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Yeast killer toxins: from ecological significance to application

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Abstract

Killer toxins are proteins that are often glycosylated and that bind to specific receptors on the surface of their target microorganism, which is then killed through a target-specific mode of action. The killer phenotype is widespread among yeast, with about 100 yeast killer species described to date. The spectrum of action of the killer toxins they produce targets spoilage and pathogenic microorganisms. Thus, they have potential as natural antimicrobials in food and for biological control of plant pathogens, as well as potential therapeutic agents against animal and human infections. In spite of this wide range of possible applications, their exploitation at the industrial level is still in its infancy. Here, we initially briefly present the biodiversity of killer toxins and the ecological significance of their production. Then their actual and possible applications in the agro-food industry are discussed, together with recent advances in their heterologous production and manipulation for development of peptide-based therapeutic agents.

Keywords: killer toxin, natural antimicrobial, interference competition, spoilage yeast, biological control, therapeutic agents

Yeast killer toxins

The antagonistic behavior of yeast was first discovered at the beginning of the last century, when the production of a volatile thermolabile extract that inhibited bacterial growth was described [for review, see 1]. Further studies showed that the antagonistic activities of yeast against other microorganisms can be attributed to a number of different properties, including competition for nutrients and space, acidification of the medium, production of ethanol, and secretion of antimicrobial compounds, such as volatile acids, hydrogen peroxide, secondary metabolites, and the so-called killer toxins.

The production of toxins is a relatively common phenotype in Nature. This has been described for bacteria, yeast and fungi as generally part of a mechanism aimed at domination of the competition within the environmental niches that they live. For yeast, the first reports on their killer phenotype dates back to just over 50 years ago, with the initial isolation of a *Saccharomyces cerevisiae* strain that inhibited the growth of other *S. cerevisiae* strains [2-4]. According to these early investigations, killer (K) yeast secreted a toxin that was lethal to sensitive (S) strains of the same or related species, but was harmless to neutral (N) strains, which were immune to their effects.

In the following years, the killer phenotype was shown to be widespread among yeast, with the description of almost 100 killer species that have been ascribed to about 46 genera [5]. Further studies have shown that the killer phenotype occurs frequently in yeast strains isolated from a variety of natural habitats (e.g., water, soil, fruit, grape must) and from different geographic origins. Most killer yeast can kill other yeast of the same or of different species and genera. Some of them are active against filamentous fungi [6-8] while some are also active against bacteria [9-12]. The spectrum of action of yeast killer toxins encompasses spoilage microorganisms relevant for the fermentative [13-17] and food and feed industries [18,19], and it also includes microbial pathogens of clinical interest [20-22], and plant pathogens [12,23-25].

Thus, they can be used as natural antimicrobials in the agro-food industry, as well as antimicrobials against animal and human infections and for biological control of plant pathogens, both in the field and for postharvest applications [26-28] (Figure 1). In addition, killer yeast and their toxins represent model systems for elucidation of the mechanisms that underlie social interactions between microorganisms [29], the regulation of polypeptide processing and secretion in eukaryotes, and expression, inheritance and maintenance of eukaryotic viruses [30].

Here, after a brief presentation of the biodiversity of killer toxins and the ecological significance of their production, their actual and possible applications in the agro-food industry are discussed, together with recent advances in their heterologous production and manipulations for development of peptide-based therapeutic agents.

Biodiversity of killer toxins

The depth of information regarding killer toxins varies depending on the characteristics of the producer and the possible biotechnological application of the toxin. The most well-characterized killer toxins with respect to their genetic determinants, biochemical characteristics, molecular targets on the sensitive cells, and mechanisms of killing are K1, K2, and K28 of *S. cerevisiae*, zymocin of *Kluyveromyces lactis*, PMKT and PMKT2 of *Pichia membranaefaciens*, PaKT of *Wickerhamomyces anomalus*, HM-1 of *Cyberlindnera mrakii* and Kpkt of *Tetrapisispora phaffii* [7,31-37]. In particular, this is because these toxins represent biological models for the study of replication and maintenance of extrachromosomal genetic elements, for dissection of the secretory pathway, and for elucidation of the mechanisms that underlie toxin immunity (K1, K28, zymocin), or because of their possible applications in the medical field (PakT), the food and feed industries (PMKT, PMKT2, KpKt, HM-1), and in the preharvest and postharvest biocontrol of plant pathogens (PMKT, PMKT2). For the remaining killer toxins, generally the localization of the genetic determinants of the killer characters and the molecular weights of the

killer toxins have been determined (Table 1), although much still needs to be done regarding their modes of action and molecular targets within the sensitive cells, and for an understanding of toxin immunity. Recently, there have been a number of reviews of killer yeast, their toxins, their genetic determinants, and their molecular mechanisms of action [5,28,38-39]. Thus, only a brief overview of what is known about ~~the~~ a selected group of killer toxins ~~are~~ is reported here and in Table 1.

