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Keywords: Lyophilization, Kpkt, *Komagataella phaffii*, natural antimicrobial, MIC

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Abstract

Kpkt is a yeast killer toxin, naturally produced by *Tetrapisispora phaffii,* with possible applications in winemaking due to its antimicrobial activity on wine-related yeasts including *Kloeckera/Hanseniaspora*, *Saccharomycodes* and *Zygosaccharomyces*. Here, Kpkt coding gene was expressed in *Komagataella phaffii* (formerly *Pichia pastoris*) and the bioreactor production of the recombinant toxin (rKpkt) was obtained. Moreover, to produce a ready-to-use preparation of rKpkt, the cell-free supernatant of the *K. phaffii* recombinant killer clone was 80-fold concentrated and lyophilized. The resulting preparation could be easily solubilized in sterile distilled water and maintained its killer activity for up to six months at 4°C. When applied to grape must, it exerted an extensive killer activity on wild wine-related yeasts while proving compatible with the fermentative activity of actively growing *Saccharomyces cerevisiae* starter strains. Moreover, it displayed a strong microbicidal effect on a variety of bacterial species including lactic acid bacteria and food-borne pathogens. On the contrary it showed no lethal effect on filamentous fungi and on *Ceratitis capitata* and *Musca domestica*, two insect species that may serve as non-mammalian model for biomedical research. Based on these results, bioreactor production and lyophilization represent an interesting option for the exploitation of this killer toxin that, due to its spectrum of action, may find application in the control of microbial contaminations in the wine and food industries.

1. Introduction

Microbial contamination is one of the major causes of food and beverages spoilage. It can occur at different stages of the production process, with negative impact on food safety and sensory properties. Among the different methods aimed at preventing food deterioration or foodborne illness, the addition of natural or synthetic ingredients is often the one of choice. In fact, although exerting variable effects on food shelf life and safety, it is generally less expensive than other methods (Carocho et al., 2015). Indeed, excessive intake of synthetic antimicrobials may result in adverse health conditions. For example, sulphites or sulphiting agents, widely utilized in food and beverages industries, are known to exert cytotoxic and carcinogenic effects towards rats and humans (Guerrero and Cantos-Villar, 2014; Iammarino et al., 2012; Suh et al., 2007). Thus, the daily intake of SO_2 should not exceed 0.7 mg/kg body weight (Scientific Committee for Food, 1996) and the search for valid alternatives to SO_2 is on the agenda of the scientific community and the food industry.

Regarding the wine industry, where SO_2 is widely employed as antioxidant and antimicrobial (Santos et al., 2012), different authors proposed the application of *Saccharomyces cerevisiae* killer starters, in partial substitution of SO_2, to control the fermentative activity of undesired wine yeasts (De Ullivarri et al., 2014; Van Vuuren and Jacobs, 1992). Indeed, the dominance of *S. cerevisiae* killer strains on the wild must microflora is not obvious and depends on a number of factors among which the killer/sensitive ratio at the inoculum, the presence of compounds that interact with the killer toxin, the spectrum of action of the killer toxin and the grape must treatments (Pérez et al., 2001). In spite of that, the killer phenotype is one of the selection criteria for wine yeast starters (Mannazzu et al., 2002) and *S. cerevisiae* killer strains are commercially available and utilized in winemaking. Interestingly, the production of killer toxins of interest for the wine industry is widespread also among non-*Saccharomyces* yeasts. As an

example *Candida pyralidae*, *Kluyveromyces wickerhamii, Tetrapisispora phaffii, Torulaspora delbrueckii, Wickerhamomyces anomalus*, produce killer toxins active on a wide range of wine-related yeasts, including *Hanseniaspora uvarum, Zygosaccharomyces bailii, Pichia membranaefaciens* and *Brettanomyces bruxellensis* (Ciani and Comitini, 2011; Ciani and Fatichenti, 2001; Comitini et al., 2004a, 2004b; Mehlomakulu et al., 2017; Villalba et al., 2016). Most of these killer toxins are active under winemaking conditions and do not inhibit *S. cerevisiae* nor the lactic acid bacteria. Others, such as those produced by *T. delbrueckii* Kbarr strains are active on *S. cerevisiae* and other wine-related yeasts. Thus, *T. delbrueckii* killer strains dominate on *S. cerevisiae* both in white (Velázquez et al., 2015) and red musts (Ramírez et al., 2016) and have been proposed in co-culture with *S. cerevisiae* to accelerate yeast autolysis and maturation in sparkling wines (Velázquez et al., 2019). Indeed, among non-*Saccharomyces* killer toxins, those active on *Dekkera*/*Brettanomyces* yeasts deserve further investigation considering the negative impact of these spoilage yeasts on wine quality (Tubia et al., 2018; Villalba et al., 2020).

In 2001 the biotechnological potential of the killer toxin produced by *Tetrapisispora phaffii* (formerly *Kluyveromyces phaffii*), was described by Ciani and Fatichenti (2001). This toxin, named Kpkt, is active on wine related yeasts of the genera *Kloeckera/Hanseniaspora*, *Saccharomycodes* and *Zygosaccharomyces* and retains its killer activity for up to two weeks under winemaking conditions (Ciani and Fatichenti, 2001; Comitini and Ciani 2010). Further works showed that Kpkt is a β-glucanase that induces ultrastructural modifications on the cell wall of the sensitive target (Comitini et al., 2004b, 2009). Accordingly, the deletion of Kpkt encoding gene results in loss of killer phenotype and β-glucanase activity (Oro et al., 2014). Since *T. phaffii* DBVPG 6076 is unsuitable for a direct utilization in grape must fermentation and produces limited amounts of Kpkt, Chessa et al. (2017) developed the molecular tools

needed for the heterologous expression of this toxin in *Komagataella phaffii* (formerly *Pichia pastoris*), a GRAS host for heterologous production of proteins of interest for the food and biopharmaceutical industries (Ahmad et al., 2014).

In the present work, with the aim of contributing to the biotechnological exploitation of Kpkt, a recombinant *K. phaffii* clone producing Kpkt was grown in bioreactor and the cell-free supernatant was lyophilized and tested for killer activity in grape must and on a wide portfolio of living organisms including bacteria, yeasts and fungi. Moreover, to gather further information on the spectrum of action of the toxin on multicellular eukaryotic targets, the lyophilized preparation of rKpkt was administered to *Musca domestica* L. (Diptera: Muscidae) and *Ceratitis capitata* Wied. (Diptera: Tephritidae), two insect species that may serve as non-mammalian model for biomedical research.

2. Materials and methods

2.1 Microorganisms and growth media. Microbial strains were: *T. phaffii* DBVPG 6076, native producer of Kpkt; *T. phaffii* TpBGL2Δ, incapable of producing Kpkt due to a deletion of *TpBGL2* gene coding for Kpkt (Oro et al., 2014), utilized as the negative control of native Kpkt production; *K. phaffii* rc#6, Mut^+ strain secreting rKpkt, obtained by transforming *K. phaffii* GS115 with pPic9TpIM; *K. phaffii* rc#24, negative control of killer activity, obtained by transforming *K. phaffii* GS115 with pPIC9 (Chessa et al., 2017); *S. cerevisiae* DBVPG 6500 utilized as the killer toxin sensitive strain in well plate assay. Other microorganisms used are reported in Tables 1 and 2.

