the Yeast Malt Broth (YMB) was used. This culture medium was composed of 0.5% peptone (Thermo Scientific, Oxoid UK), 0.3% yeast extract (Biokar, France), 0.3% malt extract (Biokar, France), 1% dextrose (Difco Laboratories, USA). The Yeast Malt Agar (YMA) media was used for plate growth, the composition was the same as YMB but with the addition of 1.5% agar (Liofilchem, Italy).

3.1.2. YMA – Methylene Blue

In order to check whether the *killer* phenotype was present in the yeasts isolated from the wine must, the YMA – Methylene Blue (YMA-MB) media was used.
To prepare YMA-MB, two different solutions were mixed: YMA medium, and a 0.2M Sodium Citrate Solution. The pH of the medium is adjusted between 4-5 with hydrochloric acid (HCl). Methylene blue was then added into the solution to achieve a final concentration of 0.15% (v/v).

3.1.3. *Killer* Toxin Production Medium

With the aim of activating the production of *killer* toxins, yeasts with this phenotype were inoculated in *killer* toxin production (KTP) medium.
KTP medium consists of YMB, 50 g/L of glycerol buffered at pH 4-5 using 50 mM-100mM Citrate Phosphate Buffer.

3.2. Yeast Strains

In this study, the spoilage yeast *Pichia guilliermondii* (sensitive yeast) was used to check the *killer* potential of yeasts isolated from wine. The *killer* yeast *Saccharomyces cerevisiae* PYCC 4620 was used as a positive control, as this yeast inhibits the sensitive yeast.

3.3. Yeast isolates recovery procedure

As mentioned above the yeast isolates were obtained in a previous study at (A. C. Lopes *et al.*, 2020), from Touriga Nacional and Aragonez musts (Figure 7). The isolation procedure was performed from 2016 to 2018 vintages. The isolates were recovered from -80ºC (YMB + glycerol (50%)) by thawed each maintenance tube on ice and 20µL of the suspension were seeded on a plate with YMA. The inoculated plate was left for 48 h at room temperature.

3.4. Evaluation of antimicrobial activity

3.4.1. Biomass method

The biomass method consisted of placing an amount of yeast cells that were to be tested for their *killer* potential in contact with the sensitive yeast, *Pichia guilliermondii*. This method was carried out using YMA-MB medium, due to the fact that this medium has the optimum pH for the production of *killer* toxins and methylene blue, which helps to visualise inhibition halos and dead cell halos, because this compound is absorbed by dead cells.

A suspension of Pichia guilliermondii was prepared by resuspending a portion of biomass in 1000 µL of PBS (137mM NaCl; 2.7mM KCl; 10mM Na_2HPO_4; 1.8mM KH_2PO_4). The suspension's turbidity was adjusted to match a 1 McFarland standard.

A sterile swab was dipped into the suspension so it could be spread over the plate of YMA-MB medium. The test yeasts biomass was collected with a loop and placed over the sensitive yeast spread, followed by an incubation at room temperature for 48 h. *Saccharomyces cerevisiae* PYCC 4620 was used as a positive control *killer* yeast.

3.5.*Killer* toxin extraction

The extraction of *killer* toxins was carried out with the aim of separating these toxins from the remaining cellular content in order to identify the proteins responsible for the *killer* phenotype. Below, all the procedures used to achieve the isolation of the *killer* toxins are described.

3.5.1. Fermentation for *killer* toxin production

Firstly, *killer* isolates were grown overnight in YMB with agitation (180 rpm) at 30 °C and then, to confirm that *killer* activity was in some way linked to the fermentation process, yeast with *killer* potential were inoculated at 10% (v/v) into KTP medium (Bajaj *et al.*, 2013), and fermentation was performed at 30 °C with agitation (180 rpm) for 24–192h. The fermented broth was centrifuged at 10,000 × *g* for 15 minutes at 4 °C. The pellet was discarded, and the supernatant (crude toxin preparation) was collected and used for verification of *killer* activity by agar diffusion, performed every 24h (Bajaj *et al.*, 2013).

3.5.2. *Killer* toxin enrichment

At this assay, *killer* yeasts were grown in YMB medium with pH changed to 4-5, to promote the toxin production, overnight at room temperature with agitation (250 rpm), followed by a centrifugation at 3100 g for 5 min. The supernatant was filtered by 0,22 µm filter, after that, a supersaturated solution of ammonium sulphate at 4ºC was added to the filtrate in a 1:1 ratio and this mix was incubated on ice for 3h. To collect the precipitated proteins was done a centrifugation at 20,800 × g for 10 min at 4º C, then the supernatant was discarded, and the proteins were suspended in 10 µL of YMB medium (pH 4-5). Finally, the *killer* toxins were incubated at room temperature or heat-inactivating at 98º C for 2 min and applied into *killer* assay plates against sensitive yeasts (Fredericks *et al.*, 2021).

3.5.3. Competition assay

The sensitive and *killer* yeast were grown simultaneously, in the same tube, and in different proportions: 1:1; 1:2; 1:3, increasing the concentration of the *killer* yeast, to understand if the *killer* toxin is only produced when the yeast is in competition. Growth was carried out overnight at room temperature with agitation (150 rpm). After a centrifugation (13,000 rpm, 10 min, 4º C), the supernatant was filtered by 0.22 µm filter, and 40 µL were introduced into 6 mm wells, that were done with the aid of a sterile Pasteur pipette in YMA-MB plate, against the sensitive yeast (Giometto *et al.*, 2021).

3.5.4. Isolation of *killer* enzyme from inhibition zone

Two types of YMA-MB medium were used for this test, one supplemented with 0.8% agar and the other with 1.25% low melting agarose, with the aim of extracting the toxin from the inhibition zone, produced using the biomass method.

The inhibition zone was removed from the plate using a scalpel and added to 100 µL of citrate-phosphate buffer (50 mM, pH 5) and liquefied at 65º C in a dry bath.

Afterwards, the medium was cooled to room temperature until it solidified, forming two phases, so the toxin that remained dissolved in the buffer solution was collected and finally tested using agar diffusion.

3.5.5. Mechanic extraction

In order to identify whether the *killer* toxins were inserted in the cell extract, the mechanical extraction procedure was used.

As described by Ge *et al.*, 2010, a small amount of glass beads (~3 spheres) were added to the cell pellet and mixed well.

To break the cell wall and to allow the protein extraction, the bead beater and the vortex genie were used, for 5 minutes at maximum speed. After that, 50 µL of citrate phosphate buffer was added, and the pellet was resuspended. Then was centrifuged at 5000 rpm, 4 ºC for 10 minutes, and the buffer containing the proteins was collected. In order to detect any *killer* activity, diffusion on agar was performed.

3.6.Evaluation of *killer* activity of protein extracts

3.6.1. Agar diffusion

The agar diffusion method was used to test the toxin production procedures. This consists in YMA-MB plates inoculated with a sensitive yeast culture, then, with the aid of a sterile Pasteur pipette, wells of 6 mm diameter were done. Previously prepared extract toxin was distributed into each well (40 µL per well). Plates were incubated at room temperature for 48 h, and the presence of inhibition zone was verified (Bajaj *et al.*, 2013).

3.6.2. Microplate assay

To check more accurately the effect of *killer* toxins, the growth of *Pichia guilliermondii* was verified by measuring the optical density. For this purpose, a microplate with 96 wells was used, and the distribution of the samples was divided through the columns. As can be observed in Figure 3.1, the first six columns were used for testing the *killer* activity of the *killer* isolates against the yeast sensitive culture of *Pichia guilliermondii*.
The remaining six columns were used for positive control, that consists in *Pichia guilliermondii* with the addition of *Saccharomyces cerevisiae* PYCC 4620, and negative control, only *Pichia*. The microplate was incubated at room temperature for 48 h, and subsequently the optical density was read at 595 nm at 0, 2, 4, 6, 24 and 48h on the SpectraMax iD3 (Molecular Devices).

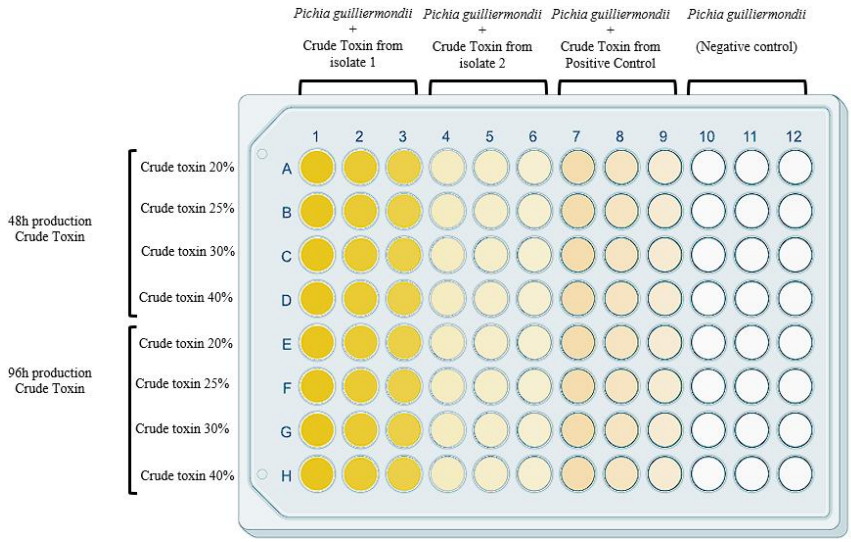


Figure 3.1 Scheme of the microplate organisation used to check the *killer* activity of the crude toxin produced at 48h and 96h. (created in Biorender)

3.6.3. Toxin Quantification

The Bradford method (Bradford, 1976) was used to quantify the *killer* toxin. This was based on method based on measuring the absorbance of the interaction between proteins and Coomassie Brilliant Blue (Bradford reagent), using bovine serum albumin (BSA) as the standard protein.

Firstly, standard solutions of BSA at different concentrations (0 ug/mL - 800 ug/mL) were prepared, and 10 uL of the standard solution and 10 uL of the solution with the unknown protein concentration were added to each well of the microplate, as shown in Figure 3.2.

200uL of Bradford reagent (1:5) was added to each well, and then the microplate was incubated at 37º C for 5 minutes. Finally, the microplate was read at 595 nm on the SpectraMax iD3 (Molecular Devices).

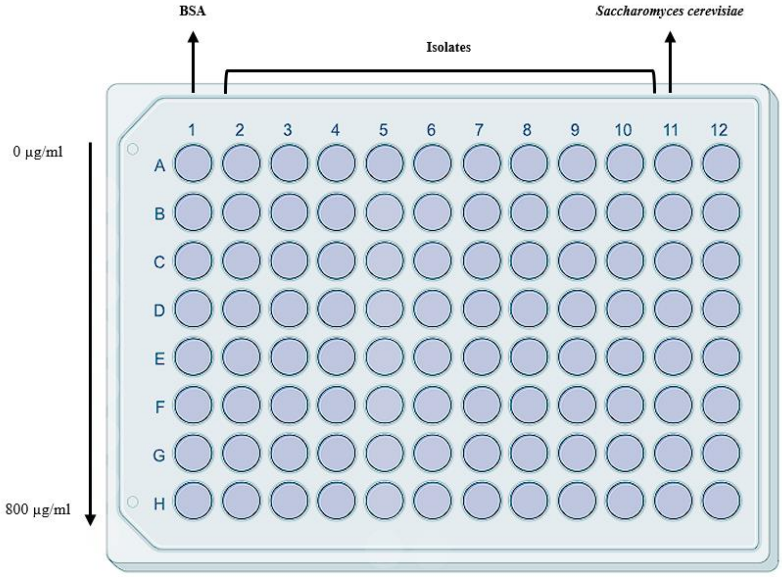


Figure 3.2 Scheme of the microplate organisation used to quantify the toxin by Bradford method. (created in Biorender)

3.7.Yeast identification

After checking which isolates from wine must showed *killer* activity, it was important to identify these yeasts in order to study the proteins responsible for this activity *against Pichia guilliermondii*.

3.7.1. DNA Extraction of Yeasts

To perform the molecular identification, the DNA of the isolated yeasts was extracted using the Wizard Genomic DNA Purification Kit (Promega).

A culture of the yeast of interest was grown for 20h in YMB Medium, after that, the culture was centrifuged at 16000 × g for 2 minutes. The supernatant was removed, and the cells were resuspended in 293 µl of 50 mM EDTA and 7,5 µl of Lyticase (75 units/µl) was added, and gently mixed. Then the samples were incubated for 60 minutes at 37° C, to permit the cell wall digestion, and were cooled at room temperature.

The samples were centrifuged, once again, with the same conditions described above, and the supernatant was removed. To the cell pellet was added 300 µl of Nuclei Lysis Solution and gently mixed, following by the addiction of 100 µl of protein precipitation solution and vortexed

vigorously at high speed for 20 seconds. The sample settled on ice for 5 minutes and was centrifuged at 16000 × g for 3 minutes. The supernatant, that was the DNA, was collected to a clean microcentrifuge tube that contained 300 µl of isopropanol at room temperature, the tube was gently mixed by inversion until the thread-like stands of DNA formed a visible mass.

The sample was centrifuged at 16000 × g for 2 minutes, the supernatant was carefully decanted and drained on clean absorbent paper, 300 µl of ethanol at 70% and the tube was gently inverted several times to wash the DNA pellet, followed by another centrifugation (16000 × g, 2 minutes), and the ethanol was carefully removed, the pellet was air-dried for 10-15 minutes and 50 µl of DNA Rehydration solution were added.

To the purified DNA sample was added 1,5 µl of RNase solution, the sample was vortexed for 1 second and was briefly centrifuged for 5 seconds to collect the liquid and was incubated at 37° C for 15 minutes.