In terms of the localization of their genetic determinants, the majority of the killer toxins characterized to date are encoded by chromosomal genes, and their production is widespread in a number of different non-*Saccharomyces* yeast, although this has also been observed within *S. cerevisiae* (Table 1, Figure 2). A few killer toxins that are produced by a handful of species are encoded by extrachromosomal double-stranded (ds)RNA virus-like particles and by extrachromosomal dsDNA virus-like elements localized in the cytoplasm (Table 1, Figure 2). The molecular weights of killer toxins range from about 1.8 kDa to >150 kDa, and while most of them consist of a single subunit (the majority of those encoded by chromosomal genes), some others can be formed by two subunits (e.g., K1, K2, K28, SMKT) or three subunits (e.g., *K. lactis*, *Pichia acaciae* killer toxins). Most killer toxins are stable at acidic pH, but some of them maintain their activity within a wide pH range, such as HM-1 of *C. mrakii* [40]. The temperature ranges of their activities also vary, depending on the natural habitat of the killer yeast. For example, marine yeast killer toxins have an optimum temperature of 15 °C, while those of Kpkt produced by a soil strain of *T. phaffii* have an optimum temperature of 25 °C. In general, the majority of killer toxins lose their activity at >40 °C, although some can resist high-temperature treatment; e.g., HM-1 remains active after 1 h at 60 °C [41]. Moreover, as reviewed by Liu et al. [38], some killer toxins change their spectrum of action and show stronger killer activity in the presence of NaCl. This is particularly seen for killer toxins produced by yeast isolated from olive brine [42] and from marine environments [43]. Indeed, all of these represent important features that can greatly affect the potential use and applicability of these yeast killer toxins.

Despite this wide diversity, the killing actions of all of the characterized killer toxins is generally mediated by a two-step mechanism (Figure 3). During the first step, the killer toxin recognizes and binds to a primary receptor on the cell wall of the sensitive target, thus indicating that the cell wall is an essential facilitator in the killing action. Accordingly, the killer toxins produced by *T. phaffi* (Kpkt), *Pichia anomala* (PiKT), and *Kluyveromyces siamensis* cannot kill spheroplasts of their sensitive targets [44-46]. Nevertheless, this is not a general rule, as PMKT and SMKT are also active on their target cells following enzymatic digestion of the cell wall [35,47]. However, it should be noted that the plasma membrane of yeast and fungi is negatively charged, while killer toxins can be more or less positively charged. Thus, the interactions between killer toxins and the plasma membrane of their sensitive target can also depend on the net cationic charge of the killer toxin [39].

As the cell wall is in general the primary site of action of killer toxins, different cell-wall components can act as the primary receptor sites. Among these, the β-1,3-D-glucans and β-1,6-D-glucans are commonly the receptors for the majority of the killer toxins characterized to date, although mannoproteins and chitin also serve as the first receptors for a number of killer toxins (Table 1).

During the second step of their actions, killer toxins are believed to be translocated to the cell membrane, where they interact with a secondary receptor. This step has been less characterized, and the secondary receptors are known, or hypothesized, for very few killer toxins (Table 1). Once the killer toxins bind to the secondary receptor and enter the sensitive cells, they kill the cells through different mechanisms (Figure 3; Table 1). These include cell-membrane permeabilization, inhibition of DNA synthesis, cell-cycle perturbation, and fragmentation of RNA [5,28,38,48,49]. In other cases, killer toxins act primarily on the cell wall of the sensitive target. Some toxins, including Kpkt and WmKt, have β-glucanase activities (Table 1) and hydrolyze the cell-wall glucans, which leads to cell lysis [44,50]. HM-1 of *C. mrakii* impairs

cell-wall biosynthesis by binding to and inhibiting β-1,3-glucan synthase, an enzyme that is localized to the plasma membrane [19,41].

Ecological significance of killer toxin production

As killer yeast can kill sensitive yeast that occupy the same ecological niche, once the killer yeast occur within a community, they would be expected to dominate the sensitive yeast, by killing them [51]. However, killer and sensitive yeast co-exist in Nature, and thus this does not appear to always be the case. To explain this phenomenon, a number of studies have investigated the ecological roles of killer yeast, and the way that their production of killer toxins affects the behaviors of both the killer and sensitive yeast in their natural environments. While investigating the benefits that can be derived from the killer phenotype, Pintar and Starmer [52] revealed that in *S. cerevisiae* the production of killer toxins has a metabolic cost, and thus reduces the fitness of the producer by ~4% [53]. On the one side, this metabolic cost negatively correlates with the competition for resources with toxin-resistant strains [54], while on the other side, it is rewarded by the benefits derived from killing the sensitive yeast [53].

Indeed, the success of killer yeast depends on a number of environmental factors, which include yeast dispersal, nutrient availability, and frequency of encounters between killer and sensitive yeast [53-55]. In structured environments (i.e., with limited dispersal, such as in solid media and soft agar), the killer yeast show self-survival behavior, as they take advantage of unused nutrients and of the nutrients released by the dead sensitive cells [29]. The mechanism that underlies this behavior is known as 'interference competition'. This is relatively common in the microbial world, and it is described as a direct negative interaction through which one microbial population actively suppresses a competing population [56]. In unstructured environments (e.g., liquid media), the toxin concentrations cannot reach lethal levels, and a greater portion of the available nutrients can be used by viable sensitive cells (Figure 4).

When nutrients are available, and under limited dispersal, the advantages that can be derived from production of a killer toxin exceed the metabolic cost of the toxin production, and the killer yeast can invade the sensitive yeast population [53]. On the contrary, under nutrient shortage, the net gain derived from toxin production is limited, and when the killer yeast are highly dispersed, the toxin they produce might not be sufficient to allow invasion of the sensitive yeast population [53,57]. Based on this evidence, the production of killer toxins appears to provide competitive advantages when nutrients are abundant, and therefore it is not just a survival strategy under nutrient shortage, as hypothesized by Ivanowska and Harwick [55].