Growth media were YEPD: 2% glucose, 1% yeast extract, 2% peptone; BMGY: 1% glycerol (w/v), 1% yeast extract, 2% peptone, 1.34% YNB w/o amino acids and 0.00004% biotin; BMMY: as BMGY with 0.5 or 1% methanol (v/v) in place of glycerol; De Man, Rogosa, Sharpe (MRS)

broth (VWR, Milano); Brain Heart Infusion (BHI, Microbiol, Cagliari); Malt extract agar (Sigma); Wallerstein Laboratory Nutrient Agar (WL, Oxoid). Media were added with 2% agar and buffered at pH 4.5 with 0.1 M citrate-phosphate buffer (0.1 M citric acid, 0.2 M Na_2HPO4) when required. Yeasts and bacteria were maintained on appropriate media at 4 °C for short term storage and at −80 °C with addition of 10% glycerol for long-term storage.

2.2 Production of native and recombinant Kpkt. Baffled flask production of recombinant Kpkt (rKpkt) was carried out as described by Chessa et al. (2017). Briefly: yeast cells were precultured in 25 ml BMGY within 250 ml baffled flasks under constant shaking (180 rpm), at 30°C. After 24 h, cells were transferred to 25 ml BMMY and grown for up to 120 h at 28 °C for the first 24 h, 25 °C for the following 24 h and 23 °C till the end of the cultivation. To induce the production of rKpkt 1% methanol was added every 24 h in a fed batch mode.

Bioreactor productions were carried out in Biostat Bplus (Sartorius, Germany) fermenter (2 L). For native Kpkt (nKpkt) production, *T. phaffii* DBVPG 6076 was inoculated in 150 ml YEPD (pH 4.5) within 250 ml baffled flask and incubated at 25 °C for 24 hours under shaking conditions (180 rpm). This pre-culture was inoculated in 1.8 L YEPD working volume within the bioreactor. Dissolved oxygen and temperature were set at 20% and 25 °C, respectively, throughout the fermentation process and stirring was in cascade with dissolved oxygen. The culture medium was buffered (pH 4.5) with automated addition of 20% phosphoric acid and 20% potassium hydroxide. After 24 h of cultivation, the cell-free supernatant was collected by centrifugation (5000 g for 10 min) and subjected to micro-filtration (0.22 µm, Millipore).

For recombinant Kpkt (rKpkt) production fed-batch high cell density fermentation (HCDF) technology was utilized. Briefly: rc#6 was inoculated in 150 ml BMGY within 250 ml baffled flask and incubated at 30 °C for at least 24 hours or until it reached optical density (OD_{600}) of

10-12 under shaking conditions (180 rpm). The pre-culture was inoculated in 900 ml of BMGY added with 0.5 ml anti foam (Antifoam 204 Sigma Aldrich) within the bioreactor. The culture medium was buffered (pH 4.5) as described above. Stirring (maximum 1100 rpm) was in cascade with dissolved oxygen which was set to 25%. The fermentation strategy was articulated into: glycerol batch phase (GBP), glycerol fed-batch phase (GFBP), methanol fed-batch phase (MFBP). After complete glycerol consumption (~24 h), indicated by a spike of dissolved oxygen (end of GBP), 10% glycerol was added (9 ml/h). When biomass reached OD_{600} of 350–400 (about 24 h) glycerol feeding was stopped. In the transition from GFBP to MFBP fermentation temperature was decreased at 20 °C and methanol induction started with the addition of 3.6 ml/L/h 100% methanol during the first 2-3 hours. Then methanol feeding rate was doubled to ~7.3 ml/hr/L initial fermentation volume. After 2 h, methanol feeding rate was increased to ~10.9 ml/hr/L initial fermentation volume till the end of the process. The cell-free supernatant was collected by centrifugation (5000 g for 10 min) and subjected to micro-filtration (0.22 µm, Millipore). Killer activity in the cell-free supernatant was evaluated by means of the well plate assay.

2.3 Well plate assay and Arbitrary Units. Cell suspensions of the target sensitive strain (OD_{600} 0.1-0.2) were prepared in sterile distilled water and aliquots of 100 µl of were spread on YEPD buffered with 0.1 M citrate-phosphate buffer (pH 4.5). 100 µl of the samples to be tested for killer activity were loaded into wells cut in the agar medium. Toxin concentration in the supernatant was evaluated in terms of Arbitrary Units (AU) as already described by Chessa et al. (2017). The calculation of the Arbitrary Units was carried out as follows. Aliquots of 10, 15, 25, 50, 75 µl of cell-free supernatant taken to 100 µl with 0.1 M citrate phosphate buffer (pH 4.5), and 100 µl of cell- free supernatant of the clones to be tested, were subjected to well

plate assay using *S. cerevisiae* DBVPG 6500 as the sensitive strain. After 3 days of incubation at 25C°, the inhibition halos generated around each well were measured and the values obtained were used to trace a calibration curve in respect to a Cartesian axes system, where the Log of the concentration of the toxin is on the abscissa and the diameter (mm) of the halo is on the ordinate. Given the linear relationship between diameter of the inhibition halo and logarithm of killer toxin concentration, it was established that 1 AU corresponds to the amount of toxin in 100 µl that produces an inhibition halo of 20 mm (as the diameter of the well), using *S. cerevisiae* DBVPG 6500 as the sensitive strain.

2.4 Lyophilization. Cell free supernatants obtained as described above (paragraph 2.2) were 80-fold concentrated by ultrafiltration through a Crossflow filter holder (10 kDa cut-off Hydrosart membrane, Sartorius). The ultrafiltered samples were aliquoted in falcon tubes (5 ml) and taken at -80°C. Lyophilization was carried out under vacuum (0.133÷0.400 mbar) at -42°C with a Labonco Freezone 4.5 system (Boston Laboratory Equipment). After 72 h the water phase was completely removed and the powder obtained was stored at 4°C. After solubilisation of the powder in sterile distilled water (100 mg/mL) total protein content of the lyophilized preparations containing Kpkt (LrKpkt) was measured according to Bradford (1976) and toxin concentration was evaluated in terms of AU. For lyophilized preparations obtained with *T. phaffii* TpBGL2Δ (NCLnKpKt) and rc#24 (NCLrKpkt) the amounts of lyophilized preparation to be used as the negative controls of killer activity were standardized in terms of total protein content.