The DNA was rehydrated by incubating the solution at 4°C and its concentration and purity was quantified by measuring its absorbance at 260 nm and 280 nm, with NanoDrop 2000/c (Thermo Fisher Scientific).

Electrophoresis (1% w/v agarose gel stained with Greensafe Premium (NZYtech)) was carried out to check the integrity of the DNA and visualized using the Kodak DC290 camera (Kodak) and the Kodak 1D software (Kodak). Finally, the DNA was stored at -20°C.

3.7.2. Amplification of ITS region

Two ITS primers were used to amplify the ITS region: ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (Integrated DNA Technologies) (Mathabatha E. Setati, Jacobson, and Bauer 2015). To 100 ng of DNA, a 50 µL reaction mixture was prepared with 5 µL of 5x Colourless GoTaq Flexi Buffer (Promega), 4 µL of 25 mM MgCl2, 0,5 µL 10 mM dNTPs, 1 µL of primer ITS1 at 10 pmol/µL, 1 µL of primer ITS4 at 10 pmol/µL, 0.125 µL of GoTaq G2 Flexi DNA polymerase (Promega) and Molecular Biology Water (Lonza) up to 50 µL.

The PCR amplification conditions consisted of a denaturation cycle of 95°C for 6 min, 35 cycles of annealing (94°C for 40 s, 53°C for 40 s, 72°C for 1 min) and an extension cycle of 72°C for 5 min.

3.7.3. Purification of PCR products

The remaining 48 µL of each PCR product already amplified for ITS region, were purified using Wizard SV Gel and PCR Clean-Up System kit (Promega), according to the manufacturer's instructions.

Briefly, 48 µL of Membrane Binding Solution were added to the PCR product. The mixture was transferred into the Minicolumn assembly and incubated at room temperature for 1 min. After being centrifuged (16,000 x *g* for 1 min at 4°C) the Minicolumn flowthrough was discarded. The Minicolumn was placed into a new collection tube and 700 µL of Membrane Wash Solution were added. The tubes were centrifuged, the flowthrough discarded, and the membrane was washed one more time with 500 µL of Membrane Wash Solution. The Minicolumn were centrifuged (16,000 x *g* for 5 min at 4°C), the flowthrough discarded, and the centrifugation repeated for 1 min (with the microcentrifuge lid off). The Minicolumn was prudently transferred to a new 1.5 mL tube and 30 µL of Nuclease-Free Water were added. After 1 min of incubation at room temperature, the tubes were centrifuged (16,000 x *g* for 1 min at 4°C) and the Minicolumn discarded.

The purified PCR products DNA concentration was quantified by measurement of its absorbance at 260 nm and 280 nm, with NanoDrop 2000/c (Thermo Fisher Scientific) and stored at –20°C.

Electrophoresis (1% w/v agarose gel stained with Greensafe Premium (NZYtech)) was used to separate each PCR product (2 µL) and visualized using the Kodak DC290 camera (Kodak) and the Kodak 1D software (Kodak).

3.7.4. Sequencing of ITS region

The sequencing process was performed at CCMAR Technologies and Services Platform (Centro de Ciências do Mar, Faro, Portugal). The sequencing process occurred in a 3130xl Genetic Analyzer (Applied Biosystems) using the BigDyeTM Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), the POP7™ Polymer (Applied Biosystems) and the pair of ITS primers: ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3') (Integrated DNA Technologies) (Mathabatha E. Setati, Jacobson, and Bauer 2015). The DNA sequences were assessed through MEGA version 11 (Tamura K, Stecher G, and Kumar S 2021) and the isolates identification was achieved using

the NCBI/GenBank nucleotide blast (BLASTn), by finding the closest match (based on the maximum identity percentage and query cover and along with the lower e-value).

3.8.Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis (SDS- PAGE)

Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis was described for the first time by Laemmli (1970) and consisted in separation of proteins based on their molecular weight.

In this work, the gel was prepared as described by Sambrook and Russell (2001). The separation gel was done at 12,5%, 11% and 10% to verify which one has the better protein bands separation and consist in H_2O, Acrylamide/bis (30%; Bio-Rad), Tris–HCl (1.5 M, pH 8.8), SDS (10%), TEMED (Bio-Rad), Ammonium persulfate (APS;10%).

Then the concentration gel (H2O, Acrylamide/bis (30%; Bio-Rad), Tris–HCl (0.5 M, pH 6.8), SDS (10%), TEMED (Bio-Rad), Ammonium persulfate (APS;10%) was made, and 35 µL of the samples were applied to the wells, at last the gel will be run at 50 mA (200 V) for 30–45 min.

3.9.Zymography

Heussen and Dowdle (1980) described zymography as a technique used to detect and quantify enzymatic activity. With the help of substrate gels and elements of SDS-PAGE, this technique makes it possible to see active enzymes in action by observing how they break down particular substrates placed within the gel.

Zymography was done by two different methods based on the procedure used by Bajaj *et al.*, 2013 (Method 1) and Souto *et al.*, 2019 (Method 2).

3.9.1. Method 1

The gel was prepared as described above. After that it was cut vertically into two halves so that each of the halves contains the *killer* toxin protein sample. One half (half I) of the native gel was stained with Coomassie brilliant blue (CBB), destained and visualized for protein band, while the other half (half II) will be used for zymogram analysis.

To do the zymogram, the protein was eluted from half II (unstained) of the native gel. A gel piece from half II of the native gel was cut from a position corresponding to the location of protein band in the stained half (half I) of the native gel; gel piece was crushed gently in citrate phosphate buffer (50 mM, pH 5) and the contents went through a centrifugation at 3000 × *g* for 5 min at 4 °C (Bajaj *et al.*, 2013). After that, supernatant was used for assaying the *killer* activity by pouring it in the wells on the YMA-MB plate pre-spread with the sensitive yeast of interest. The plates will be incubated at 30 °C for 24 h, and then observed for inhibition zone around the well.

3.9.2. Method of 2

This method was realized based on Souto *et al.* (2019) with some adaptions. The SDS-PAGE was prepared, but two gels were made, one of which contained 0.2% cells of a sensitive yeast.

The gel without cells was stained with Coomassie brilliant blue (CBB), destained and visualized for protein band.

On the other hand, the gel with the sensitive cells, was washed, using a solution of 25 mM Tris (pH 7.5) and 1% Triton X-100, while shaking for 30 minutes, repeating 3 times. After that, in order to permit the enzymes' renaturation, the gel was incubated, with a 25 mM Tris (pH 7.5) and 0.1% Triton X-100 solution, overnight, under constant agitation (Souto *et al.*, 2019). Finally, the gel was stained using a solution of 1% of methylene blue for 2-3h and destained in distilled water, to intensify the activity zones, that are indicative of toxin action (Souto *et al.*, 2019).

3.10. Protein Purification

After zymography, the fractions that presented *killer* activity purified. The purification aims to isolating a specific protein of interest from a complex mixture of cellular components. This way it was easier to study the proteins of interest in detail, in terms of structure, function and research.

3.10.1. Protein Precipitation and Quantification

The sample containing the proteins was precipitated to separate it from the rest of the cell components.

The precipitation carried out by using 500 µL of acetone. This solution was incubated at -20º C for 1h, and after that, was centrifuged (18,000 x *g* for 30 min at 4°C). The pellet containing the proteins was separated from the supernatant.Afterwards, the tube was air-dried, and the dried pellet was sent for sequencing at Mass Spectrometry Unit (described below).

Protein quantification was carried out by measuring absorbance at 260 nm and 280 nm using the NanoDrop 2000/c (Thermo Fisher Scientific), to this purpose citrate-phosphate buffer (50 mM, pH 5) was added to the dried pellet.

3.10.2. Protein identification by MALDI-TOF/TOF

After selecting the most visible protein bands on SDS-PAGE, Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight/Time-of-Flight (MALDI-TOF/TOF) was used to identify the proteins present in these bands. This method combines MALDI ionisation with TOF mass analysers in a configuration that promotes higher sensitivity, speed and resolution. A critical step in the search for biomarkers was the sequencing of profiled peptides using MALDI-TOF/TOF Mass Spectrometry (MS), which adds another level of information content to these proteomic profiles (Leung & Pitts, 2008).

The protein bands of interest were cut into "cubes" of approximately 1-2 mm^3 and were sent for sequencing at Mass Spectrometry Unit (UniMS), ITQB/iBET, Oeiras, Portugal. The protein bands were reduced, alkylated and digested with trypsin (Promega) overnight at 37ºC. The tryptic peptides were desalted and concentrated using POROS C18 (Empore, 3M) and eluted directly onto the MALDI plate using 1 µl of 5 mg/ml CHCA (alpha-cyano-4-hydroxycinnamic acid, Sigma) in 50 % (v/v) acetonitrile and 5 % (v/v) formic acid.

The data was acquired in positive reflector MS and MS/MS modes using a 5800 MALDI-TOF/TOF (AB Sciex) mass spectrometer and using TOF/TOF Series Explorer Software v.4.1.0 (Applied Biosystems). External calibration was performed using CalMix5 (Protea).

The twenty-five most intense precursor ions from the MS spectra were selected for MS/MS analysis.

The raw MS and MS/MS data were analyzed using Protein Pilot Software v. 4.5 (ABSciex) with the Mascot search engine (MOWSE algorithm). The search parameters were as follows: monoisotopic peptide mass values were considered, maximum precursor mass tolerance (MS) of 50 ppm and a maximum fragment mass tolerance (MS/MS) of 0.3 Da. The search was performed against UniProt protein database UniProt with taxonomy restriction to genus of interest. Cysteine carbamidomethylation (C) was set as fixed modifications, methionines oxidation (M), asparagine and glutamine deamidation (NQ) and the transformation of N terminal glutamine to pyroglutamic acid (N-term Q) were set as variable modifications.

Protein identification was only accepted when significant protein homology scores were obtained and at least one peptide was fragmented with a significant individual ion score ($p<0.05$).

3.10.3. Peptide mapping by LC-MS

The proteins were characterised using Liquid Chromatography-Mass Spectrometry (LC-MS). This method consists of enzymatically digesting the protein into smaller peptide fragments, which are then separated by liquid chromatography (LC) and identified by mass spectrometry (MS). Peptide mapping can be used to verify the primary structure of proteins and detect possible post-translational modifications (Yang *et al.*, 2023).

The liquid chromatography was carried out at ITQB/iBET, Oeiras, Portugal as well as the Mass spectrometry data generated by the Mass Spectrometry Unit (UniMS). The files generated by mass spectra (ZenoTOF 7600 mass spectrometer) were converted using ProteoWizard's msconvert.exe tool (v. 3.0.23111-67c7064). Subsequently, searches were carried out using MSFragger (v.4.0), Philosopher (v.5.1.0), and FragPipe (v.21.1).

The database was created from Metschnikowia protein sequences (Taxon ID: 27320), retrieved through UniProt (31052 entries), considering reverse protein sequences such as decoys and common contaminants (cRAP proteins sequences from gpmDB and contaminants from MaxQuant). Protein identifications were considered with FDR<1%.

4. Results and Discussion

4.1. Screening for *killer* phenotypes of indigenous yeasts isolated from grape musts

The initial step in this study was to evaluate all the isolates collected in a previous study by (Lopes *et al.*, 2020) for their ability to inhibit or kill the wine spoilage yeast *Pichia guilliermondii*.

This yeast is recognized as susceptible to *killer* toxins produced by various *killer* yeast species (Lopes *et al.*, 2009).

For this purpose, the YMA-MB medium was employed, onto which *Pichia guilliermondii* was spread along with the biomass of the isolates under investigation. In this assay, the presence of clear zones (halos) around *killer* yeast colonies indicates inhibition of the sensitive yeast.

In this work, sixty isolates have been recovered and tested by the biomass method and nine of these isolates showed *killer* activity. Figure 4.1 shows the isolates that revealed antimicrobial activity. As can be seen, from A to D (Figure 4.1), the isolates that caused an inhibition halo: 16MNAGPC6R1 (I1), 16MNAGPC6R2 (I2), 16MNAGPB6R4 (I3) and 16MNTNPIR8 (I4).

Since the culture medium contains methylene blue, this compound was absorbed by the cells, which lost viability. Thus, five isolates were identified that exhibited this effect on *Pichia guilliermondii* cells. From E to I (Figure 4.1) are the isolates that showed a halo of dead cells, 16MNAGPI66B4 (I5), 17MNTNPIP8 (I6), 16MNAGPB6Br4 (I7), 16MNAGPC6R5 (I8) and 18MNAGPIBo1 (I9). The positive control test in J, using the *killer Saccharomyces cerevisiae* PYCC 4620, demostrated an inhibition halo.

As can be seen, isolates I1 and I2 were the ones that showed the most significant inhibition halo of *Pichia guilliermondii*. On the other hand, the largest halo of dead cells was exhibited by the isolates I5 and I7.