For the frequency of encounters, Rivero et al. [58] reported that in high cell density cultures, killer and sensitive wild isolates of *S. cerevisiae* are engaged in a "reward–punishment mechanism" that regulates the production and exploitation of nutrients for the 'public good' while counteracting the insurgence of opportunistic strains. According to this mechanism, an apparently 'altruistic' fraction of the killer population undergoes cell lysis, which releases Hsp12p for the public good as it can improve the fitness of the yeast (which will potentially be available to both killer and sensitive yeast). To take better advantage of this public good, the killer cells also show contact-dependent killer toxin secretion, to thus decrease the fitness of the sensitive cells. However, in turn, these sensitive cells can overexpress Pau5p, a membrane protein that provides stress resistance, and can counteract the effects of the killer toxin, creating a balance between the killer and sensitive yeast, and completing this "reward–punishment loop" [58]. Recently, Deschaine et al. [59] observed that when killer and sensitive biofilm-forming strains grow in mixed communities, the killer strain can surround the biofilm-forming strain and prevail at the edge between the colonies. However, the competitive advantage provided by the increased spatial use of biofilm-forming strains offers them an escape from the killer toxin, with the possibility of outgrowth at the edge of at least one section of the colony.

The frequency of the killer phenotype ranges from 5% to 30% [12,53,60], although it can reach >50% of isolates in some species [61]. A survey of 136 wild isolates of *Saccharomyces*

indicated that within this genus, the frequency of the killer-toxin-resistant phenotype is much higher (25%) than that of the killer phenotype (10%), which suggests that the acquisition of the resistant phenotype is a consequence of the cohabitation with killer competitors in natural environments [62]. Indeed, the co-evolution between killer and sensitive yeast results in rapid appearance of aneuploid toxin-resistant mutants. These derive from the sensitive strain, which can take advantage of variations in gene dosage that can lead to activation of beneficial recessive alleles [54]. Interestingly, when such resistant mutations become fixed in the previously sensitive population, the benefits that can be derived from production of killer toxins are less, which leads to selection of killer strains with decreased killing activity, accompanied by more beneficial use of the available resources [54].

Yeast ploidy can also affect the results of these killer/ sensitive interactions. McBride et al. [63] reported that while *S. cerevisiae* K1 killer toxin produced by killer haploid cells can kill diploid sensitive cells, it does not kill haploid sensitive cells. This is important to ensure horizontal transmission of the killer virus during mating, and therefore to spread the virus into different genetic backgrounds.

Hence, overall in natural environments, the co-existence of killer, sensitive and resistant strains is the result of establishment of their dynamic equilibrium.

The active role of killer yeast as natural antimicrobials

In recent years, increased consumer awareness of the safety and health-promoting effects of food and beverages has been an important driving force for research and development of natural antimicrobials. Indeed, on the one hand, it is widely recognized that the overall quality of products of the agro-food industry is negatively related to the development of spoilage and pathogenic microorganisms. On the other hand, the possibility to substitute chemical

preservatives with natural antimicrobials parallels the increased consumer interest in minimally processed food and 'greener' food and beverage additives.

As a result, the number of studies that have focused on the roles of bioactive molecules produced by antagonistic microorganisms continues to increase, and the application of natural approaches to counteract undesired microorganisms in food matrices is receiving considerable attention. In addition, the antagonistic behavior of yeast against other microorganisms has attracted increasing attention and stimulated their application as general biocontrol agents for crop protection and as therapeutic agents for use in human and veterinary medicines.

Thus, the exploitation of killer yeast or their toxins as natural antimicrobials that was at first confined to the research laboratory is now supported by the strong market demand for yeast with antimicrobial activities that can be profitably used to counteract proliferation of undesired microorganisms, and thus to reduce the use of chemical antiseptic agents [64]. Accordingly, a number of patents regarding the use of killer yeast or their toxins have been registered (Table 2).

Killer yeast in preharvest and postharvest control of fungal diseases

A promising field of application of killer yeast as biocontrol agents is preharvest and postharvest control of phytopathogenic fungi. Fungal diseases of crops cause significant losses in food production worldwide. It is estimated that globally about 33% of fruit and vegetable production is wasted, mainly due to postharvest fungal diseases [65]. In this scenario, and with the need for sustainable agriculture, the biological control of fungal diseases using antagonistic microorganisms represents a possible alternative to the use of fungicides. Just over 20 years ago, Walker and co-authors [66] reported that *P. anomala* can markedly inhibit the growth of certain wood-decay basidiomycetes and plant pathogenic fungi. Later, it was shown that *Pichia membranifaciens* can protect grapevines against the causal agent of gray mold disease, *Botrytis cinerea* [6,67], while *Candida sake* and *Pantoea agglomerans* protect apples and pears against *Penicillium expansum* and *Botrytis cinerea* [68].

Indeed, *W. anomalus* (formerly *P. anomala*) killer strains have great potential as postharvest biocontrol agents, as they are active against *Colletotrichum gloeosporioides* [69], *Penicillium digitatum*, *Penicillium italicum* and *B. cinerea* [25,70]. Similarly, *Debariomyces hansenii* can counteract growth of *Monilinia fructigena* and *Monilinia fructicola* [71]. Indeed, *W. anomalus* and *D. hansenii* can inhibit growth of various sensitive yeast strains and several phytopathogenic fungi and have been granted Qualified Presumption of Safety status by the European Food Safety Authority, which would authorize their use as novel microorganisms in food preservation [71]. Thus, the use of killer yeast that compete with pathogenic epiphytic microorganisms appears to represent a suitable tool to reduce the application of chemical antimicrobials, and thus to generate benefits for humans [72,73].