2.5 Determination of rKpkt activity in grape must. Vermentino (residual sugars 190 g/L; total acidity 5.7 g/L; pH 3.5; tartaric acid 3.5 g/L; malic acid 3.3 g/L; nitrogen components (APA) 120

188 ppm) and Cannonau (residual sugars 300 g/L; total acidity 4.8 g/L; pH 3.4; tartaric acid 6 g/L;
189 malic acid 1.3 g/L; nitrogen components (APA) 100 ppm) grape musts were provided by Tenute
190 Sella & Mosca (Loc. I Piani, Alghero). Vermentino grape must was clarified (NTU about 100)
191 while Cannonau was not. The two musts were aliquoted in 24-well microtiter plates (1.5 ml in
192 each well). To evaluate the effect of the lyophilized preparations on pure cultures of wine
193 related yeasts, Vermentino grape must was pasteurized (80 °C for 5 min), inoculated with
194 1×10^6 cell/mL and added with LrKpkt (2 AU/mL). After 48 h growth at 25 °C under constant
195 shaking (50 rpm) viable plate count was carried out on YEPD. To evaluate the effect of the
196 lyophilized preparations on wild wine microflora and on *S. cerevisiae*, fresh Vermentino and
197 Cannonau grape musts, and the same musts inoculated at the industrial scale with two *S.*
198 *cerevisiae* commercial starter strains, were treated with LrKpkt (2, 1 and 0.5 AU/mL) and
199 incubated for up 48 h growth at 25 °C under constant shaking (50 rpm). Viable plate count was
200 carried out on WL (uninoculated fresh grape must) or YEPD (inoculated fresh grape must)
201 immediately after toxin addition (T0) and after 24 and 48 h (T1 and T2, respectively).
202 In all trials, equivalent amounts in terms of total protein content of NCLrKpkt were utilized as
203 negative control (NC) and, to exclude any possible effect of NCLrKpkt on yeast growth, also
204 pasteurized grape must as such (AS) was subjected to viable plate count. At least three
205 technical replicates from at least two independent experiments were carried out.
206
207 **2.6 Spectrum of action of rKpkt**. For bacteria and yeasts, dose response assays were carried
208 out in 96-well microtiter plates. Briefly, $1x10^6$ cells/mL for yeasts and $1x10^5$ cells/mL for
209 bacteria, were inoculated in microtiter wells containing 200 µl medium (YEPD for yeast, MRS
210 for lactic acid bacteria and BHI for food-borne pathogens) added with decreasing
211 concentrations of LrKpkt (two-fold serial dilution) in sterile distilled water and expressed in

212 terms of AU/mL (2, 1, 0.5, 0.25, 0.125, 0.062, 0.031, 0.015, 0.007, 0.003) and comparable
213 amounts of NCLrKpkt (NC). The microtiter plates were incubated in agitation at 25 °C for yeasts,
214 at 37 °C for bacteria, for 24 h and growth was measured automatically every 30 min at OD_{600}
215 using a SPECTROstar nano microplate spectrophotometer (BMG Labtech, Ortenberg,
216 Germany). Each of the resulting growth curves was fitted by the primary model of Baranyi and
217 Roberts (1994) wrapped in DMFit Excel Add-in, downloaded from
218 http://www.combase.cc/index.php/en/tools, that was utilized also to evaluate the maximum
219 specific growth rate (μ), the duration of lag phase (λ), and the generation time (g). The minimal
220 inhibitory concentration (MIC), and the minimal concentration affecting growth (MCAG) were
221 evaluated based on μ values. The minimal microbicidal concentration (MMC) was further
222 confirmed by track-dilution method (Jett et al., 1997). Results are means ± standard deviation
223 of three technical replicates of two independent experiments. For filamentous fungi, 100 μl
224 aliquots of sterile distilled water containing 20 AU/mL of LrKpkt, were loaded into wells cut in
225 the centre of Malt extract agar plates (pH 4.5) and three aliquots of 10 μl containing 10^2 conidia
226 in water were placed on top of a triangle at equal distances from the well directly onto the
227 agar, to allow the expansion of the fungus in all direction and detect a possible growth
228 inhibition dictated by the presence of the LrKpkt in the middle of the plate. Equivalent amounts
229 of NCLrKpkt, in terms of total protein content, were utilized as negative control.

230 For insect bioassays, oral toxicity of recombinant and native Kpkt was evaluated on newly
231 emerged adults of the medfly *C. capitata* and of the housefly *M. domestica* provided by the
232 insect rearing facility of the Department of Agriculture of the University of Sassari (Italy) as
233 described in Ruiu et al. (2015) and Marche et al. (2017). Briefly, insects were administered a
234 liquid diet incorporating either LrKpkt and LnKpkt. According to the experimental design, the
235 bioassays involved four groups of 10 newly emerged adult flies for each treatment. Each group

was maintained within a plastic cage (10 × 15 × 5 cm) at 25°C and 60% relative humidity. Lyophilized preparations were rehydrated in sterile distilled water, before being added with 30% saccharose. Aliquots of 75 µl of lyophilized preparations containing 2 AU of nKpkt and rKpkt were administered daily to each fly group by micropipette. As negative control, flies were fed comparable amounts of NCLrKpkt and NCLnKpkt. As untreated control, analogous groups of flies were fed with the sole saccharose solution (30%). Insects were inspected daily and mortality was assessed after 5 days (Ruiu et al., 2007; Ruiu et al., 2015). Bioassays involved four replicates and each experiment was repeated at least twice.

2.7 Data analysis

One-way ANOVA was carried out for the evaluation of the effect of rKpkt for each sampling time. The critical value for significance level (p) was set at 0.05 and the Tukey test was used for post-hoc comparisons. All statistical analyses were performed using R-studio for Windows, version 10 (RStudio, PBC, USA).

3. Results

3.1 Production of native and recombinant Kpkt. Bioreactor cultivation of *T. phaffii* DBVPG 6076 confirmed the production of native Kpkt (nKpkt) with 7.7±0.14 UA/mL after 24 h of growth. Baffled flasks and bioreactor cultivation of rc#6 led to the production of significantly higher ($p<0.001$) amounts of killer toxin. In particular, 16.8±0.11 AU/mL of recombinant killer toxin (rKpkt) were obtained after 120 h of methanol induction in baffled flask while bioreactor production resulted in a time-dependent increase in toxin production with 2.3±0.07, 8.2±0.12 and 17.1±0.21 AU/mL at 24, 48 and 70 h, respectively. Thus, rc#6 Kpkt productivity proved significantly higher ($p<0.05$) than that of *T. phaffii* DBVPG 6076 in terms of AU/mL

3.2 Lyophilization of rKpkt. Following the lyophilization of a 30 mL aliquot of 80-fold concentrated cell-free supernatant of rc#6, 2.15 g of powder containing rKpkt were obtained. The lyophilized preparation, named LrKpkt, was solubilised in water and in 0.1 M citrate-phosphate buffer (pH 4.5) and subjected to well plate assay to evaluate possible differences in killer activity in the two solvents. Since no differences were observed, sterile distilled water was utilized to solubilize LrKpkt for the following experiments. Based on well plate assay, it was found that while 30 mL of ultrafiltered cell-free supernatant (UFrKpkt) contained 3870 AU, the lyophilized preparation deriving from 30 ml of ultrafiltered cell-free supernatant contained 1032 AU of rKpkt with a 73% reduction of killer activity in terms of AU. Regarding specific activity, this was 3.88 AU/mg of protein for UFrKpkt and 0.75 UA/mg protein for LrKpkt. Comparison of long-term stability of UFrKpkt and LrKpkt showed that while UFrKpkt completely lost killer activity within 6 weeks at -80°C, LrKpkt displayed no significant variations of killer activity for up to six months at 4°C. The lyophilized preparation of *T. phaffii* DBVPG 6076, named LnKpkt, contained 891 AU of native Kpkt with a specific activity of 0.80 AU/mL. As expected, the lyophilized preparations of rc#24 and *T. phaffii* TpBGL2Δ, named NCLrKpkt and NCLnKpkt, respectively and utilized as negative controls, showed no killer activity.