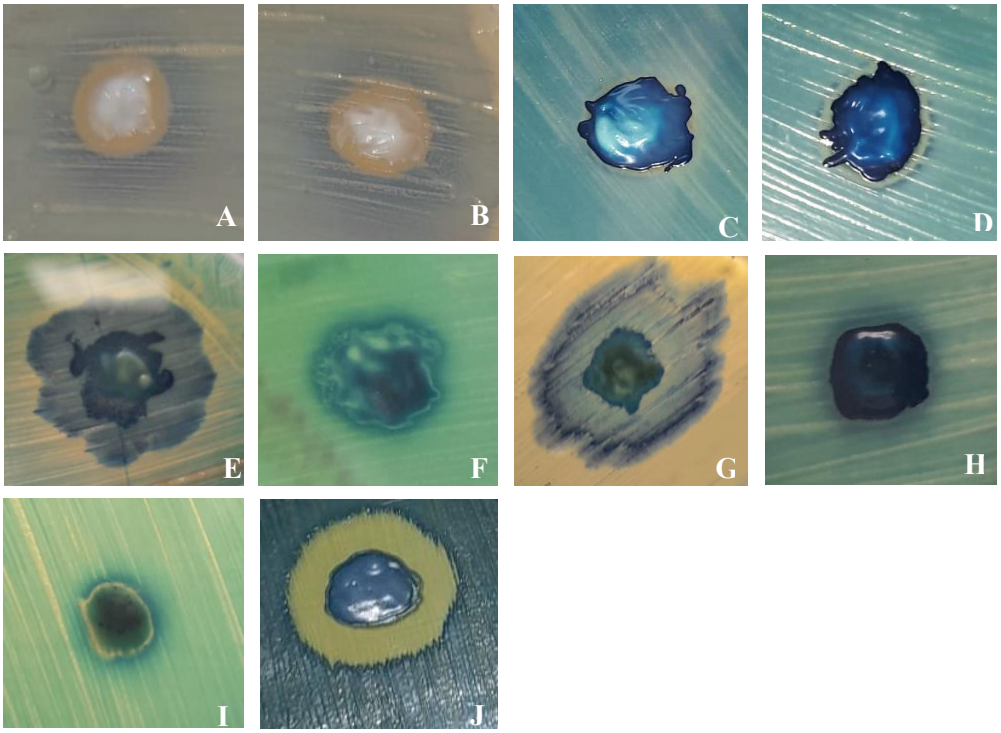


Figure 4.1 Isolates that caused *killer* effects on *Pichia guilliermondii*. **A)** Isolate 1 (I1); **B)** Isolate 2 (I2); **C)** Isolate 3 (I3); **D)** Isolate 4 (I4) **E)** Isolate 5 (I5); **F)** Isolate 6 (I6); **G)** Isolate 7 (I7); **H)** Isolate 8 (I8); **I)** Isolate (I9); **J)** *Saccharomyces cerevisiae* (C+).

4.2. Toxin Production

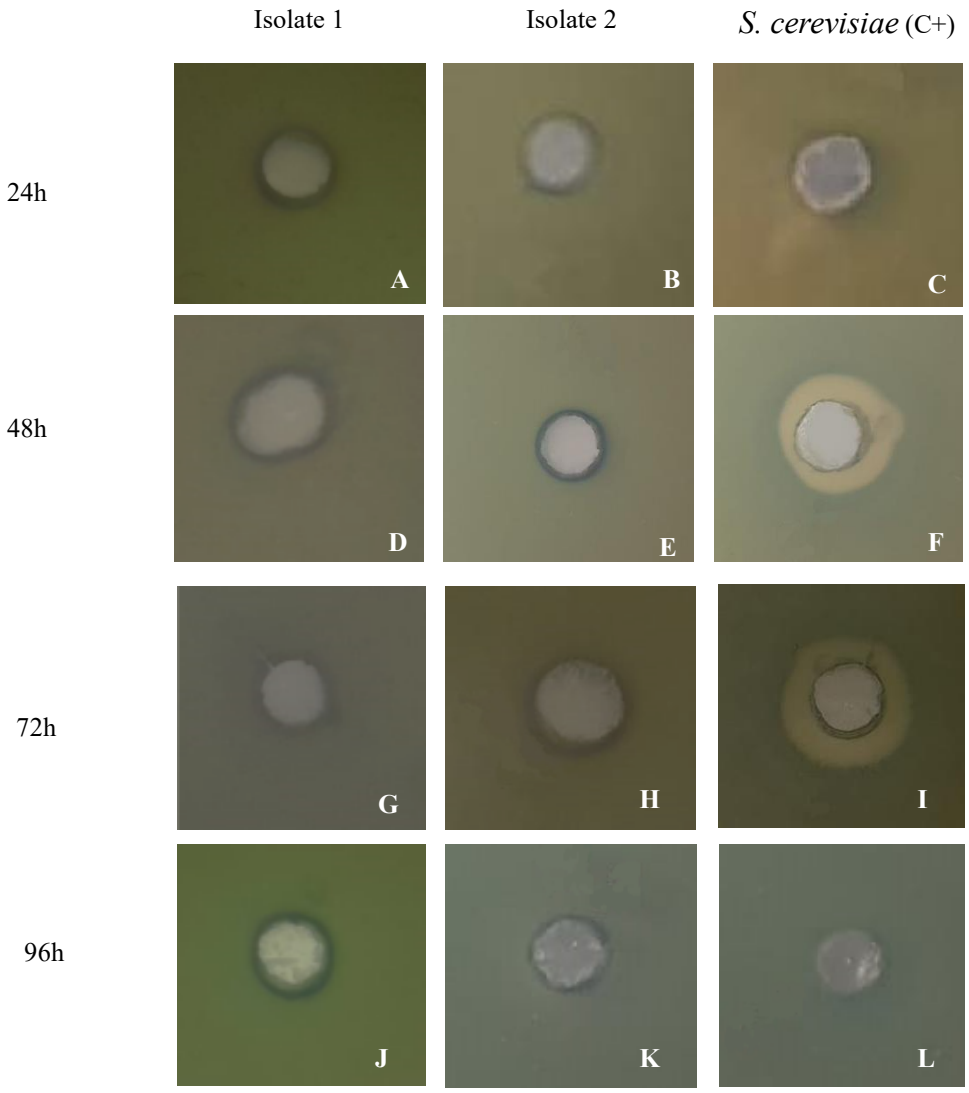
4.2.1. Fermentation for *Killer* production

To check whether the production of *killer* toxins was derived from fermentation, the isolates that showed activity in the previous test were inoculated into fermentation medium, and then, the supernatant resulting from the fermentation (crude toxin) was tested by agar diffusion. Due to the unfavorable results of this technique, only the positive control and isolates 1 and 2 were subjected to testing.

Figure 4.2 shows the tests that were carried out every 24 hours, from 24h until 192h. Isolate 1 is shown in the first column of Figure 4.2 from A to V, isolate 2 in the second column from B to W and finally the positive control in the third column from C to X.

The results for the different hours are shown along the lines, 24 hours from A to C, 48 hours from D to F, 72 hours from G to I, 96 hours from J to L, 120 hours from M to O, 144 hours from P to R, 168 hours from S to U and finally 192 hours from V to X (Figure 4.2).

As can be seen in F and I (Figure 4.2) corresponding to the positive control and at 48h and 72h respectively, they showed inhibition halos. No other inhibition halo was seen over the remaining hours or for isolates 1 and 2.



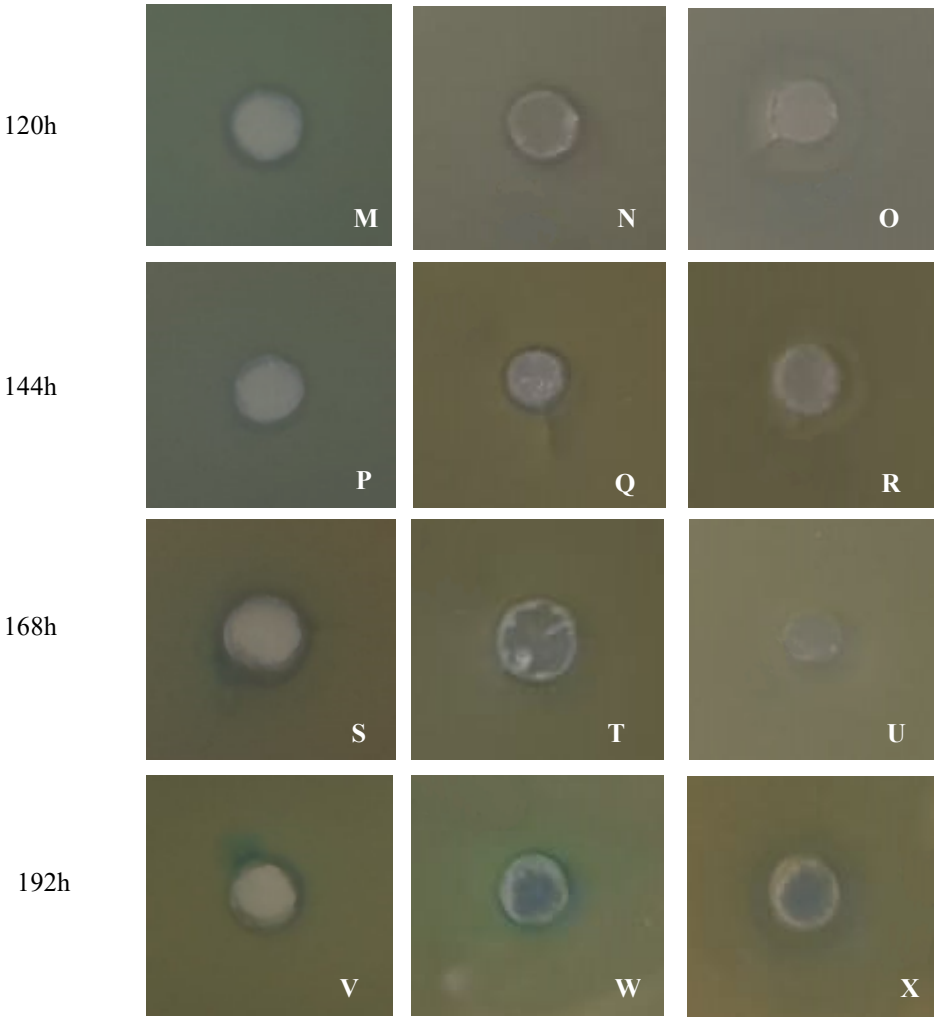


Figure 4.2 Agar diffusion test for *killer* toxin production by fermentation. The hours are arranged in a horizontal alignment. **A) -C)** 24h; **D) -F)** 48H; **G) -I)** 72h; **J) -L)** 96h; **M) - O)** 120h; **P) - R)** 144h; **S) -U)** 168h; **V) -X)** 192h.
The isolates and the positive control are arranged in a vertical alignment. **A) -V)** Isolate 1; **B) - W)** Isolate 2; **C) -X)** *Saccharomyces cerevisiae* (C+).

For this reason, a microplate was prepared as shown in Figure 3.1 and then the absorbance at 600 nm was measured. The graphs in Figures 4.3 and 4.4 show the results of the absorvance corresponding to the growth curve of *Pichia guilliermondii* with the presence of the crude toxins produce in 48h and 96h by the isolates 1 and 2 and *Saccharomyces cerevisiae* (C+).

The graphs corresponding to the toxin production test at 48 hours (Figure 4.3) show that, increasing the concentration of the toxin, did not inhibit the growth of *Pichia guilliermondii*, as the differences in growth shown by the negative control, positive control and isolates 1 and 2 are not significant.

In graph D (Figure 4.3), where the toxin concentration is 40%, it is possible to see a clearer difference between the negative control, positive control and isolates 1 and 2. Although there was a difference, it was not significant, which is why the toxin produced at 96 hours was checked.

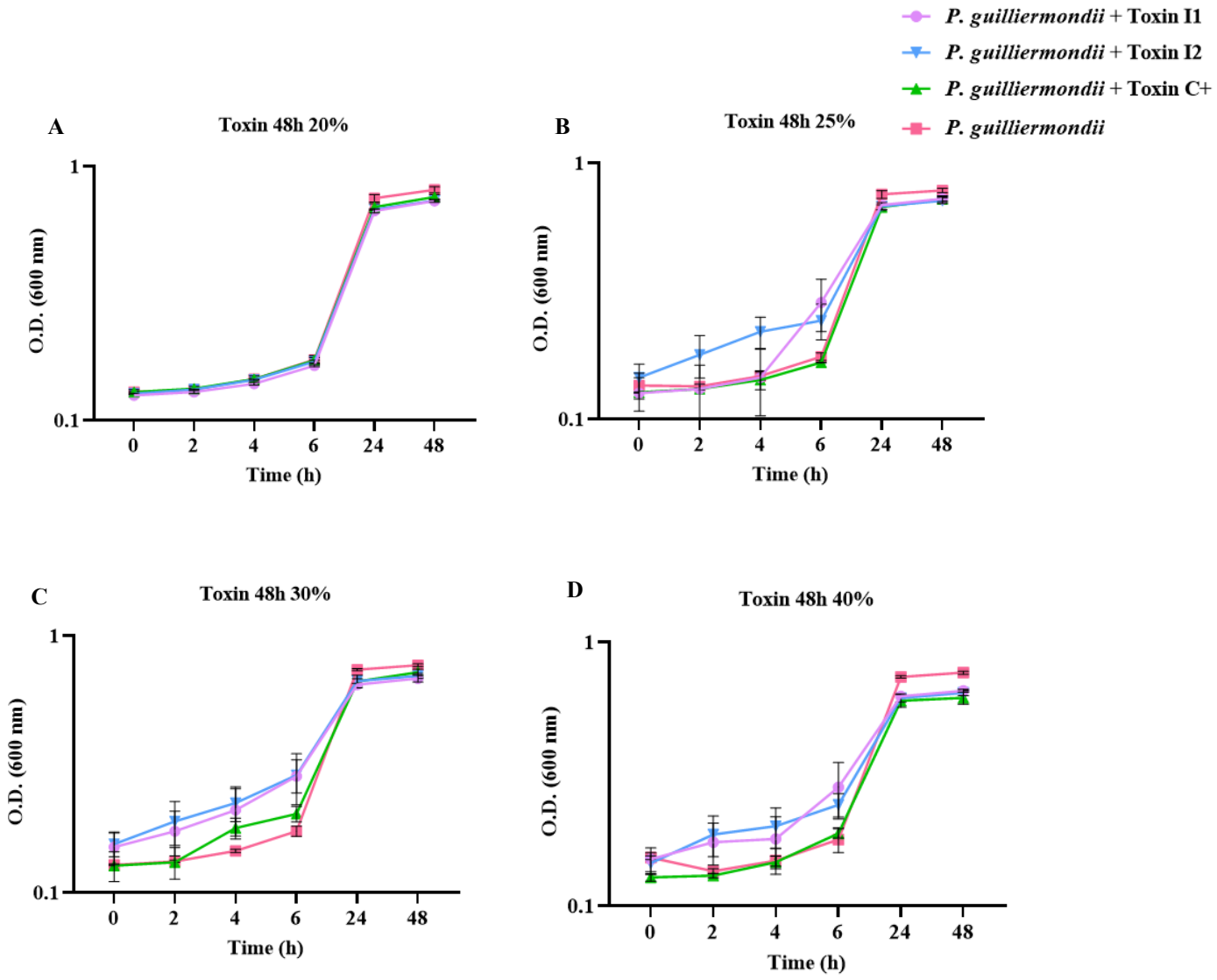


Figure 4.3 Growth curve of *Pichia guilliermondii* with the presence of *killer* toxins produced in 48 hours from the isolates 1, 2 and positive control at different concentrations: A) 20% Toxin; B) 25% Toxin; C) 30% Toxin; D) 40% Toxin
The negative control is *Pichia guilliermondii*, with absence of toxins.
There were no significant differences.