Killer yeast in fermented food and feed

Killer yeast might have biotechnological applications in fermented food and feed production also due to their adaption to extreme environments. Studies on the microflora of fermenting olives revealed the presence of a rich yeast community that contributes to the fermentation process, but that can also be involved in spoilage during fermentation, storage and packing [74,75]. Most of the yeast strains isolated from spontaneously fermenting olive brines have the killer phenotype and maintain their killer activities under the selective conditions of olive brines; e.g., *P. membranaefaciens* killer strains are particularly active at pHs and salt concentrations that are seen in fermentation processes [42,76]. In addition, at the sodium chloride concentration in olive brines, the toxicity of killer yeast can be enhanced. This enlarges the spectra of action of some killer yeast, and appears to be due to an additional selective advantage conferred to the killer strains in competition with sensitive cells.

Debariomyces hansenii killer strains isolated from olive brines cannot only kill a number of sensitive yeast, but are also active on *Lysteria monocytogenes*, *Bacillus cereus*, and *Salmonella typhimurium* [77]. Thus, even though the occurrence of foodborne pathogens in

fermented olives is not likely, the use of yeast starters that can counteract spoilage yeast and food-borne pathogens can contribute to improved quality and safety of olives and other fermented vegetables [77]. This might also be the case for unpasteurized fermented vegetables, such as sauerkraut or other vegetables that can undergo yeast spoilage during storage [78].

As well as fermented vegetables, dairy products can benefit from the availability of killer toxins that can counteract lactate-using spoilage yeast. Accordingly, a killer strain ascribed to *Williopsis saturnus* var. *saturnus* that was isolated from dairy products appears to be a promising biopreservative for the control of cheese spoilage [79].

For the feed industry, the silage process might benefit from the killer yeast system. During the feeding step, silage is exposed to the air, which leads to aerobic deterioration. During this aerobic deterioration, aerobic microorganisms, including yeast, can degrade lactic acid to CO_2 and break down proteins and free amino acids to amines and ammonia. Indeed, 25 years ago, Kitamoto et al. [80] suggested the use of killer yeast belonging to the genera *Kluyveromyces*, *Saccharomyces* and *Hansenula* for inhibition of growth of lactate-using spoilage yeast, and for the prevention of aerobic deterioration in silage. Subsequently, Lowes et al. [19] proposed ~~that~~ the utilization of the killer toxin produced by *C. mrakii* (Table 1, HMK) ~~can be used~~ as a biocontrol agent against spoilage yeast both in yoghurt production and silage maintenance. More recently, Bajaj et al. [81] reported that the killer toxin produced by *Pichia kudriavzevii* RY55 has good antimicrobial activity also against several pathogenic bacteria that are of significance in human health, including *Escherichia coli*, *Enterococcus faecalis*, *Klebsiella* sp., *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Pseudomonas alcaligenes*. Thus, this toxin is of great interest as a new candidate for food preservation.

Killer yeast in winemaking

Wine can undergo spoilage at different stages of the winemaking process as a result of the presence of undesired yeast. Generally, spoilage yeast are controlled using sulfur dioxide (SO_2),

a chemical antiseptic agent that can cause harm to consumers. On this basis, the World Health Organization has highlighted the need to reduce the content of SO_2 in food products, indicating that the daily intake of SO_2 should not exceed 0.7 mg/kg body weight [82]. Following these indications, over the last few decades, winemakers have adopted different strategies to reduce the SO_2 in wine. Van Vuuren and Jacobs [13] suggested the use of killer starters that can be used to complete grape must fermentation and to control growth and persistence of undesirable yeast. Todd et al. [14] proposed the use of killer and sensitive starter yeast during sparkling wine production to accelerate the onset of yeast autolysis. More recently, sequential inoculation of two *Saccharomyces* killer strains that can produce active and stable toxins under wine-making conditions have been proposed as a new strategy to control spoilage yeast species [11].

Also, non-*Saccharomyces* yeast or their toxins might also find application in the wine industry. The killer toxin naturally produced by *T. phaffii* is active on spoilage yeast of the genera *Kloeckera*/ *Hanseniaspora* and *Zygosaccharomyces* and can maintain its killer activity in wine for up to 14 days under such technological conditions [83]. Thus, it was hypothesized that Kpkt can partially replace SO_2 during grape must fermentation, to reduce the sulfite content in wine. Other killer toxins secreted by several non-*Saccharomyces* yeast are active against a wide range of spoilage yeast, which include *Dekkera* and *Brettanomyces* (Table 3). These last~~,~~ ~~however,~~ produce unpleasant odors during wine fermentation, ageing and storage, which can significantly affect the quality of the final product and result in considerable economic loss to the winemakers [87]. Interestingly, these killer toxins are not affected by the pH, temperature, and ethanol concentrations that are typical of winemaking conditions. Furthermore, they do not inhibit the fermenting *S. cerevisiae* nor the lactic acid bacteria, and are therefore hypothesized not to have negative impact on alcoholic and malolactic fermentation.

The killer phenotype is included among the selective criteria in the development of wine yeast starters, and at present, different *S. cerevisiae* killer strains are commercially available and

are actually used in winemaking on an industrial scale. In contrast, as far as we are aware, the use of the yeast killer toxins themselves has not been reported for the wine industry to date.