3.3 Effect of LrKpkt on pure cultures of wine-related yeasts in pasteurized Vermentino grape must. Pure cultures of *D. bruxellensis*, *H. uvarum*, *K. apiculata*, *L. thermotolerans*, *S. cerevisiae, Sacch. ludwigii*, and *W. anomalus* were inoculated in pasteurized Vermentino grape must and treated with LrKpkt. As shown in Figure 1, no residual viability was detected after 48 h treatment at 25°C, thus indicating that LrKpkt maintains its killer activity in grape must. On the contrary, NCLrKpkt (NC) showed no effect on cell viability, that was comparable to that

284 observed in grape must as such (AS).

285

286 **3.4 Effect of LrKpkt on wild yeast microflora and inoculated starter strains in fresh grape**
287 **must.** Increasing concentrations of LrKpkt were added to fresh Cannonau (red) and
288 Vermentino (white) grape musts and residual yeast viability was evaluated at different
289 sampling times. Regarding Cannonau, cell densities at T0 were $3\times10^6\pm1.8\times10^5$ CFU/mL and
290 $2.7\times10^6\pm1.9\times10^5$ CFU/mL in grape must as such (AS) and in grape must added with NCLrKpkt
291 (NC), respectively. At T1 and T2 a dose-dependent effect of the toxin was observed with
292 significant reductions of viable plate counts ($p<0.05$) (Figure 2). However, while at T1 and T2
293 cell viability was above 60% in respect to AS when 0.5 and 1 AU/mL LrKpkt were applied, at
294 the highest concentration (2 AU/mL) LrKpkt determined more than 90% reduction of cell
295 viability. As compared to Cannonau, Vermentino grape must showed lower viable plate count
296 at T0 with $3\times10^4\pm2.12\times10^3$ and $4.8\times10^4\pm1.27\times10^3$ CFU/mL in AS and NC, respectively. Under
297 these conditions, LrKpkt showed a dramatic effect on viable plate count, no matter the
298 concentration used. In particular, residual viability was below 1% at T1 while slight increases
299 in viable plate count were observed at T2 (Figure 2).
300 Finally, LrKpkt was added to Vermentino and Cannonau grape musts inoculated with *S.*
301 *cerevisiae* EC1118 (Lalvin EC1118) and *S. cerevisiae* Okay (Lalvin ICV OKAY®), respectively. At
302 T0, the two musts showed comparable cell densities with about 4×10^7 cell/mL. LrKpkt addition
303 to Vermentino resulted in a dose-dependent effect on *S. cerevisiae* EC1118 (Figure 3). At T1,
304 0.5 and 1 AU/mL exerted the same effect ($p<0.05$) on the starter strain, that showed more
305 than 70% cell viability in respect to AS, while 2 AU/mL resulted in about 50% cell viability as
306 compared to AS. At T2, the starter strain fully restored cell growth at 0.5 AU/mL LrKpkt while
307 higher concentrations of LrKpkt determined significant reduction in cell viability ($p<0.05$).

308 Regarding Cannonau, no differences in viable plate count on YEPD were observed for *S.*
309 *cerevisiae* Okay between the thesis without and with LrKpkt (Figure 4).
310
311 **3.5 Spectrum of action of LrKpkt on bacteria, yeast and filamentous fungi.** To further explore
312 the spectrum of action of the recombinant toxin, a rich portfolio of bacteria, yeasts and
313 filamentous fungi was challenged with known amounts of LrKpkt. Regarding bacteria, results
314 reported in Table 1 indicate that among the Lactobacilli, *L. rhamnosus* was the most resistant
315 to rKpkt (MIC=1 AU/mL and MMC= 2 AU/mL), while the two *L. plantarum* strains were affected
316 by lower concentrations of the toxin (MIC=0.5 AU/mL and MMC= 1 AU/mL) (Table 1). Among
317 the food-borne pathogens, *L. monocytogenes* was the most resistant to LrKpkt (MIC =1 AU/mL)
318 although being killed by 2 AU/mL (Table 1, Figure 5). *E. coli*, *S. aureus* and *S. bongori* were
319 inhibited and killed by lower concentrations of LrKpkt (MIC=0.5 AU/mL and MMC=1 AU/mL).
320 With the exception of *E. coli*, that showed the highest MCAG (0.25 AU/mL), the other food-
321 borne pathogens were affected by lower concentrations of LrKpkt and showed increases in the
322 duration of the lag phase and of the generation time at 0.125 AU/mL.
323 Regarding yeasts, results reported in Table 2 indicate both inter- and intraspecific variability in
324 the cellular response to LrKpkt. The MIC ranged from a minimum of 0.25 (*Z. bailii* UNISS 38) to
325 a maximum of 2 AU/mL (*W. californica* DiSVA 366) with most of the strains being inhibited by
326 0.5 or 1 AU/mL.
327 In respect to the MMC, with the exception of *Z. bailii* UNISS 38 that was killed by 0.5 AU/mL,
328 and *W. californica* DiSVA 366 that survived 2 AU/mL, 56% of the remaining strains were killed
329 by 2 AU/mL and 44% by 1 AU/mL (Table 2, Figure 5). The MCAG ranged from a minimum of
330 0.25 AU/mL (about half of the *S. cerevisiae* and almost all *L. thermotolerans* tested, *S.*
331 *paradoxus* UNISS 96 and UNISS 137, *D. bruxellensis* DiSVA 692, *Z. bailii* UNISS 38) to a maximum

of 1 AU/mL (*M. pulcherrima* UNISS 19) while the remaining strains were affected by 0.5 AU/mL of LrKpkt.

LrKpkt was tested also onto on pure cultures of nine filamentous fungi, isolated from Vermentino grape must identified as *Galactomyces spp*., *Fusarium spp*., *Aspergillus spp*., *Cladosporium spp*., *Rhizopus spp*., *Penicillium spp*. (V. Balmas, University of Sassari, personal communication). Results obtained showed no activity of the toxin on these filamentous fungi.

3.6 Activity of LrKpkt on insects. Considering its wide spectrum of action on yeasts and bacteria, LrKpkt was tested also on the medfly *C. capitata* and on the house fly *M. domestica* to evaluate its possible toxicity on two representative invertebrate species with very different biological and ecological features. Due to the observed differences in the spectrum of action of recombinant and native toxins (Chessa et al., 2017), the effect of LnKpkt was also considered in insect bioassays. As a result, average insect mortality ranged between 15 and 35% for the medfly and between 15 and 25% for the house fly with no significant differences among treatments (LrKpkt, LnKpkt, NCLrKpkt, NCLnKpkt) and the untreated controls for both species (*C. capitata* $p = 0.3982$; *M. domestica* $p = 0.7135$). Thus, it was shown that LrKpkt and LnKpkt exert no lethal effect on the two insects.