The results with the toxin produced at 96 hours (Figure 4.4) showed the same results as at 48 hours, although when the toxin was at 40% concentration (figura 4.4 D) there was a slight difference in the growth of *Pichia guilliermondii*.

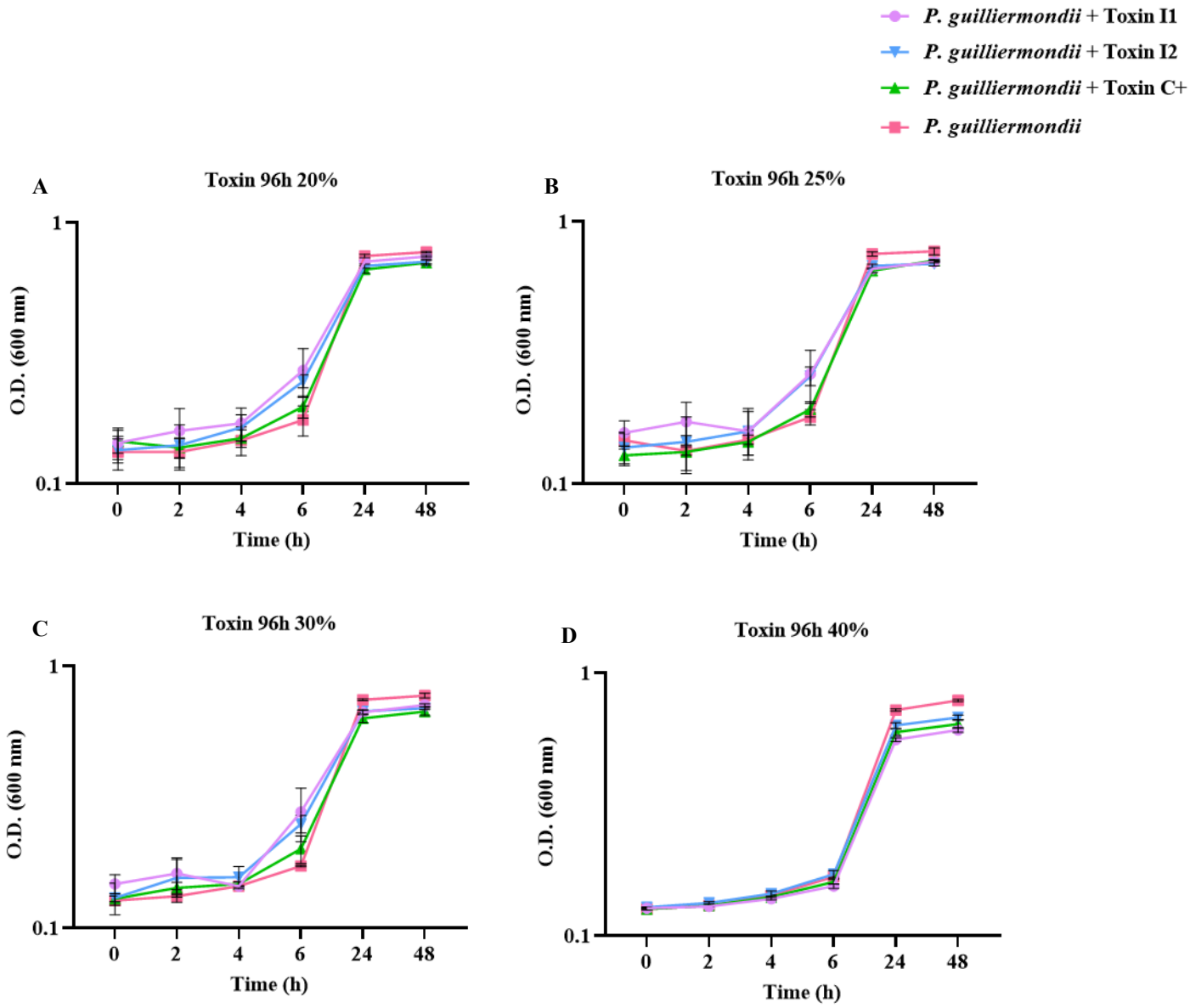


Figure 4.4 Growth curve of *Pichia guilliermondii* with the presence of *killer* toxins produced in 96 hours from the isolates 1, 2 and positive control at different concentrations: A) 20% Toxin; B) 25% Toxin; C) 30% Toxin; D) 40% Toxin
The negative control refers to *Pichia guilliermondii*, with absence of toxins.
As with the toxin produced in 48 hours, there were no significant differences in the growth of the isolates either.

When comparing the results obtained in the agar diffusion test (Figure 4.2) with those from the graphs (Figures 4.3 and 4.4), it is interesting to note that, in the graphs, it was not possible to distinguish the effect of the positive control and isolates 1 and 2. However, in the agar diffusion test, differences were observed, but only at 48h and 72h (Figure 4.2 F and I). This suggests that the positive control ceases to produce *killer* toxins as fermentation progresses.

Since consistent toxin production was not achieved in the *killer* toxin fermentation assay, alternative techniques were explored, as detailed below.

4.2.2. *Killer* toxin enrichment

The enrichment for *killer* toxin production was carried out using a medium that promotes this activity, with the pH adjusted to between 4 and 5. The *killer* yeast was inoculated in YMB medium with a pH between 4 and 5, and following overnight growth, it was tested on a plate inoculated with the sensitive yeast.

Unexpectedly, the toxin of positive control was not produced or was inactive, as no inhibition halos were observed in A and B (Figure 4.5). In B and D (Figure 4.5), the results were as expected, since the toxin was supposed to be inactive due to incubation at 98º C.

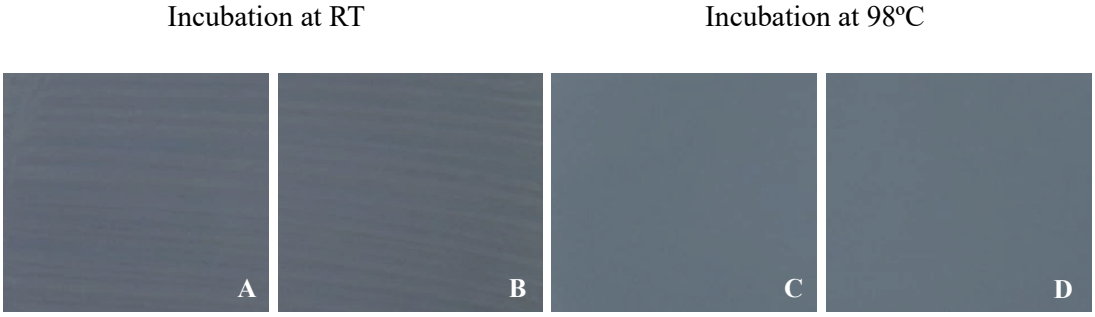


Figure 4.5 Test of *Killer* toxin enrichment. A) and B) contained the same amount of toxin, 10 µL.
C) and D) contained 50 µL. A) and C) were incubated at room temperature (RT), on the other hand, B) and D) were incubated at 98º C, to inactivate the *killer* activity. The result was the same with different amounts of toxin and different incubation conditions, showing that the toxin is not active in A) and C).

Given the results obtained in this test, it was hypothesized that this toxin would only be produced when the *killer* yeast is under stress, such as in a competitive environment.

4.2.3. Competition assay

The competition test began with the simultaneous inoculation of the sensitive yeast and the *killer* yeast. This inoculation was carried out in different proportions in order to see if one of them produced more toxin.

The results, displayed in Figure 4.6, from the tests conducted between the positive control, *Saccharomyces cerevisiae* PYCC 4620, and the sensitive yeast, *Pichia guilliermondii*, show that no toxin production occurred when the cultures were grown together in a liquid medium. These co-culture tests suggest that toxin production does not seem to be influenced by competition between the *killer* yeast and the sensitive yeast.

Figure 4.6 Competition assay results. *Pichia guilliermondii* and positive control in the proportion: A) 1:1; B) 1:2; C) 1:3. No inhibition halos formed, indicating that *killer* toxin production does not appear to be influenced by competition.

4.2.4. Isolation of *killer* enzyme from inhibition zone

Since none of the tested assays were yielding the expected results, it was decided to isolate the toxin from the inhibition halos produced using the biomass method. For this, the 1.5% agar in YMA-MB medium was replaced with 0.8% agar and 1.25% low melting agarose, with the aim of facilitating easier elution for subsequent toxin separation.

Once again, only the positive control was tested, as the result was not as expected. After elution of the inhibition halo area in buffer, it was tested using agar diffusion. The zone of inhibition was more easily extracted from the agarose low melting, and so showed a better halo of inhibition, although the differences are not very significant (Figure 4.7).

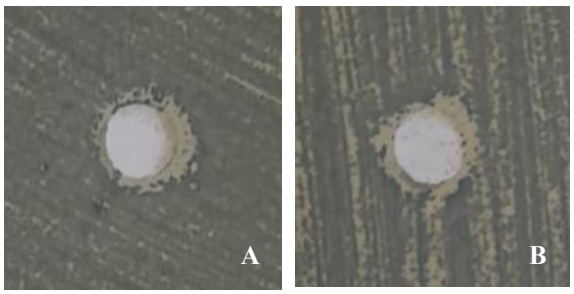


Figure 4.7 Toxin from inhibition zone. A) Medium with 0.8% agar; B) Medium with 1.25% Low melting agarose. In B) it was possible to extract the toxin better, but the halo of inhibition produced was very small compared to the halo produced in the biomass test.

For this reason, and because this method is not sustainable for all yeasts that exhibited *killer* activity, since some did not produce inhibition halos and others did not have a sufficient inhibition area to allow isolation, a search for another method became necessary.

4.2.5. Mechanic extraction

Mechanical extraction was carried out by breaking the cell wall with the help of glass beads, as, contrary to what would be expected, these yeasts also showed the production of the toxin at the level of the cell extract.

First, the yeasts were inoculated in YMB medium and after overnight growth, a centrifugation was performed in which the supernatant was removed and tested in agar diffusion and the pellet went through the mechanical extraction process. The result of this extraction was eluted in buffer and centrifuged again in order to separate the remaining cellular contents from the proteins of interest. Therefore, proteins eluted in buffer were tested in agar diffusion.

Figure 4.8 shows the inhibition halos produced by the cell extracts of the isolates. The most significant results in this test were isolates 1 and 2, represented in A and B (Figure 4.8), respectively. The remaining isolates represented from C to E (Figure 4.8) showed not very significant inhibition halos. The positive control is represented in F (Figure 4.8) and showed a halo of inhibition when the cell extract was tested.

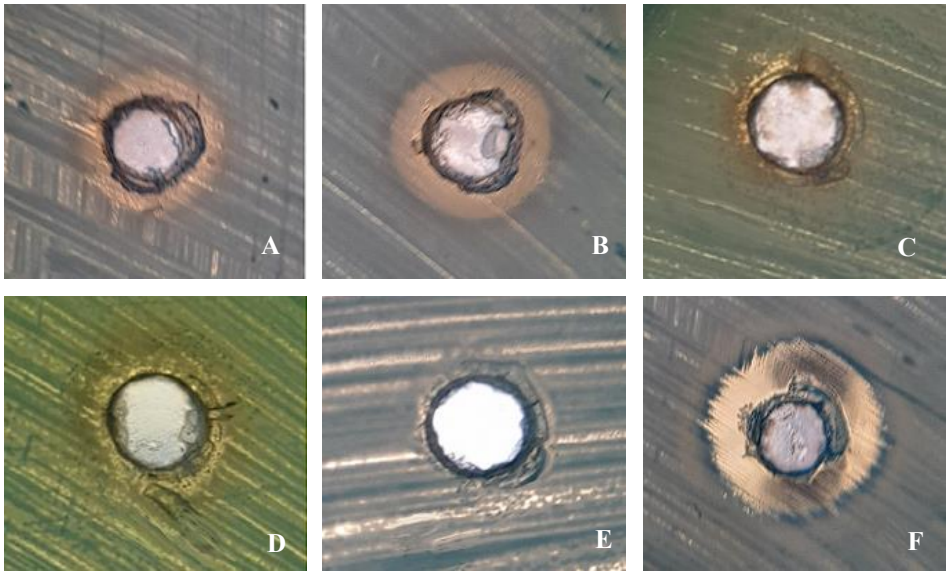


Figure 4.8 Inhibition of growth produced by the cell extracts of the isolates and positive control. A) I1; B) I2; C) I3; D) I4; E) I6; F). *S. cerevisae* as positive control. Isolate 2 showed the best results.