Recombinant production of killer toxins

Different modes of use can be envisaged for killer yeast or for their killer toxins, which depend on the application concerned (Figure 1) and on the toxin levels secreted by the native producer. Indeed, when the direct use of a killer yeast is impractical, the application of purified preparations of native or recombinant killer toxins can be considered. Remarkably, although yeast killer toxins have been under investigation for decades, their heterologous production has been poorly explored to date.

To the best of our knowledge, the first reports on heterologous yeast killer toxin production was the expression of KHS and KHR killer toxins [88-89] that are naturally produced in low amounts by weak killer strains of *S. cerevisiae* [90]. These pioneering studies were mainly aimed at the characterization of these toxins, and the determination of their primary structures, and their processing and secretion. However, they did pave the way for further studies, and a few years later, Kimura et al. [91] expressed *C. mrakii* killer toxins HM-1 and WmKt in *S. cerevisiae*. Further studies have indicated that *S. cerevisiae* is not the only possible host for heterologous expression of killer toxins. K28 and zygocin were successfully expressed in *Sch. pombe* [92-93]. More recently, fluorescent variants of K28 killer preprotoxin have been produced in *Pichia pastoris* [94] to investigate K28 binding to the surface of sensitive cells and its trafficking in yeast. To gather further information on the cellular targets involved in the K1 mechanisms of toxicity and immunity, Gier et al. [37] expressed K1 toxin derivatives in *S. cerevisiae*.

While most of these studies mainly addressed the mechanisms underlying toxin production, immunity, and secretion, other studies were aimed at producing killer toxins with a

view to their possible applications as antimicrobials in the food and beverages industries. Lowes et al. [19] proposed the heterologous expression of HMK in *Aspergillus niger* to obtain a recombinant protein that was active as a biocontrol agent against spoilage yeast in both yoghurt and silage production. In particular, silage is a product that is particularly susceptible to yeast contamination once it is exposed to air, and they showed that recombinant HMK can delay its spoilage by aerobic yeast. Moreover, they reported that HMK is also effective on spoilage yeast that can be introduced into yoghurt following addition of fruit, honey, and nuts. Another study reported the heterologous production of Kpkt killer toxin [95], with a view to its biotechnological exploitation in the wine industry. This recombinant Kpkt showed a wider spectrum of action than the native Kpkt. In particular, it also had marked effects on *Dekkera bruxellensis*, thus widening the possible applications of this toxin in the control of spoilage yeast during wine ageing in barrels.

As several enzymes currently applied to food processing are produced by recombinant microorganisms [96], a future for recombinant yeast killer toxin production and use can be foreseen.

From killer toxins to peptide-based antimicrobials

As well as representing a powerful tool as natural antimicrobials, killer toxins can serve as the starting point for the development of peptide-based therapeutic agents, to provide new antifungal drugs with high biological activities that are associated with low toxicities and high specificities. To date, a crucial step has been the development of anti-idiotypic single-chain antibodies that represent the internal image of the active domain of a killer toxin and show killer activity. In particular, this was indicated by studies on the exploitation of the antimicrobial activity of PaKT, the killer toxin that is naturally produced by *P. anomala* ATCC 96603 [97]. PaKT is active on different human pathogens [34,97], but its parenteral administration as a therapeutic agent is not

feasible as it is antigenic, toxic, and labile at physiological pH and temperature [98]. To circumvent this problem, PaKT-neutralizing monoclonal antibodies were raised and used to obtain anti-idiotypic antibodies of PaKT. One of these (ScFvH6) that showed *in-vitro* and *in-vivo* killer activity on a broad range of pathogens was expressed in *Streptococcus gordonii* to obtain a recombinant vector of the bioactive molecule [99]. Then, a search of the biologically active fragments of ScFvH6 for possible therapeutic use lead to the synthetic killer decapeptide known as KP [100]. KP represents the internal image of PaKT, and it is stable *in vivo* and active *in vitro* and *in vivo* against unrelated pathogens, such as viruses, bacteria, yeast, algae, and protozoa [101-103]. Moreover, it confers plant pathogen resistance when expressed in *Nicotiana benthamiana* [104], thus defining it as a powerful therapeutic agent with applications in medical fields and in preharvest control of fungal pathogens.

Likewise, to expand the array of applications of HM-1, recombinant anti-idiotypic antibodies that represented the internal image of this killer toxin were produced [105]. These anti-idiotypic antibodies were active on important fungal pathogens, such as *Candida albicans* and *Cryptococcus neoformans* [105]. Further work led Kabir et al. [106] to individuate two peptides that showed higher killer activities compared ~~to~~ with the native HM-1 killer toxin: SP3, SP6. Remarkably, the amino-acid sequence of the SP6 peptide has a high degree of homology with the amino-acid sequence of the killer decapeptide KP described above. This thus suggests that the common features of these two peptides represent necessary requisites for their antifungal activities [106].

Concluding remarks

Several studies have indicated killer yeast as possible starters for food and beverage fermentation, and their killer toxins as promising natural antimicrobials with a plethora of different applications. However, while killer starters are currently used in winemaking, there are

several constraints regarding the use of native or heterologous killer toxins as natural antimicrobials. Thus, although a number of patents for exploitation of killer toxins have been registered to date, their use has not taken off on an industrial scale.

An important limitation to the use of killer toxins in the food and feed industries is certainly the scarcity of studies regarding their effects on human and animal consumers. A deeper understanding of the mechanisms of action of killer toxins will allow more specific applications to be devised and developed, and make these killer toxins more effective toward different sensitive targets. The possibility to crystallize killer proteins to study their three-dimensional structure and to raise specific antitoxin-antibodies represents another tools that will be effective to increase our knowledge and to make these toxins more applicable.