4. Discussion

The main constraints to the biotechnological exploitation of yeast killer toxins are related to the difficulty to raise their production at the industrial scale, to their ease of use and to the lack of information regarding their safety and spectrum of action (Mannazzu et al., 2019). To contribute to address these issues, in this work the production of rKpkt was scaled up and the cell free supernatant containing rKpkt was lyophilized to obtain a ready-to use antimicrobial

356 compound that was tested on a wide portfolio of biological targets.

357 Following that reported for the heterologous expression of genes under the control of *AOX*1
358 promoter, bioreactor cultivation of rc#6 was articulated into three phases aimed at repressing
359 (GBP), de-repressing (end of GFBP) and fully inducing (MFBP) *AOX*1 promoter (Inan and
360 Meagher, 2001; Juturu and Wu, Liu et al., 2019; Sun et al., 2018; Viña-Gonzalez et al., 2018;
361 Zhang et al., 2000). Accordingly, glycerol feeding in the first two phases led to reach a high
362 biomass concentration, while methanol feeding in the third phase resulted in a final
363 concentration of rKpkt that was comparable to that obtained in baffled flask. Moreover, scale
364 up of the fermentation process determined no reduction in the yield of rKpkt that, contrary to
365 that expected, more than doubled that obtained with the native producer *T. phaffii* DBVPG
366 6076.

367 Lyophilization of 80-fold concentrated cell-free supernatant of rc#6, although determining
368 significant decreases in killer activity in terms of AU and specific activity, appeared an
369 interesting option for the management of the toxin. In fact, it led to the obtainment of a dried
370 preparation that showed longer shelf life as compared to UFrKpkt, was easily soluble in sterile
371 distilled water and could be stored at 4°C, all properties that facilitates its biotechnological
372 exploitation.

373 Indeed, LrKpkt greatly affected the wild wine yeast microflora, although with significant
374 differences between Cannonau and Vermentino grape musts. Considering that the efficacy of
375 yeast killer toxins depends, at least in part on the cell density of the target strains (Pérez et al.,
376 2001; Velàzquez et al., 2015), the differences observed in fresh uninoculated Vermentino and
377 Cannonau grape musts could be due to the significant differences in the microbial load of the
378 two grape musts at T0. However, it should be noted that also when Vermentino and Cannonau
379 grape musts were inoculated with the same cell density of commercial starter strains, LrKpkt

exerted a concentration-dependent effect in Vermentino and no significant effect in Cannonau. Considering that the two starter strains displayed equal sensitivity to LrKpkt in terms of MIC and MMC, also a grape must effect on LrKpkt toxicity could be hypothesized. Thus, on the one side, the lower toxicity of LrKpkt in Cannonau in respect to that observed in Vermentino could depend, at least in part, on the interactions between the killer toxin and the phenolic compounds in the red must (Ozdal et al., 2013). On the other side, considering that Vermentino grape must had been thoroughly clarified prior to inoculation while Cannonau had not, also the presence of grape debris in the red must might have interfered with LrKpkt activity, in accordance with that found by Pérez et al. (2001) for *S. cerevisiae* K2 killer toxin.

LrKpkt showed a wider spectrum of action than previously thought (Chessa et al., 2017) being active also on Gram-positive and Gram-negative bacteria. Other authors reported on the activity of yeast killer toxins on a plethora of Gram-positive and Gram-negative pathogenic and non-pathogenic bacteria (Al-Qaysi et al., 2017; Bajaj et al., 2013; Guyard et al., 2002: Izgu and Altinby, 1997; Meneghin et al., 2010; Psani and Kotzekidou, 2006; Waema et al., 2009). Studies on the killer toxin produced by *Pichia anomala* ATCC 96603 showed that the monoclonal antibodies representing the internal image of the toxin are active on bacteria possibly through the interaction with the cell surface polysaccharides (Conti et al. 2000, Conti et al. 2002). While the mechanisms of action of LrKpkt on yeasts has already been at least in part elucidated that on bacteria still needs to be investigated. Nonetheless, the results here presented indicate that rKpkt similar to other yeast killer toxins, may represent a possible approach to the control of Gram-positive and Gram-negative contaminants and food borne pathogens.

Regarding eukaryotic targets, while no effect was observed on filamentous fungi, the width of rKpkt spectrum of action on wine-related yeasts was confirmed in grape must. Moreover, with the exception of *W. californica,* all of them were killed by LrKpkt and showed significant

impairments of cell growth when treated with sub-lethal doses of LrKpkt thus suggesting that by finely tuning LrKpkt concentration it is possible to obtain either a microbicidal or a microbiostatic effect.

Contrary to that observed for toxins produced by entompathogenic microorganisms employed in the biocontainment of harmful insect species (Ruiu, 2018), LrKpKt and LnKpKt proved not toxic *C. capitata* and *M. domestica. C. capitata* is one of the most important agricultural pests developing on fruits of a broad plant host range in tropical and sub- tropical regions and is a potential vector of bacterial food-borne pathogens (Sela et al., 2005). *M. domestica* lives in close contact with organic matrices of different origin, such as excrements, food residues and cadavers, thus continuously interacting with various human pathogenic microorganisms (Scott et al., 2014) and is often employed as a biological model in medically-related studies. Indeed, the lack of toxicity of LrKpKt and LnKpKt on the two insects clearly indicated that recombinant or natural Kpkt do not represent potential tools for the management of these insect pests. On the other hand, these results provided important information on the toxicity range of native and recombinant Kpkt that appear not to be active against these multicellular eukaryotic model organisms whose employment in biomedical research is well documented (Kouloussis et al., 2017). Thus, although LrKpkt toxicity needs to be evaluated on human cell lines, this result adds further support to the use of these toxins in the food industry.