The supernatants were tested (Figure 4.9) and some of the isolates showed growth inhibition, although the halos produced were not as significant as those shown in Figure 4.8.

Isolates 3 and 9 (Figure 4.9 A and D, respectively) showed the most significant halos when compared to the other isolates (Figure 4.9 B, C and E)

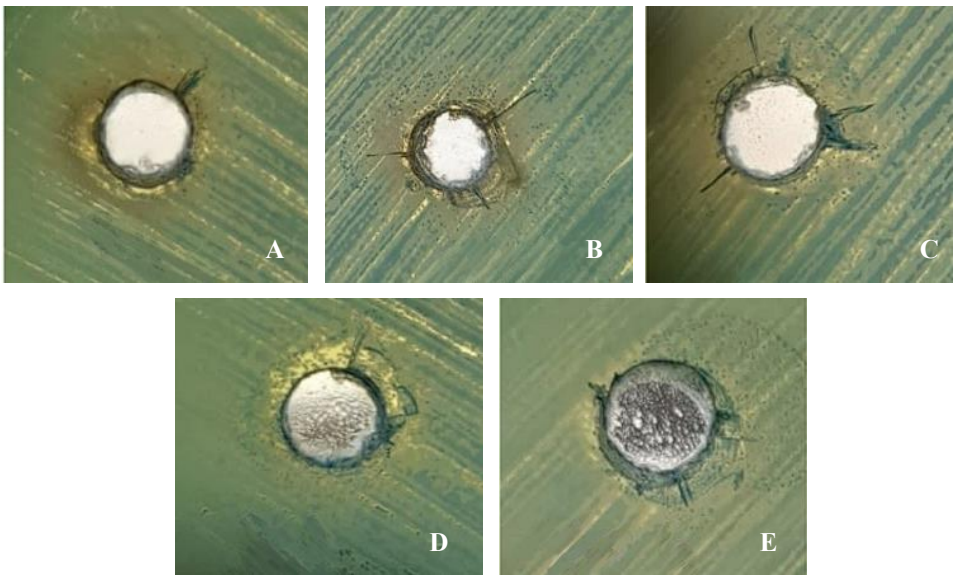


Figure 4.9 Halo of inhibition produced by the supernatant. A) I3; B) I8; C) I5; D) I9; E) I7. Isolate 3 showed the best results.

In this method, it was possible to isolate the toxins from yeasts that showed *killer* activity. These results suggest that not all tested yeasts produced toxins in the same manner, indicating that they may not belong to the same species or even the same genus, as might have been expected. Consequently, the identification of the yeast isolates was carried out.

4.3.Isolates Identification

4.3.1. DNA extraction

It was important to identify the yeasts isolated from the wine must in order to understand what had already been studied about them, particularly their *killer* potential, and to identify which proteins were responsible for the *killer* phenotype.

The first step was to extract the DNA using the Wizard Genomic DNA Purification Kit (Promega), as described above. After the extraction, the DNA was quantified, as shown in Table 4.1, and an electrophoresis gel was used to check the integrity of the DNA (Figure 4.10).

As can be seen in Table 4.1, the A260/A280 ratio was used to check the quality of the DNA, which means without the presence of contaminants such as protein or phenolic compounds. DNA is of good quality if the values of this ratio are between approximately 1.8 and 2. In terms of the isolates shown in Table 4.1, it can be seen that only two of them (isolates 7 and 9) had lower than expected DNA quality, which is also reflected in the quantity of DNA, which was lower than that of the other isolates. On the other hand, isolate 5 also had a lower quantity of DNA, but with good quality.

Table 4.1 Quantification of DNA. The purity of the DNA was based on the A260/A280 ratio. Values between 1.8 and 2, the DNA is considered pure.

Isolates	1	2	3	4	5	6	7	8	9
DNA (ng/µL)	102,5	39,9	103,5	190,6	41,5	116	35,2	117,2	20,9
A260	2,049	0,797	2,069	3,813	0,829	2,335	0,704	3,544	0,417
A280	1,024	0,451	1,038	1,907	0,465	1,199	0,461	1,790	0,250
A260/A280	2,00	1,77	1,99	2,00	1,78	1,95	1,53	1,98	1,67

The electrophoresis gel used for checking DNA integrity visually confirms that the DNA is not contaminated or degraded. It is crucial to assess the quality of the DNA before carrying out other techniques, such as polymerase chain reaction (PCR). This way, if high molecular weight smear or a single thick band appears, we can consider the DNA to be of good quality. In the gels it is also important to use a negative control in order to validate the quality of the experimental result, which must not show any bands.

In the electrophoresis gel shown in Figure 4.10, the negative control only contained the loading buffer without the presence of DNA and showed no band. As for the isolates, the result was as expected since they all showed a single band. Isolates 7 and 9, as verified by the A260/A280 ratio, showed lower integrity, which was confirmed visually by the weak bands in each of them.

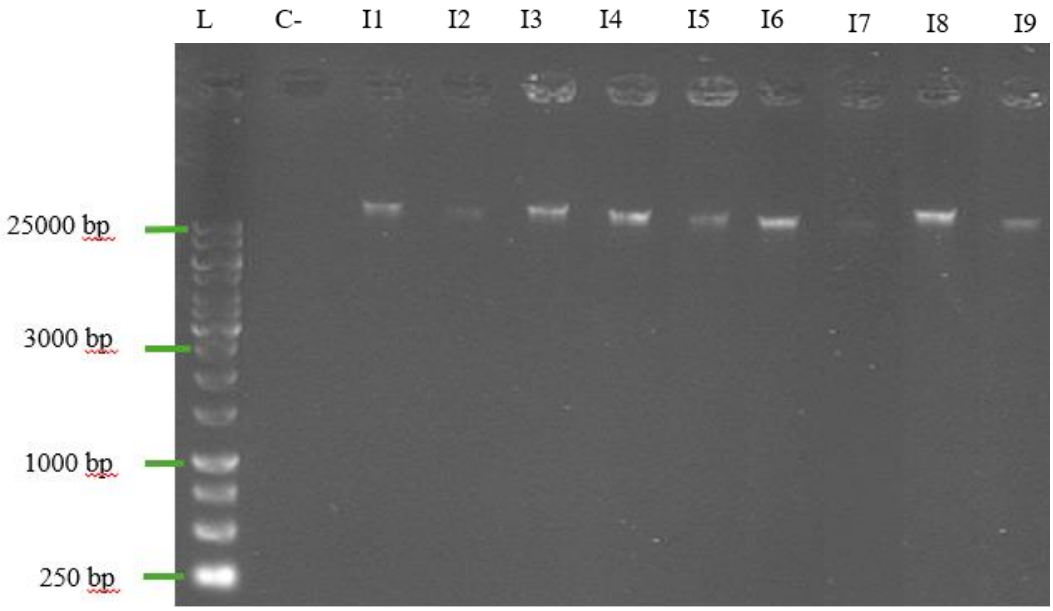


Figure 4.10 Electrophoresis gel of the Genomic DNA. As expected, each isolate showed a single band, and the negative control was clean.
Ladder (L): O'GeneRuler 1KB Plus (Thermo Scientific).
C^-: Only loading buffer with the absence of DNA.

4.3.2. Purification of PCR products

The ITS region of the DNA was amplified using a PCR reaction in which the ITS1 and ITS4 primers were used. These primers are normally used to identify fungi.

The results of the quantification of the PCR products are shown in Table 4.2. As explained in the previous point, it was essential to check the quality of the DNA, in this case amplified for the ITS region.

When the quality of the PCR products was checked, there were no significant differences in terms of the A260/A280 ratio, except for isolate 9, which showed higher integrity, in contrast to what was previously seen in the integrity of the DNA before it underwent PCR. Regarding the amount of DNA, isolate 6 stood out the most.

Table 4.2 Quantification of PCR products by spectrophotometry. The absorbance at (A260) estimate DNA concentration. The quality of the DNA was performed by the A260/A280 ratio.

Isolates	1	2	3	4	5	6	7	8	9
DNA (ng/µL)	40,1	40,0	54,3	46,5	44,4	67,0	46,4	45,1	35,4
A260	0,802	0,799	1,085	0,929	0,888	1,340	0,927	0,902	0,708
A280	0,448	0,448	0,620	0,520	0,505	0,751	0,525	0,520	0,379
A260/A280	1,79	1,79	1,75	1,79	1,76	1,78	1,77	1,74	1,87

As with checking the integrity of the DNA, the PCR products were also visualised on the electrophoresis gel (Figure 4.11). The negative control, which contained all the reagents used in the reaction except the DNA, was clean. As expected, given the results of the A260/A280 ratio, it was possible to visualise single, well-defined bands.

Isolate 6 stood out from the other isolates because it had a different size, thus higher number of base pairs (~400bp). This may have been because this isolate belongs to a relatively different species from the other isolates.

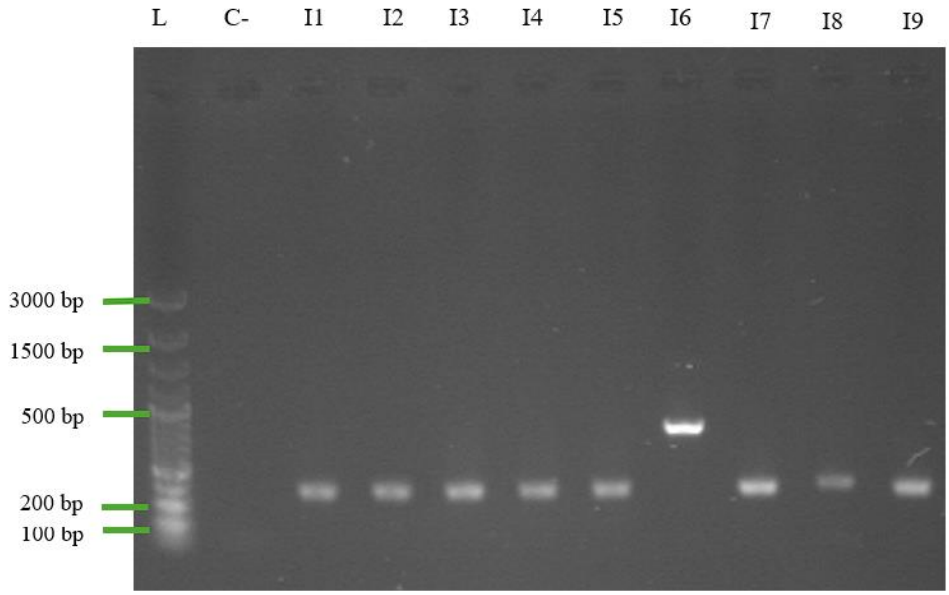


Figure 4.11 PCR analysis on electrophoresis gel. Each isolate showed a single band, and the negative control was clean. The isolate 6 showed a band with different base pairs (~400 bp) from the other isolates, which may indicate that it belongs to a species that is more distant from the species of the other isolates.
Ladder - O'GeneRuler 100 bp Plus (Thermo Scientific)
C-: Only loading buffer with the absence of DNA.

4.3.3. Sequencing results

The PCR products were sequenced according to the ITS region. The results obtained for ITS1 and ITS4 were aligned using MEGA software, the aligned sequence was identified using BLASTn, the identification of the isolates is shown in Tables 4.3 and 4.4.

The isolates (1 to 4) shown in Table 4.3 were identified as belonging to the species *Metschnikowia pulcherrima*. Table 4.4 shows the remaining isolates: isolates 5 and 7 correspond to *Metschnikowia cibodasensis*, isolate 6 to *Saccharomyces uvarum*, isolate 8 to *Metschnikowia chrysoperlae* and finally isolate 9 to *Pichia terricola*.

Relating the identification of the isolates to the results obtained in the biomass method (Figure 4.1), it was possible to verify that the isolates that caused growth inhibition halos in *Pichia guilliermondii* are from the same species.

However, the inhibition halos produced when tested against *Pichia guilliermondii* were different from each other, which may indicate that although they belong to the *Metschnikowia pulcherrima* species, they may belong to different strains.

On the other hand, the isolates that produced the dead cell halo correspond to the remaining isolates belonging to other species.

The yeast Metschnikowia pulcherrima is one of the most studied, however this yeast is known to be a non-traditional *killer* yeast because its *killer* phenotype is linked to the pulcherrimin pigment (Morata *et al.*, 2019). It was therefore necessary to carry out a protein study to check for the existence of proteins with *killer* toxin function.

Table 1.3 Isolates identification. The first 5 results presented on blastn were selected, and the first result for each isolate was considered the most correct.