Another issue regards the need to gather more information on the mechanisms that underlie killer toxin production in yeast. A deeper knowledge of the costs and benefits associated with toxin production not only in *S. cerevisiae,* but also in the complex world of the non-*Saccharomyces* yeast will prove useful. These non-*Saccharomyces* yeast are characterized by the production of a vast array of killer toxins that have different target spectra and modes of action, and they will prove useful to further elucidate the regulation of killer toxin production, also with a view to their biotechnological exploitation. Indeed, killer toxins are generally secreted at low concentrations by their native producers. This hampers the implementation of fermentation processes that are aimed at their production on an industrial scale, and defines the need for development of recombinant production systems that can increase their production and facilitate their purification. The development of killer toxins into a new generation of antimicrobial agents with useful applications in the agro-food sector will provide a further boost to the study of these killer yeast and their killer toxins in the future.

Disclosure of interest

No potential conflict of interest was reported by the authors.

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Figure legends

Figure 1. Biotechnological applications of killer yeasts and their toxins.

Figure 2. Localization of killer toxins genetic determinants. CHR: chromosomal genes; dsRNA VLP: extrachromosomal double-stranded (ds)RNA virus-like particles; dsDNA VLE: extrachromosomal ~~(ds)~~double stranded DNA virus-like elements.

Figure 3. Mechanisms of action of killer toxins. Killer toxins killing action is generally mediated by a two-steps mechanism. In the first step the killer toxin binds a primary receptor on the cell wall of the sensitive target. In the second step the killer toxin interacts with a secondary receptor and kills the target cell through the following different mechanisms: -(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 G1/S phase or completion of G1 phase; (D) fragmentation of RNA in terms of 18S and 25S rRNA, or tRNA.

Figure 4. Competitive advantage and metabolic cost of killer toxin production by yeast as a function of nutritional and environmental conditions. The success of the killer yeast depends on yeast dispersal, nutrient availability, and frequency of encounters between killer and sensitive yeast.

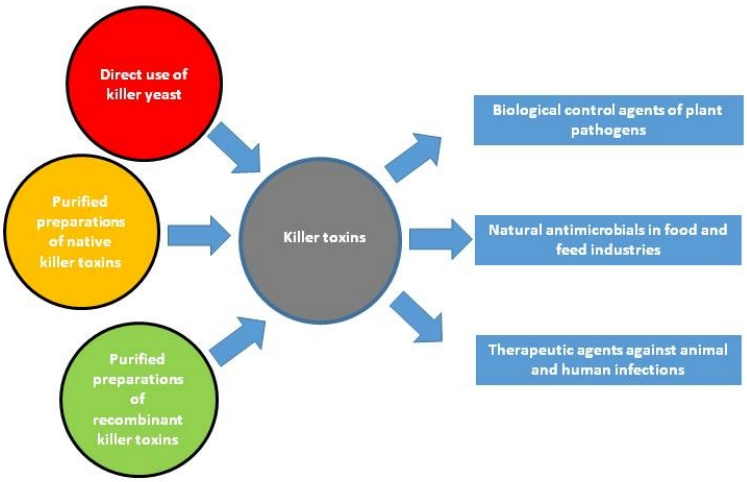


Figure 1

254x190mm (96 x 96 DPI)

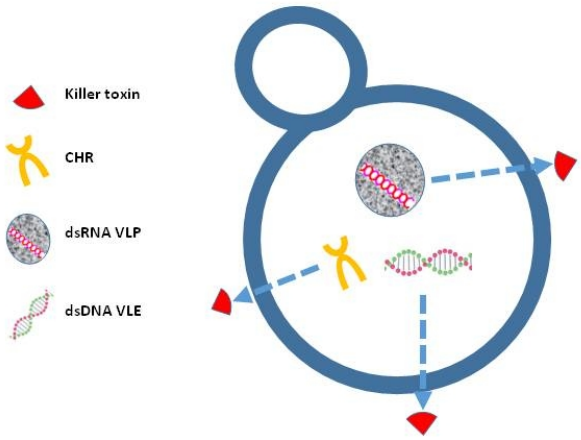


Figure 2

254x190mm (96 x 96 DPI)

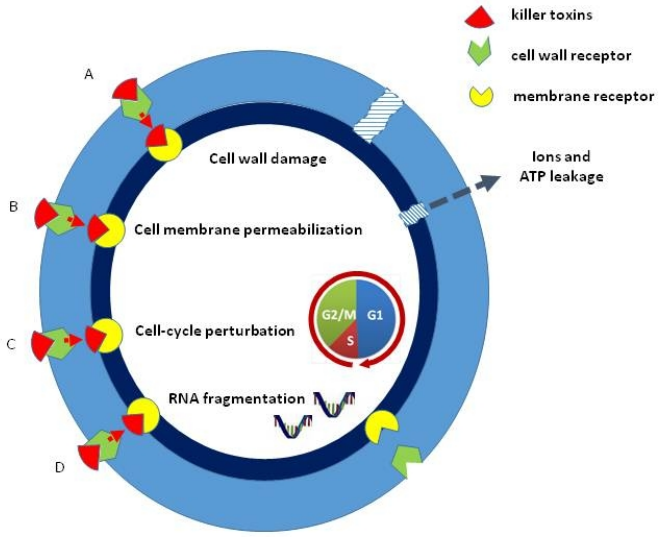


Figure 3

254x190mm (96 x 96 DPI)