In conclusion, killer yeasts may contribute to improve wine quality by counteracting the fermentative activity of undesired spoilage yeasts. Thus, their utilization is recommended and rather common in the wine industry. Moreover, their toxins may be active also on bacteria in typical food fermentation environments, thus making them attractive tools for the management of microbial contaminations also in the food industry. Here, with the aim of

428 further exploring the feasibility of the biotechnological exploitation of yeast killer toxins, the
429 heterologous expression of Kpkt and the bioreactor production of rKpkt were obtained.
430 Moreover, to produce a ready-to-use preparation of the recombinant killer toxin, the cell-free
431 supernatant of the *K. phaffii* recombinant killer clone was 80-fold concentrated and
432 lyophilized. The long term stability of lyophilized preparation of rKpkt at 4 °C, and the fact that
433 it maintains its killer activity upon solubilisation in water, indicate that lyophilization
434 represents an interesting option for handy storage and utilization of this killer toxin that, due
435 to its spectrum of action, may find application in the control of microbial contaminations in
436 the wine and food industries.
437
438 **Disclosure of interest**
439 The authors declare that they have no known competing financial interests or personal
440 relationships that could have appeared to influence the work reported in this paper.
441
442 **Funding**
443 This work was partially granted by ”Fondo di Ateneo per la Ricerca 2019 (P.I I.M.)”. G.C. PhD
444 grant was supported by Ministero dell’Università e della Ricerca, program PON-RI (2014-2010)
445 Azione I.1 “Dottorati innovativi con caratterizzazione Industriale” (PI I.M.).
446
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596 **Legends to Figures**

597 **Figure 1.** Effect of LrKpkt on pure cultures of wine-related yeasts in pasteurized Vermentino
598 grape must. Cell viability was evaluated by viable plate count 48 h after toxin addition. AS:
599 grape must as such without LrKpkt; LrKpkt: grape must with 2 AU/mL LrKpkt; NC: grape must
600 with NCLrKpkt (lyophilized supernatant of rc#24, utilized as negative control). Same dilution
601 (10^{-3}) is reported for each strain and condition. Results are representative of three technical
602 replicates of two independent experiments.

603

604 **Figure 2.** Viable plate count of fresh Cannonau (A) and Vermentino (B) grape musts upon
605 LrKpkt treatment. Viable plate count was carried out on WL at T1 and T2 (24 and 48 h after
606 toxin addition, respectively). Cell viability was expressed as percentage in respect to grape
607 must as such (AS); NC: grape must with NCLrKpkt (lyophilized supernatant of rc#24, utilized as
608 negative control); 0.5, 1 and 2 AU: grape must with 0.5, 1 and 2 AU/mL of LrKpkt, respectively.
609 Results are means ± standard deviation of three technical replicates of two independent
610 experiments. Same superscript letters indicate results not significantly different for each
611 sampling time ($p< 0.05$).

612

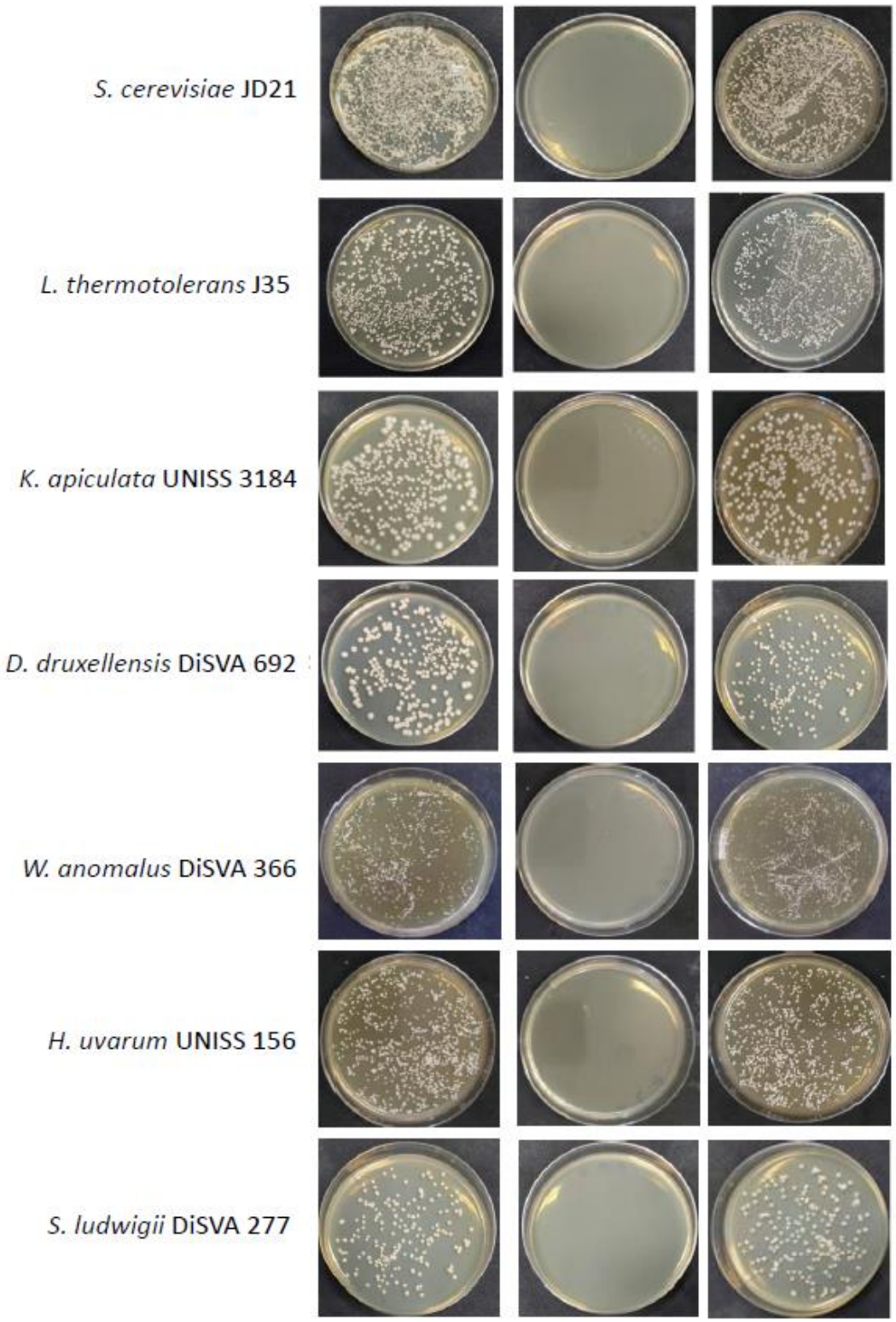
613 **Figure 3.** Effect of LrKpkt in fresh Vermentino grape must inoculated with *S. cerevisiae* EC1118.
614 Viable plate count was carried out on YEPD at T1 and T2 (24 and 48 h after toxin addition,
615 respectively). In (A) cell viability is expressed as percentage in respect to grape must as such
616 (AS). Results are means ± standard deviation of three technical replicates of two independent
617 experiments. Same superscript letters indicate results not significantly different for each
618 sampling time ($p< 0.05$). In (B) the effect of increasing concentrations of LrKpkt on viable plate
619 count is shown at T2. Same dilution (10^{-5}) is reported for all conditions. NC: grape must with

NCLrKpkt (lyophilized supernatant of rc#24, utilized as negative control); 0.5, 1, 2 AU: grape must with 0.5, 1, 2 AU/mL LrKpkt.

Figure 4. Effect of LrKpkt in fresh Cannonau grape must inoculated with *S. cerevisiae* Lalvin ICV Okay. Viable plate count was carried out on YEPD at T1 and T2 (24 and 48 h after toxin addition, respectively). In (A) cell viability is expressed as percentage in respect to grape must as such (AS). Results are means ± standard deviation of three technical replicates of two independent experiments. Same superscript letters indicate results not significantly different for each sampling time ($p< 0.05$). In (B) the effect of increasing concentrations of LrKpkt on viable plate count is shown at T2. Same dilution (10^{-5}) is reported for all conditions. NC: grape must with NCLrKpkt (lyophilized supernatant of rc#24, utilized as negative control); 0.5, 1, 2 AU: grape must with 0.5, 1, 2 AU/mL LrKpkt.