Isolate	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident
1	*Metschnikowia pulcherrima* NRRL Y-7111 ITS region; from TYPE material	*Metschnikowia pulcherrima*	123	123	80%	4,00E-29	100.00%
	Metschnikowia chrysoperlae ATCC MYA-4304 ITS region; from TYPE material	*Metschnikowia chrysoperlae*	123	123	80%	4,00E-29	100.00%
	Metschnikowia shishimaru ITS region; from TYPE material	*Metschnikowia shishimaru*	100	100	80%	3,00E-22	93.75%
	Metschnikowia pimensis ATCC MYA-4306 ITS region; from TYPE material	*Metschnikowia pimensis*	83.4	83.4	80%	5,00E-17	89.06%
	Metschnikowia gelsemii CBS 10509 ITS region; from TYPE material	*Metschnikowia gelsemii*	64.1	64.1	48%	3,00E-11	94.87%
2	*Metschnikowia pulcherrima* NRRL Y-7111 ITS region; from TYPE material	*Metschnikowia pulcherrima*	150	238	100%	6,00E-37	100.00%
	Metschnikowia chrysoperlae ATCC MYA-4304 ITS region; from TYPE material	*Metschnikowia chrysoperlae*	150	150	50%	6,00E-37	100.00%
	Metschnikowia shishimaru ITS region; from TYPE material	*Metschnikowia shishimaru*	145	145	50%	3,00E-35	98.77%
	Metschnikowia pimensis ATCC MYA-4306 ITS region; from TYPE material	*Metschnikowia pimensis*	145	145	50%	3,00E-35	98.77%
	Scolecopeltidium wangtianshuiense IFRD 9302 ITS region; from TYPE material	*Scolecopeltidium wangtianshuiense*	76.8	76.8	27%	1,00E-14	97.73%
3	*Metschnikowia pulcherrima* NRRL Y-7111 ITS region; from TYPE material	*Metschnikowia pulcherrima*	119	119	81%	5,00E-28	98.46%
	Metschnikowia chrysoperlae ATCC MYA-4304 ITS region; from TYPE material	*Metschnikowia chrysoperlae*	119	119	81%	5,00E-28	98.46%
	Metschnikowia shishimaru ITS region; from TYPE material	*Metschnikowia shishimaru*	85.3	85.3	66%	1,00E-17	94.34%
	Metschnikowia pimensis ATCC MYA-4306 ITS region; from TYPE material	*Metschnikowia pimensis*	79.5	79.5	81%	7,00E-16	87.69%
	Metschnikowia chrysomelidarum ATCC MYA-4344 ITS region; from TYPE material	*Metschnikowia chrysomelidarum*	41.1	41.1	42%	3,00E-04	91.43%
4	*Metschnikowia pulcherrima* NRRL Y-7111 ITS region; from TYPE material	*Metschnikowia pulcherrima*	123	123	80%	4,00E-29	100.00%
	Metschnikowia chrysoperlae ATCC MYA-4304 ITS region; from TYPE material	*Metschnikowia chrysoperlae*	123	123	80%	4,00E-29	100.00%
	Metschnikowia shishimaru ITS region; from TYPE material	*Metschnikowia shishimaru*	100	100	80%	3,00E-22	93.75%
	Metschnikowia pimensis ATCC MYA-4306 ITS region; from TYPE material	*Metschnikowia pimensis*	83.4	83.4	80%	5,00E-17	89.06%
	Metschnikowia gelsemii CBS 10509 ITS region; from TYPE material	*Metschnikowia gelsemii*	44.9	44.9	36%	2,00E-05	93.10%

Table 4.4 Isolates identification. The first 5 results presented on blastn were selected, and the first result for each isolate was considered the most correct. Isolate 9 showed only two results.

Isolate	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident
5	*Metschnikowia cibodasensis* UICC Y-335 ITS region; from TYPE material	*Metschnikowia cibodasensis*	119	119	93%	5,00E-28	96.05%
	Metschnikowia reukaufii CBS 5834 ITS region; from TYPE material	*Metschnikowia reukaufii*	114	114	93%	3,00E-26	94.74%
	Metschnikowia chrysomelidarum ATCC MYA-4344 ITS region; from TYPE material	*Metschnikowia chrysomelidarum*	71.8	71.8	83%	1,00E-13	85.07%
	Metschnikowia rancensis CBS 8174T ITS region; from TYPE material	*Metschnikowia rancensis*	68.0	68.0	83%	2,00E-12	86.57%
	Metschnikowia vanudenii CBS 9134 ITS region; from TYPE material	*Metschnikowia vanudenii*	66.1	66.1	83%	8,00E-12	88.24%
6	*Saccharomyces uvarum* CBS 395 ITS region; from TYPE material	*Saccharomyces uvarum*	142	142	96%	6,00E-35	98.70%
	Saccharomyces eubayanus PYCC 6148 ITS region; from TYPE material	*Saccharomyces eubayanus*	142	142	96%	6,00E-35	98.70%
	Saccharomyces kudriavzevii ATCC MYA-4449 ITS region; from TYPE material	*Saccharomyces kudriavzevii*	142	142	96%	6,00E-35	98.70%
	Saccharomyces mikatae ATCC MYA-4448 ITS region; from TYPE material	*Saccharomyces mikatae*	142	142	96%	6,00E-35	98.70%
	Saccharomyces bayanus CBS 380 ITS region; from TYPE material	*Saccharomyces bayanus*	137	137	92%	3,00E-33	98.65%
7	*Metschnikowia cibodasensis* UICC Y-335 ITS region; from TYPE material	*Metschnikowia cibodasensis*	123	123	87%	4,00E-29	97.14%
	Metschnikowia reukaufii CBS 5834 ITS region; from TYPE material	*Metschnikowia reukaufii*	117	117	87%	2,00E-27	95.71%
	Metschnikowia rancensis CBS 8174T ITS region; from TYPE material	*Metschnikowia rancensis*	58.4	58.4	80%	2,00E-09	87.50%
	Metschnikowia vanudenii CBS 9134 ITS region; from TYPE material	*Metschnikowia vanudenii*	56.4	56.4	51%	6,00E-09	90.24%
	Metschnikowia chrysomelidarum ATCC MYA-4344 ITS region; from TYPE material	*Metschnikowia chrysomelidarum*	56.4	56.4	80%	6,00E-09	84.38%
8	*Metschnikowia chrysoperlae* ATCC MYA-4304 ITS region; from TYPE material	*Metschnikowia chrysoperlae*	125	125	98%	9,00E-30	96.20%
	Metschnikowia pulcherrima NRRL Y-7111 ITS region; from TYPE material	*Metschnikowia pulcherrima*	123	123	87%	4,00E-29	97.14%
	Metschnikowia pimensis ATCC MYA-4306 ITS region; from TYPE material	*Metschnikowia pimensis*	81.4	81.4	81%	2,00E-16	90.77%
	Metschnikowia shishimaru ITS region; from TYPE material	*Metschnikowia shishimaru*	73.7	73.7	55%	4,00E-14	95.45%
	Metschnikowia chrysomelidarum ATCC MYA-4344 ITS region; from TYPE material	*Metschnikowia chrysomelidarum*	41.1	41.1	42%	3,00E-04	91.43%
9	*Pichia terricola* CBS 2617 ITS region; from TYPE material	*Pichia terricola*	102	102	66%	8,00E-23	100.00%
	[*Candida*] *berthetii* NBRC 10266 ITS region; from TYPE material	*[Candida] berthetii*	35.3	35.3	22%	0.015	100.00%

4.4.SDS-PAGE

SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis) is a widely used technique in molecular biology for separating proteins based on their molecular weight. This will permit further analysis of the protein profile of these *killer* yeasts, identifying and characterizing the *killer* toxins, that these yeasts produce.

To do this, gels were first made with different concentrations in order to achieve the best separation of the protein bands.

SDS-PAGE was only carried out on the isolates with the best inhibition halos (isolates 1 and 2) and best dead cells halos (isolates 5 and 7) or in the case of isolate 3, because it showed *killer* activity in both the supernatant and the cell extract.

Three different gel concentrations were tested: 12.5% (Figure 4.12), 11% (Figure 4.13) and 10% (Figure 4.14).

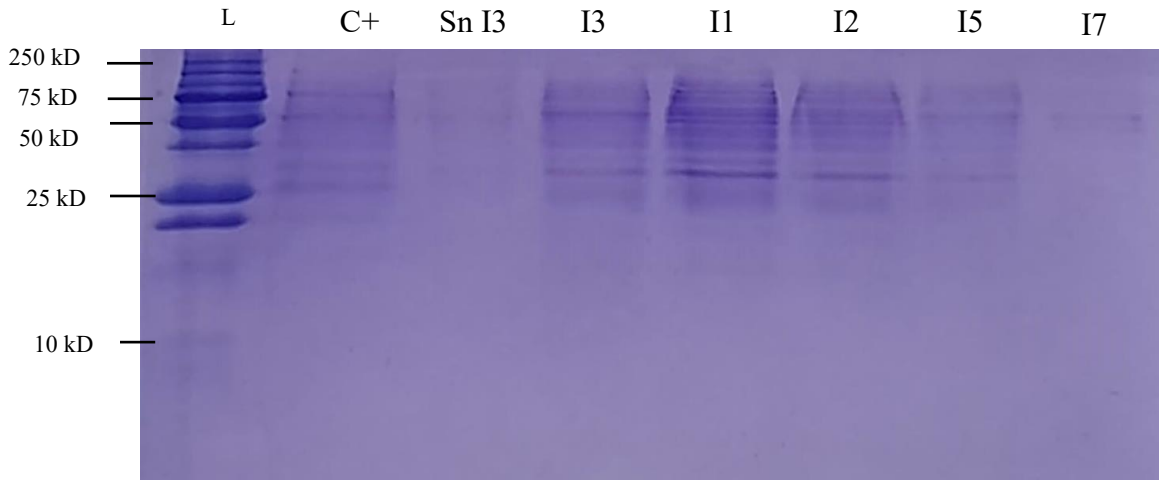


Figure 4.12 SDS-PAGE 12,5%.
Ladder (L): Precision Plus Protein All Blue Standards (Bio Rad)
Sn: Supernatant

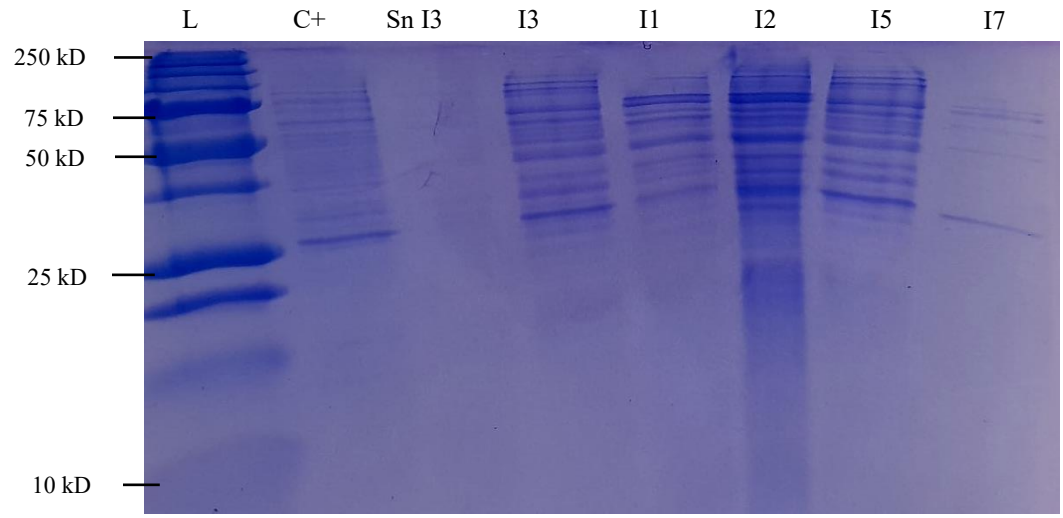


Figure 4.13 SDS-PAGE 11%. Although more separated than in the previous gel, the bands were still very close together.
Ladder (L): Precision Plus Protein All Blue Standards (Bio Rad)
Sn: Supernatant

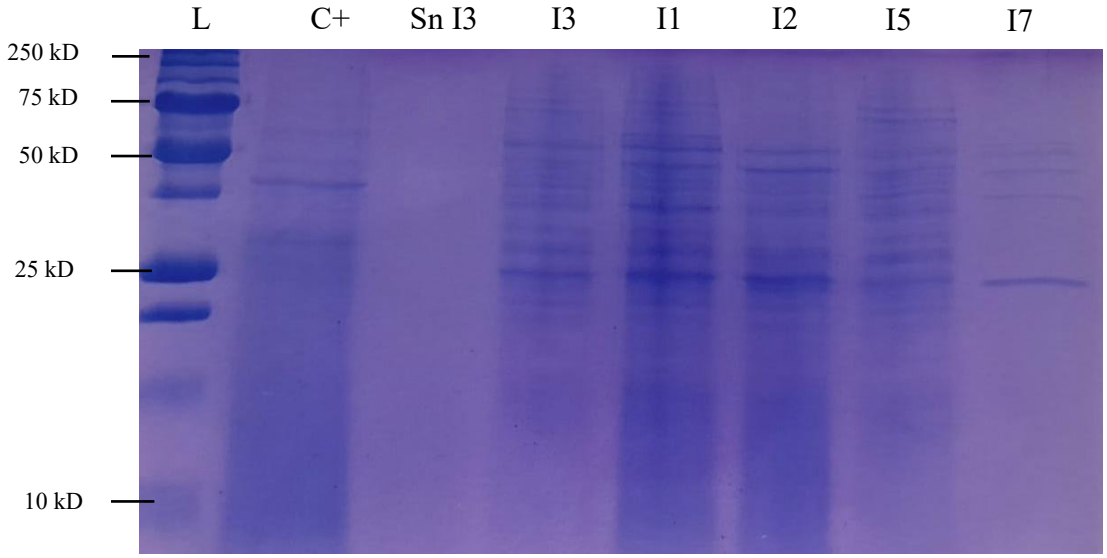


Figure 4.14 SDS-PAGE 10%. This concentration of gel showed the most adequate separation of bands and for this reason it was used in the other gels made throughout the work.
Ladder (L): Precision Plus Protein All Blue Standards (Bio Rad)
Sn: Supernatant

Using a 12.5% polyacrylamide gel, the bands appeared very close to each other. Although they were more separated than in the previous 11% gel, the bands were still quite close. Therefore, we also tested a 10% gel. After experimenting with the three different polyacrylamide gel concentrations, it was observed that the 10% gel provided the best results, displaying the most distinctly separated bands.

SDS-PAGE analysis can be used to compare different protein profiles in different strains, so it is important to note that the protein profiles presented by the positive control and isolates 5 and 7 are notably different from the protein profiles of isolates 1, 2 and 3.
However, it is possible to see that isolates 1, 2 and 3, which all correspond to *Mestchnikowia pulcherrima*, have very similar protein profiles but some differences, which may indicate that they belong to different strains.