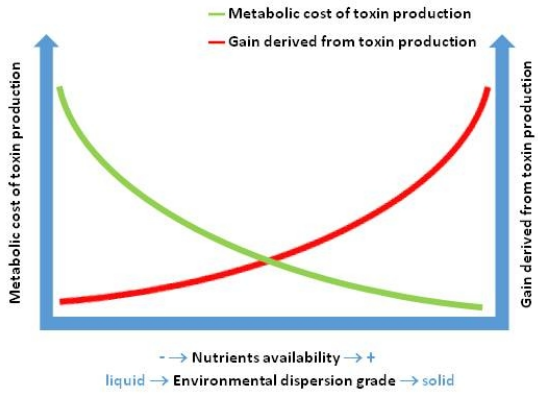


Figure 4

254x190mm (96 x 96 DPI)

Table 1. Known details of selected killer toxins and their biodiversity.

Killer yeast strain	Former designation	Toxin		Cell target		Mode of action
		Name	Molecular weight (kDa)	Primary	Secondary	
Chromosomal genes						
Saccharomyces cerevisiae 111		KHR	20	nd	nd	nd
Saccharomyces cerevisiae 115		KHS	75	nd	nd	Membrane permeabilization
Candida nodaensis PYCC3198		CnKT	nd	nd	nd	nd
Candida saturnus IFO0117	*W. saturnus. var. saturnus*	HYI	9.5	nd	nd	nd
Cyberlindnera mrakii IFO0895	*W. saturnus var mrakii; H. mrakii*	HM-1 or HMK	10.7	β-glucan	nd	Inhibition of β-1,3-glucan synthase
Cyberlindnera mrakii MUCL41968	*W. saturnus var. mrakii*	WmKT	85	β-glucan	nd	Cell-wall damage
Cyberlindnera mrakii NCYC500	*W. saturnus var. mrakii*	K500	~1.8	nd	nd	nd
Debariomyces hansenii CYC1021		nd	23	β-1,6-glucan	nd	nd
Kluyveromyces marxianus NCYC587	*K. fragilis*	K6	42	nd	nd	nd
Kluyveromyces wickerhamii DBVPG6077		KwKT	72	nd	nd	nd
Millerozyma farinosa KK1	*P. farinosa*	SMKT	6.6 (α), 7.9 (β)	nd	nd	Membrane permeabilization
Pichia kluyveri DBVPG5286		nd	19	nd	nd	Membrane permeabilization
Pichia membranifaciens CYC1106		PMKT	18	β-1,6-glucan	Cwp2p	Membrane permeabilization
Pichia membranifaciens CYC1086		PMKT2	30	Mannoprotein	nd	Cell-cycle arrest

Schwanniomyces occidentalis ATCC44252		nd	7.4(α), 4.9(β)	Mannoprotein	nd	Membrane permeabilization
Tetrapisispora phaffii DBVPG6076	*K. phaffii*	KpKT	33	β-1,3/ β-1,6-glucan	nd	β-glucanase activity
Wickerhamomyces anomalus BCA15, BCU24, BS91		nd	nd	β-1,3/ β-1,6-glucan	nd	β-glucanase activity
Wickerhamomyces anomalus NCYC434	*P. anomala*	Panomycocin	49	β-1,3-glucan	nd	Hydrolysis of β-1,3-glucan
Wickerhamomyces anomalus DBVPG 3003	*P. anomala*	PiKT	<8	β-1,6-glucan	nd	nd
Wickerhamomyces anomalus YF07b	*P. anomala*	nd	47	nd	nd	β-1,3-glucanase activity
Wickerhamomyces anomalus ATCC 96603	*P. anomala*	PaKt	85	β-1,3-glucan	nd	nd
Extrachromosomal double-stranded RNA virus-like particles						
Hanseniaspora uvarum 470		nd	18	β-1,6-glucan	nd	nd
Saccharomyces cerevisiae		K1	9.5 (α), 9(β)	β-1,6-glucan	Kre1p	Membrane permeabilization
Saccharomyces cerevisiae		K2	38.7	β-1,6-glucan	nd	Membrane permeabilization
Saccharomyces cerevisiae		K28	10.5 (α), 11 (β)	α-1,3-mannoprotein	Erd2p	DNA synthesis inhibition and cell-cycle block
Saccharomyces cerevisiae		Klus	nd	nd	nd	nd
Zygosaccharomyces bailii		Zygocin	10.4	Mannoprotein	nd	Membrane permeabilization
Extrachromosomal double-stranded DNA virus-like elements						
Debaryomyces robertsiae CBS6693	*W. robertsiae*	DrT	>100	Chitin	nd	rRNA fragmentation, cell-

						cycle perturbation
Kluyveromyces lactis IFO1267		Zymocin	97(α), 31(β), 28(γ)	Chitin	Ipt1p	Exo-chitinase activity; tRNA fragmentation; cell-cycle perturbation
Babjeviella inositovora NRRLY18709	*P. inositovora*	PiT	>100	Chitin	nd	rRNA fragmentation
Millerozyma acaciae NRRLY18665	*P. acaciae*	PaT	110(α), 39(β), 38(γ)	Chitin	nd	tRNA fragmentation, cell-cycle perturbation

Sources: Klassen et al. [5], Schaffrath et al. [28], Liu et al. [38], Belda et al. [49].

nd, not determined/ not known.