Figure 5. Dose-response effect of LrKpkt on cell viability. Bacterial and yeast cells were grown in the presence of 0.5, 1 and 2 AU/mL LrKpkt (A, C) and comparable amounts of NCLrKpkt (lyophilized supernatant of rc#24, utilized as negative control) (B, D). Residual cell viability after 24 h incubation was evaluated with the track dilution method. Results are representative of three technical replicates of two independent experiments.

644



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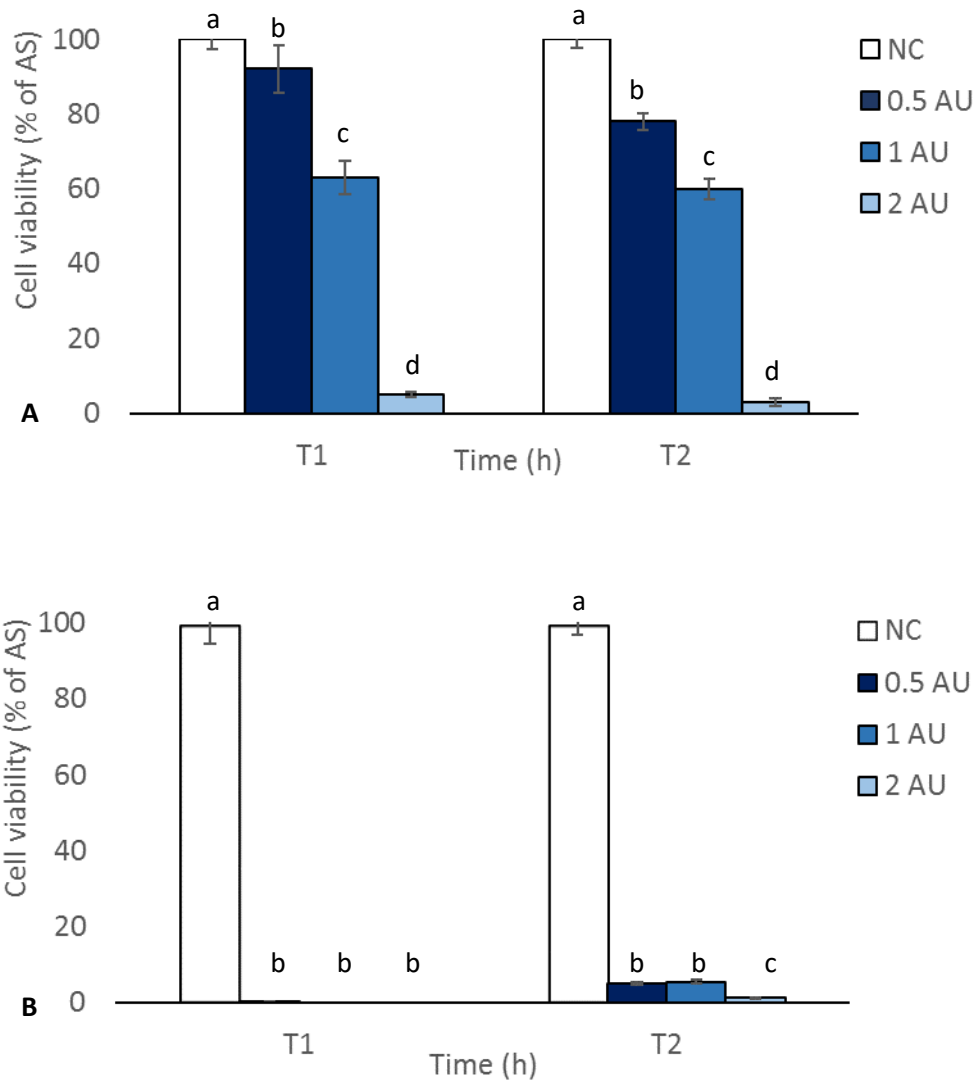
646 Figure 1

647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Cell viability (% of AS)
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40
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a
b
c
d
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b
c
d
T1
Time (h)
T2
NC
0.5 AU
1 AU
2 AU
B
Cell viability (% of AS)
100
80
60
40
20
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T1
Time (h)
T2
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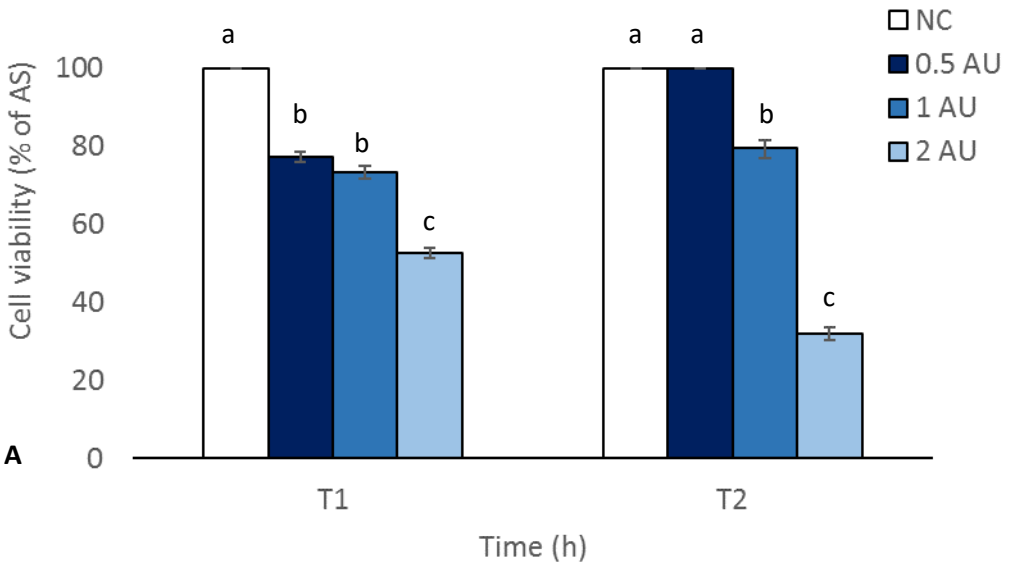
Figure 2