4.5. Zymography

Zymography is a powerful electrophoretic technique used to detect enzyme activity, including that of proteases and other hydrolytic enzymes, on a substrate-containing gel. In the context of studying *killer* toxins produced by yeast, zymography can be particularly useful for identifying and characterizing enzyme activities that are responsible for the antimicrobial properties of these toxins.

In this study, the zymography assay was used to evaluate the inhibition of *Pichia guilliermondii* growth by the enzymatic activity of *killer* proteins, using two methods:

- Method 1 consisted of removing the protein fractions from SDS-PAGE and then testing them in agar diffusion to see if the fraction inhibited the growth of the sensitive yeast (Bajaj *et al.*, 2013).
- Method 2 consisted of visualizing the inhibition of growth of the sensitive yeast when it was incorporated into the polyacrylamide gel (Souto *et al.*, 2019).

4.5.1. Method 1

In this test, the fractions of interest were removed from the unstained half of the gel, using the stained gel as a reference for their location. The fractions were crushed in buffer and centrifuged to separate the proteins from the gel. The proteins were then tested in agar diffusion. The positive control and the isolates 1, 2, 3 were considered, as they showed the best results in the extracted protein tests (point 4.2.5.).
To do this, 3 proteins were chosen from the SDS-PAGE as described below for each of the yeasts that were tested with the protein cell extract.

4.5.1.1.Positive Control

The gel fractions selected from the positive control are shown in Figure 4.15, these fractions were then cut into the unstained gel at the corresponding location.

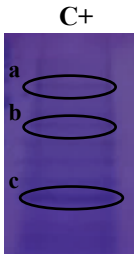


Figure 4.15 SDS-PAGE of Positive Control (C+; *Sacchamromyces cerevisiae*) with the three fractions used at agar diffusion test: a, b and c.

The fractions were tested in agar diffusion (Figure 4.16), as well as the cell extract containing the proteins, and it was observed that none of the fractions caused a halo of inhibition, while in the extract we could observe a large halo of inhibition (fig. 4.16 D). This could mean that none of the fractions contained activity or that the concentration of toxin in the fraction was not sufficient.

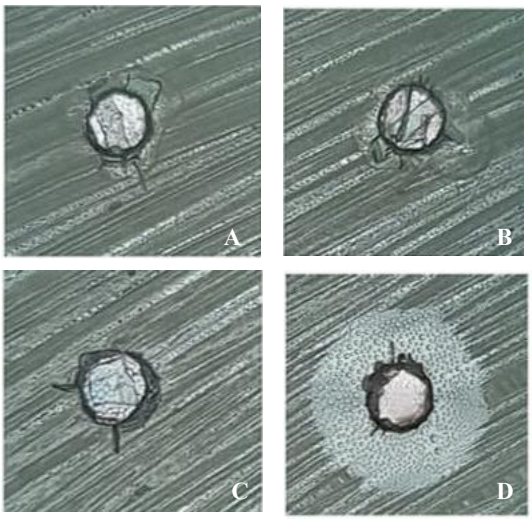


Figure 4.16 Agar diffusion test with Positive control. A) Fraction a; B) Fraction b; C) Fraction c; D) Cell extract.

4.5.1.2. Isolate 1

As with the positive control, the procedure used for isolate 1 was the same. The selected fractions are represented in Figure 4.17.

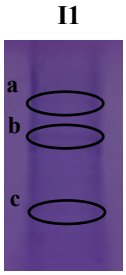


Figure 4.17 SDS-PAGE of Isolate 1 with the three fractions used at agar diffusion test: a, b and c.

As well as the positive control, isolate 1 also did not cause growth inhibition in *Pichia guilliermondii* (Figure 4.18). The cell extract caused an inhibition halo, as expected (fig. 4.18 D)), but smaller than the halo caused by the positive control.

As observed previously, the absence of halos may be attributed to the low concentration of the toxin, or possibly because these fractions do not contain the proteins responsible for the *killer* toxin activity. This inference is supported by the fact that the cell extract used for the SDS-PAGE did exhibit an inhibition halo.

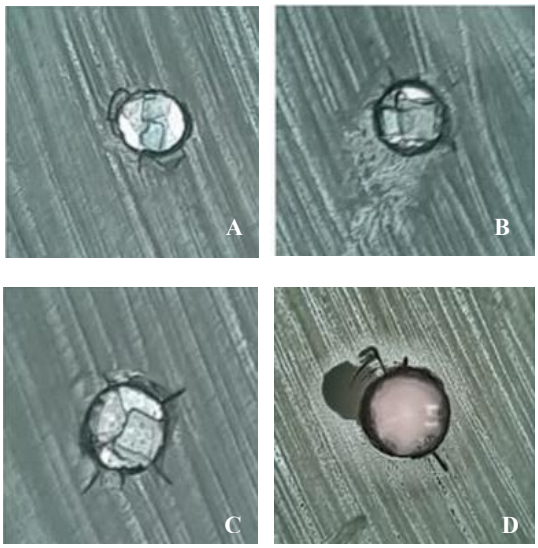


Figure 4.18 Agar diffusion test with Isolate 1. A) Fraction a; B) Fraction b; C) Fraction c; D) Cell extract.

4.5.1.3.Isolate 2

Regarding isolate 2, which was fractionated on SDS-PAGE as shown in Figure 4.19, the fractions were tested, as before, to determine if they were responsible for inhibiting *Pichia guilliermondii*.

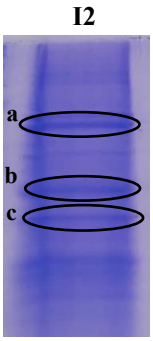


Figure 4.19 SDS-PAGE of Isolate 2 with the three fractions used at agar diffusion test: a, b and c.

Contrary to the results obtained previously, isolate 2 showed activity in two of the fractions (Figure 4.20).

Fractions a and b (Figure 4.20 A and B, respectively) caused some inhibition in the growth of the sensitive yeast. The inhibition halo produced by fraction b was more significant. The cell extract containing the proteins also showed a halo of inhibition.

Since fractions a and b demonstrated the presence of a *killer* phenotype, it was necessary to identify them, in order to determine what mechanism this *killer* yeast employs to inhibit the sensitive yeast.

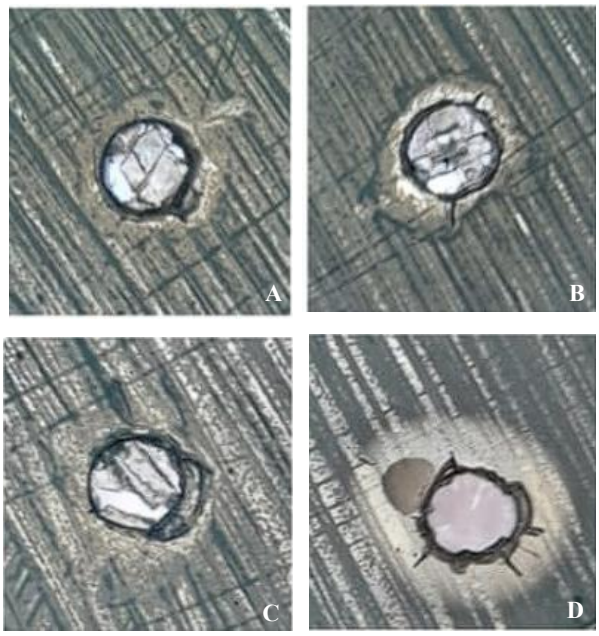


Figure 4.20 Agar diffusion test with Isolate 2. A) Fraction a; B) Fraction b; C) Fraction c; D) Cell extract.

4.5.1.4.Isolate 3

As with the other isolates, isolate 3 also had the three SDS-PAGE fractions selected (Figure 4.21). Again, the fraction extracts were placed in wells to be tested for their *killer* activity.

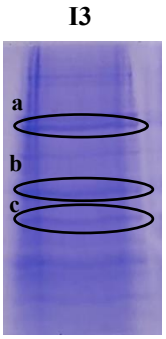


Figure 4.21 SDS-PAGE of Isolate 3 with the three fractions used at agar diffusion test: a, b and c.

As previously observed with the positive control and the 1 isolate, the isolate 3 also showed no inhibition halos when fractions a, b and c were tested (Figure 4.22), as expected the cell extract inhibited the growth of the sensitive yeast. Again, this can be explained by the reasons given above.

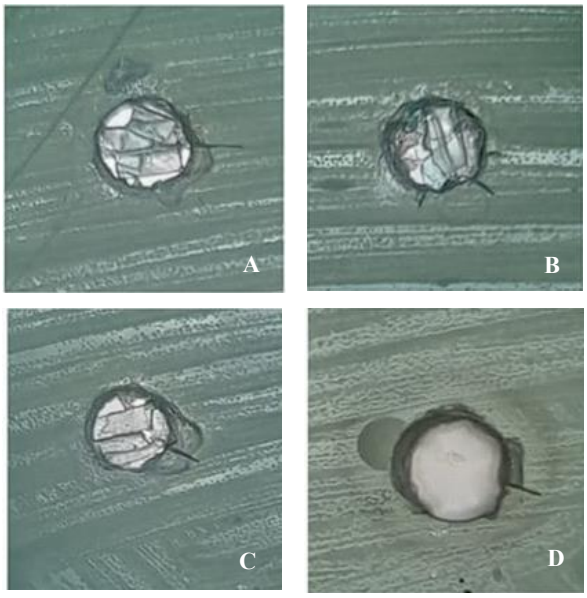


Figure 4.22 Agar diffusion test with Isolate 3. A) Fraction a; B) Fraction b; C) Fraction c; D) Cell extract.

After the tests, it was possible to confirm the hypothesis that isolates 1, 2 and 3 are from different strains of Metschnikowia pulcherrima, because the clearest bands are not the same in all of them.

Since isolate 2 showed activity in fractions a and b, it was crucial to identify the proteins within these fractions and determine whether they were associated with the production of the pulcherrimin pigment.

4.5.2. Method 2

This method was adapted from the one proposed by Souto. It consisted of an SDS-PAGE gel that was added with an inoculum of *Pichia guilliermondii* and stained with methylene blue, so that the toxins were supposed to inhibit the presence of Pichia in their location and the gel was colourless in this area. The rest of the gel would be coloured because the dead *Pichia guilliermondii* cells would absorb the pigment, as explained above.

Figure 4.23 shows that there was no inhibition of *Pichia guilliermondii* cells, as the entire gel is stained blue, with no colourless areas.

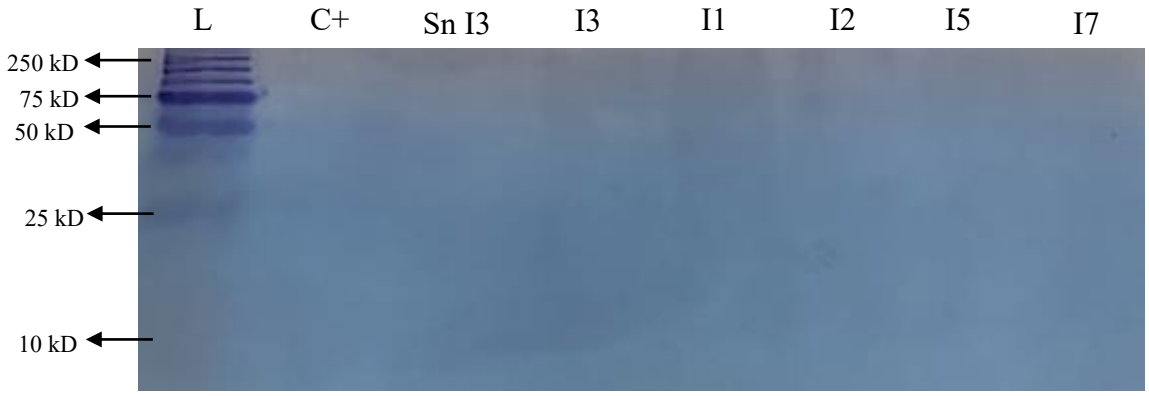


Figure 4.23 Zymography was done using SDS-PAGE stained with methylene blue to identify the fraction responsible for *killer* activity by a colourless area. No colourless bands are observed.
Ladder (L): Precision Plus Protein All Blue Standards (Bio Rad)
Sn: Supernatant

4.6.Protein quantification

Protein quantification was carried out using the Bradford method. This technique consists of using a standard curve generated using known concentrations of bovine serum albumin (BSA) to determine the concentration of other proteins.

Table 4.5 shows the results of the protein quantification of the isolates under study and the positive control.

These results provide crucial information on the results presented earlier, helping in the experimental analysis and interpretation. Isolate 2 had a greater amount of extracted protein, which may explain the more significant outcomes in terms of inhibiting the growth of *Pichia guilliermondii*. This was evident, not only in the tests carried out using the complete extract, but also when the fractions derived from the SDS-PAGE were tested.

Table 4.5 Protein quantification of cell extracts from isolates

Isolate	Protein (mg/mL)
1	0,249
2	0,502
3	0,115
4	0,035
5	0,061
6	0,143
7	0,076
8	0,072
9	0,354
Positive Control	0,211

4.7.Protein Purification

Protein purification was a critical step in this study, as it was essencial to isolate the proteins responsible for the *killer* phenotype and subsequently identify them.

To this end, isolate 2, corresponding to *Metschnikowia pulcherrima*, was selected, since throughout the work, this isolate showed more significant *killer* activity.

4.7.1. Protein Precipitation and Quantification

The selection of proteins was based on the zymography assay carried out using method 1 (Bajaj *et al.*, 2013). The fractions removed from the SDS that showed *killer* activity, as shown in Figure 32, went through the process of precipitation and protein quantification.