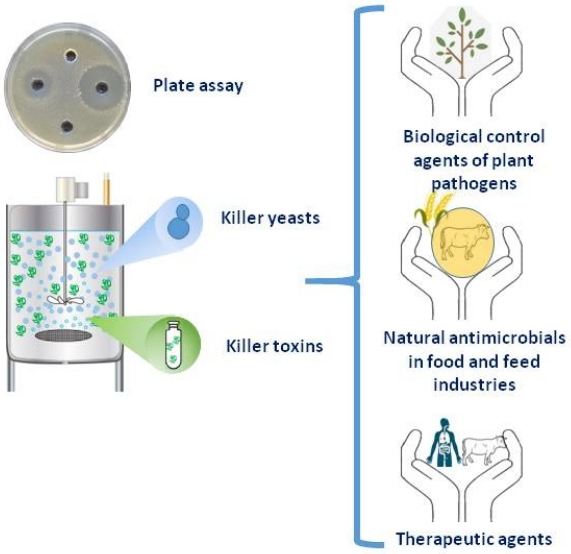
Table 2. Patents regarding the exploitation of killer yeasts and killer toxins.

Title of patent	Number	Source	Brief description	Year
Process for the preparation of killer toxins	WO2016199090	https://worldwide.espacenet.com/	Method for obtaining a preparation containing a killer toxin useful against phytopathogenic fungi on fruit plants, crops and domesticated plants	2016
Killer yeast Xiaoqu koji and preparation method thereof	CN103540538	https://worldwide.espacenet.com/	Preparation of Xiaoqu (starter for the fermentation of cereal wines) containing killer yeast	2014
Method for purifying killer protein	JP2007228937	https://patentscope.wipo.int/	Method for the purification of a killer protein	2007
Non-lactate-assimilating yeast for improving aerobic stability of silage	EP1194541B1	https://patents.google.com/	Utilization of a killer yeast unable to assimilate lactate to counteract spoilage yeasts in silage	2004
Toxin-related antibodies with antimicrobial and antiviral activity	WO2003095493	https://patentscope.wipo.int/	Anti-idiotypic antibody that retains yeast killer toxin activity and/or antiviral activity	2003
Methods of enhancing the preservation of forage materials with particular "killer" yeast strains that inhibit the growth of wild yeasts	NZ516456	https://worldwide.espacenet.com/	Utilization of *Saccharomyces exiguus* killer yeast or of its toxin to improve aerobic stability of silage	2002
Mixed starter culture and uses thereof	EP1476021A1	https://patents.google.com/patent/EP1476021A1/en	Composition of starter cultures containing a fermentative microorganism and at least one killer yeast that counteracts spoilage microorganisms or pathogens in different fermented foods	2002
Novel antifungal agents and fungicides, method for the production thereof and their use	CA2372935A1	https://patents.google.com/	Recombinant production of killer toxins Wicaltin and Zygocin to be used as antifungal agents and in plant protection	2001
Use of yeast-derived killer toxins to treat or prevent diarrhea, particularly in piglets and especially where caused by	DE19912439	https://worldwide.espacenet.com/	Use of killer toxins produced by fermentation of killer yeast, for prevention and/or treatment of diarrhea	2000

Escherichia coli				
Killer protein	JP10075789	https://patentscope.wipo.int/	New killer protein useful as component for antimicrobial agents	1998
Novel killer yeast	JPH0662836	https://worldwide.espacenet.com/	Killer strains of *Candida stellata* and *Hanseniaspora occidentalis* active against *Hansenula* or *Kloeckera*	1994
Cloning of the zymocin gene and use of zymocin in beverages	WO1994020620A3	https://patents.google.com/	Preservation of carbonated drinks by using a killer toxin (Zymocin) active against a wide range of different yeast strains and cloning of Zymocin gene	1994
Novel yeast having killer activity	JPS6219079	https://worldwide.espacenet.com/	Use of *Candida vinea* having stable killer activity in a wide temperature range	1987
Yeast containing plasmids providing killer characteristics	US4418150	https://worldwide.espacenet.com/	Use of recombinant *Saccharomyces cerevisiae* showing killer toxin-resistance and killer activity toward wide range of yeasts	1983
Killer yeast and its preparation	JPS58158180	https://worldwide.espacenet.com/	Preparation of a high-quality yeast harboring the killer factor by sporulating a homothallic yeast, fusing the resultant monoploid with a nuclear-fusionless yeast mutant having killer plasmid, and selecting from the produced yeasts	1983

Table 3. Non-*Saccharomyces* killer yeasts with promising applications in the wine industry.

Killer yeast	Strain	Sensitive targets	Reference
C. piralidae	IWBTY1140	*B. bruxellensis*	[84]
K. wickerhamii	DBVPG6077	*Brettanomyces/ Dekkera*	[16,85]
T. phaffii	DBVPG6076	*Hanseniaspora/ Kloeckera; Saccharomycodes ludwigii, Zygosaccharomyces bailii, Zygosaccharomyces rouxii*	[15,17,44]
T. delbruecki	NPCC1033	*H. uvarum, B. bruxellensis, P. guillermondii, P. membranaefaciens*	[86]
W. anomalus	DBVPG3003	*Brettanomyces/ Dekkera*	[16,45]
W. anomalus	Cf20	*B. bruxellensis, D. anomala, P. membranaefaciens, P. guillermondii*	[11]



graphical abstract

254x190mm (96 x 96 DPI)