Firstly, the proteins in fractions a and b (Figure 4.24) were extracted from the SDS-PAGE, as before in the zymography test, and then they went through the precipitation process, which allowed the proteins of interest to be separated from the rest of the cellular components.

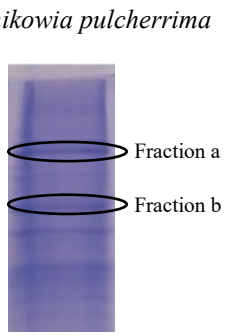


Figure 4.24 SDS-PAGE of *Metschnikowia pulcherrima* with the 2 fractions select for identification.

After the precipitation process, the proteins were eluted in buffer so that they could be quantified. This step was crucial because the analytical procedure used, MALDI-TOF/TOF (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight/Time-of-Flight), required an

amount of 2.0 pmol/µL protein to be effective. The quantification results are shown below in Table 4.6.

Table 4.6 Protein quantification of gel fractions.

Gel Fraction	Protein Quantification (mg/mL)
Fraction a	0,032
Fraction b	0,062

4.7.2. Protein identification by MALDI-TOF/TOF

Using MALDI-TOF/TOF for the analysis of *killer* toxins provides a sophisticated method for the rapid and accurate identification and characterization of these bioactive proteins. This technique is highly valuable in the study of *killer* toxins from yeasts, enabling precise mass determination and protein sequencing essential for elucidating their structure and possible functional mechanisms.

Fractions a and b were identified using the MALDI-TOF/TOF technique. The subsequent analysis of the fractions showed that the protein corresponding to fraction **a** is Phosphotransferase, on the other hand, fraction **b** represents a Transaldolase (Table 4.7).

Table 4.7 Identification of gel bands.

Gel Bands	Protein hits	Protein Score	Sequence Coverage (%)	Identified peptides
Fraction a	Phosphotransferase	83	13	K.MISGYYLGEVLR.L K.LHEPYIMDTSFPSR.V K.ETGDYLAIDLGGTNLR.V K.YDIQIDAESPRPGQQAFEK.M R.LKYDIQIDAESPRPGQQAFEK.M
Fraction b	Transaldolase	102	14	K.VTLISPFVGR.I K.VSYIDDESEFR.F K.DAQTLLAELENR.F K.YGYNTIVMGASFR.N K.DAQTLLAELENRFK.-

The results did not meet expectations as these proteins are not associated with *killer* phenotypes. However, this could be the case because, although only one band was visible, it was possible that the fraction contained more than one protein.

Phosphotransferases are enzymes involved in the transport and phosphorylation of sugars. On the other hand, transaldolases, participates in the pentose phosphate pathway and is essential to produce NADPH and ribose-5-phosphate.

Given that Mestchnikowia pulcherrima is known for its *killer* phenotype, which is derived from the pigment pulcherrimin that can precipitate iron, thereby creating a hostile environment for some microorganisms reliant on iron for growth, it was hypothesized that these fractions might correspond to proteins associated with this pigment. However, as outlined in the previous paragraph, phosphotransferase and transaldolase are not responsible for this process. It was therefore important to analyse the proteome in an attempt to identify which proteins might be linked to *killer* activity.

4.7.3. Peptide mapping by LC-MS

Peptide mapping using Liquid Chromatography-Mass Spectrometry (LC-MS) is a powerful technique employed in the identification and characterization of proteins, with *killer* activity.

The complete proteome analysis identified two proteins linked to *killer* activity, Super*Killer* protein 3 and Super*Killer* protein 8, shown below in Table 4.8.

Table 4.8 Description of *Killer* Proteins identified by LC-MS.

Protein	Gene	Length	Protein Description	Coverage	Total Peptides
tr|A0A4P6XWK2|A0A4P6XWK2_9ASCO	MPUL0E06430	1379	SuperKiller protein 3	1,74	2
tr|A0A4P6XMB7|A0A4P6XMB7_9ASCO	MPUL0B03470	386	SuperKiller protein 8	3,89	1

The Super*killer* (SKI) genes were initially discovered by mutations that resulted in the enhanced expression of the endogenous double-strand RNA '*killer*' virus in yeast (Wang *et al.*, 2005). The SKi genes were first described in *Saccharomyces cerevisiae* by Vodkin *et al.* (1974).

However, when the genome of the yeast Metschnikowia pulcherrima was described for the first time by (Gore-Lloyd *et al.*, 2019), the genes encoding the Super*Killer* proteins were also found. In this study, the analysis of the complete proteome also led to the identification of the Ski3 and Ski8 proteins.

Yeast's nuclear genome contains the SKI genes (J. T. Brown *et al.*, 2000), which explains the fact that when the supernatant of the isolates was tested in the fermentation tests for toxin production (Figure 4.2), they showed no inhibition halos.

The Ski complex consists of three components; Super*killer* 2 (Ski2), Super*killer* 3 (Ski3), and Super*killer* 8 (Ski8); has hetero-tetrameric stoichiometry and is made up of two copies of Ski8 and one copy each of Ski2 and Ski3 (Synowsky & Heck, 2008), as explained previously.

In this analysis it was not possible to identify the SKi2 protein, but according to (J. T. Brown *et al.*, 2000), the Ski 3 and Ski 8 proteins are able to interact even in the absence of the Ski 2 protein.

The Ski complex is responsible for 3'-mRNA degradation pathway. There is a hypothesis that suggests that the Ski complex attracts the exosome to its substrate, and then the substrate is degraded. Furthermore, the Ski complex has been demonstrated to safeguard the cell from viral replication by inhibiting the production of external mRNA transcripts (Synowsky & Heck, 2008).

As is well known, mRNA degradation plays an important role in cell cycle regulation, since correct mRNA renewal is therefore essential for the fidelity of cell cycle regulation. This shows that this mechanism is part of the *killer* toxins' modes of action, as it disrupts the cell cycle, blocking its normal progression.

On the other hand, the yeast *Metschnikowia pulcherrima* is characterised by the production of the pigment pulcherrimin, which precipitates iron present in the environment, making it hostile for microorganisms that depend on iron to survive (Morata *et al.*, 2019). Since iron is a facilitator of electron transfer during oxidation-reduction reactions and is therefore essential for all cells, pulcherrimin may be the key to *killer* activity in *Pichia guilliermondii* (Prokopiv *et al.*, 2013). However, the proteins responsible to produce pulcherrimin were not found in the proteome analysis, this may indicate that these proteins are produced outside the nucleus, being localised in the supernatant.

5. Conclusion and future perspectives

The main objectives of this work were to detect the *killer* phenotype in yeast isolates from wine must and to identify the proteins involved in this phenomenon.

Based on this assessment, the first step was to identify the isolates that showed *killer* activity, using YMA-MB medium to screen all the isolates from the wine must, using *Saccharomyces cerevisiae* as a positive control.

It was possible to identify 9 isolates that showed this phenotype, and these were distinguished by two characteristics: 4 isolates caused growth inhibition of *Pichia guilliermondii* and, on the other hand, 5 isolates caused cell death of *P. guilliermondii* (Figure 4.1).

Subsequently, it was important to carry out a more detailed study of the *killer* toxins produced by these yeast isolates, and it was necessary to remove the toxins and test them in isolation. Several procedures were tested for this purpose.

The first test carried out was described by Bajaj *et al.* (2013) and consisted of collecting the crude toxin produced in the fermentation medium over 192 hours, and this toxin was tested every 24 hours. The results were not as expected, as toxin production was supposed to be increasing, as were the inhibition halos produced by the toxins. As can be seen in Figure 4.2, only the positive control caused an inhibition halo at 48h and 72h, suggesting that toxin production does not occur during fermentation.

A new procedure proposed by Fredericks *et al.* (2021) was tested, which consisted of inoculating the isolates of interest in YMB medium with a pH between 4 and 5, the toxin was collected and incubated in two different conditions, at room temperature and at 98º C for two minutes. Again, it was not possible to verify growth inhibition of *P. guilliermondii*, indicating that the toxin production of these isolates did not occur in the cytoplasm.

It was hypothesised that toxin production only occurs when these yeasts are in competition, and for this reason, the procedure described by (Giometto *et al.*, 2021) was tested and again no growth inhibition occurred.

Given that none of the procedures were producing the desired results, it was thought that one possibility would be to isolate the toxin from the inhibition halos. However, this technique had some limitations, such as: it was only possible to remove the inhibition halo from isolates that showed a considerable inhibition halo, and for isolates that caused a halo of dead cells it was not possible to carry out this technique. Although it was possible to obtain a halo of

inhibition using this procedure, these halos were not very significant and given the limitations it was necessary to use another procedure.

Finally, the mechanical extraction technique proposed by Ge *et al.*, (2010)was tested, making it possible to collect the toxins present in the cytoplasm (supernatant) and from the nucleus (cell extract). The desired results were obtained using this technique, so it was possible to move on to the next step, SDS-PAGE.

But first, the isolates had to be identified in order to analyse the proteins responsible for *killer* activity more accurately, so the isolate that stood out the most was identified as *Mestchikowia pulcherrima.*

The SDS-PAGE technique was used to separate proteins according to molecular weight, so that it is possible to analyse the protein profile of different strains, purify and identify proteins, carry out comparative analyses between species and verify the mechanisms of action of proteins.

The gel showed that isolates 1, 2 and 3, all corresponding to *M. pulcherrima*, had significantly different protein profiles, suggesting that they belong to different strains.

This gel is also essential for zymography, as this technique is only possible after separating the proteins. Zymography is a technique that makes it possible to visualise and analyse the antimicrobial activity of *killer* toxins in a very direct way, allowing an increase in knowledge about these toxins. Two different procedures were carried out, as described by Bajaj *et al.* (2013) and Souto *et al.* (2019).

The first method proposed by Bajaj *et al.* (2013) consisted of removing a band of interest from the SDS-PAGE, after which the proteins were separated from the gel and tested in agar diffusion. In this test, only isolate 2 showed significant results in fractions a and b (Figure 4.20).

Subsequently, the protocol suggested by Souto *et al.* (2019) was carried out, which was based on the SDS-PAGE gel carried out with a substrate that would drive the enzymatic activity of *killer* toxins. In this case, the substrate used was *P. guilliermondii* inoculum, the gel would be stained with methylene blue, and the gel would show a colourless zone at the site of enzyme activity, since no *P. guilliermondii* growth would occur in this area. As can be seen in Figure 4.23, no enzymatic activity could be seen in this technique.

Fractions a and b (Figure 4.24) were selected using zymography, as these fractions showed inhibition halos against *P. guilliermondii*, and identified using the MALDI-TOF/TOF technique. The results were phosphotransferase and transaldose. This identification was

unexpected as these enzymes have no connection with *killer* activity, but it was a possible result given the fact that more than one protein could be found in a fraction.

In the end, the proteome was analysed, it was possible to identify two proteins (SKi 3 and SKi 8) that cause mRNA degradation in sensitive yeasts, impacting the cell cycle and subsequent cell death.

However, this yeast can produce pulcherrimin, a pigment that precipitates the iron present in the medium, making the medium unsuitable for iron-dependent microorganisms but the proteins that encode this pigment were not identified in the proteome analysis. This could mean that these proteins are encoded in the cytoplasm and would therefore be in the supernatant and not in the cell extract.

Since this isolate of *Mestchnikowia pulcherrima* did not inhibit *Pichia guilliermondii* when the supernatant was tested, two hypotheses arise: one is that pulcherrimin is not produced, under the conditions tested, and another is that the mechanism for causing its production depends on other cellular components.

This work showed that isolates from wine must have the ability to inhibit the spoilage yeast *P. guilliermondii*, so in the future it would be important to check for isolates capable of inhibiting other wine spoiling yeasts, such as *Brettanomyces bruxellensis*.
On the other hand, in the wine industry it would be important to check the effects on fermentation caused by the combination of *killer* yeasts, in this case *M. pulcherrima*, and yeasts normally used for this purpose such as *S. cerevisiae*.

Also, in the context of winemaking, if the aim is to eliminate *Pichia guilliermondii*, *M. pulcherrima* yeast can be used instead of the sulphites currently used, so it is important to check the effect of adding this yeast over and above the amount normally present, in terms of aroma, flavour and mouthfeel.

This study could also be important in terms of finding a new way to combat the challenge posed by antimicrobial resistance. As mentioned earlier, resistance by bacteria and fungi to these agents has become an increasing concern in terms of global health and it is therefore imperative to find new mechanisms to combat this problem. *Killer* yeasts could be the key to overcoming this challenge.

Therefore, in the future, it would be important to test isolates with a *killer* phenotype on pathogenic microorganisms that are of higher concern to global health in terms of antimicrobial resistance, such as Enterobacterales and *Acinetobacter baumannii*, in the case of bacteria

(Figure 1.10), or *Candida auris* and *Aspergillus fumigatus*, in the case of fungi (Figure 1.11). And to understand how *killer* yeasts act on these microorganisms, and how these mechanisms can be enhanced to have the capacity to replace traditionally used microbial agents and combat or remedy antimicrobial resistance.

Finally, in particular for the Ski3 and Ski8 proteins found in *M. pulcherrima*, it would be important to study and analyse the characteristics of these proteins in more depth, understanding the intrinsic mechanisms of the proteins together or in isolation and whether they are capable of inhibiting the growth of sensitive microorganisms, given that in this study these proteins were identified in the proteome, but were not tested in isolation.

To summarise, this work has been important in identifying the *killer* potential of isolates from wine must and understanding which proteins are responsible for this characteristic.
These findings underscore the potential of *killer* yeasts as novel, natural antimicrobial agents, enhancing their use in wine fermentation and preservation, and potentially improving both wine quality and protection against spoilage organisms. Moreover, this discovery highlights their capability to address not only wine spoilage but also antimicrobial resistance in pathogenic microorganisms, promising a more sustainable, healthy and natural approach.

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