Cell viability (% of AS)
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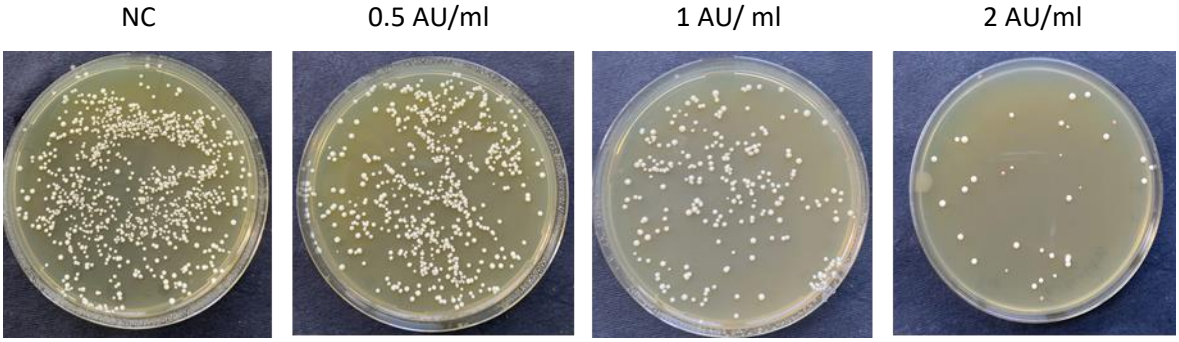
T1
T2
Time (h)

A


NC
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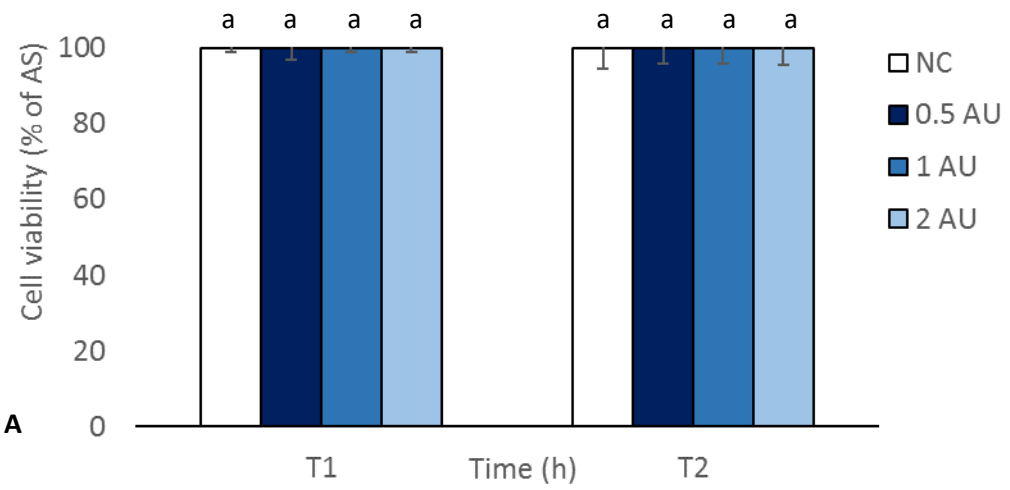
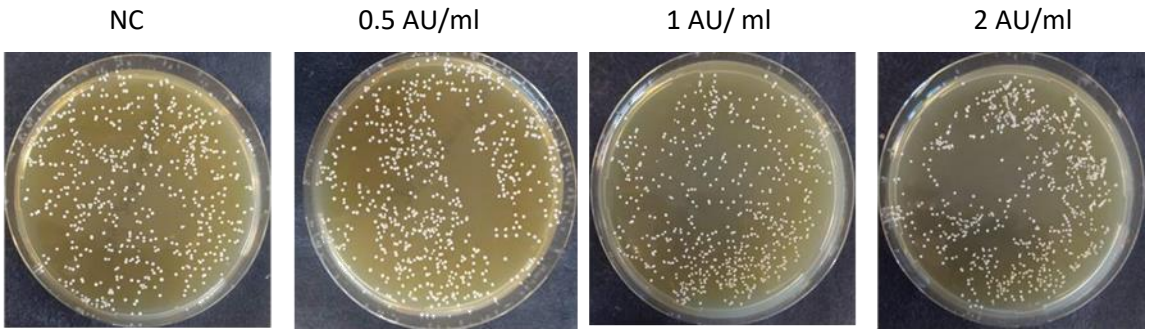
1 AU/ ml
2 AU/ml

B

Figure 3



A



B

Figure 4

Figure 4

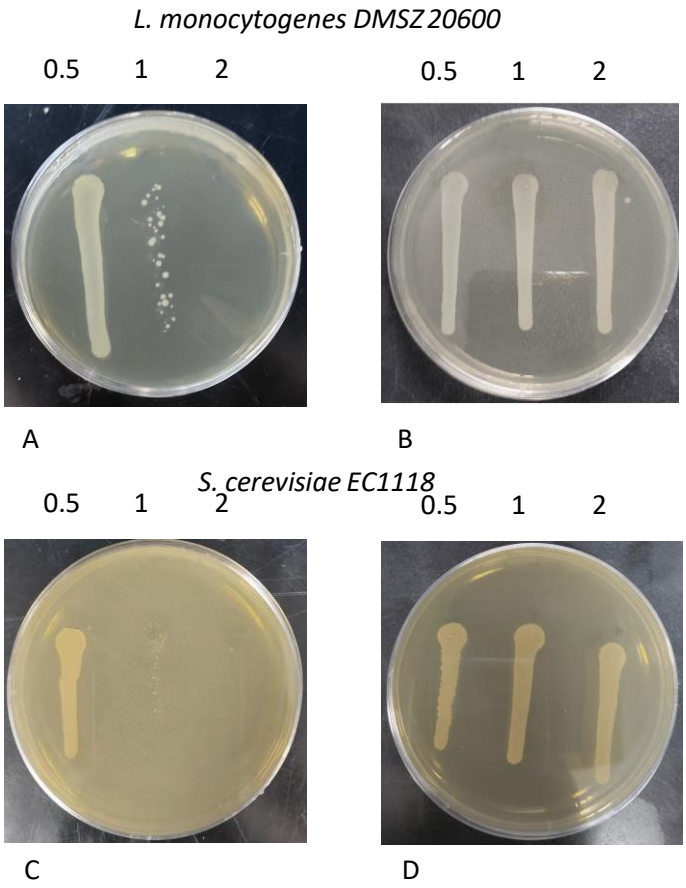

L. monocytogenes DMSZ 20600
0.5 1 2
0.5 1 2
A
B
S. cerevisiae EC1118
0.5 1 2
0.5 1 2
C
D

Figure 5

744

745

746 **Table 1**. Evaluation of rKpkt toxicity on bacteria.

747

Species	MMC (AU/mL)	MIC (AU/mL)	MCAG (AU/mL)	λ MCAG (min)	g MCAG (min)	λ NC (min)	g NC (min)
E. coli DMSZ 30083	1	0.5	0.25	314±18.7[a]	48±6.5[a]	43±5.8[b]	23±2.3[b]
L. monocytogenes DMSZ 20600	2	1	0.125	294±47.0[a]	61±3.9[a]	161±25.5[b]	38±3.8[b]
L. monocytogenes UNISS B	2	1	0.125	340±15.3[a]	99±1.1[a]	229±13.5[b]	96±3.6[a]
L. monocytogenes UNISS C	2	1	0.125	294±29.2[a]	114±7.7[a]	228±9.8[b]	86±3.3[b]
L. monocytogenes UNISS E	2	1	0.125	338±25.0[a]	87±5.5[a]	220±12.2[b]	74±4.1[b]
S. bongori DSMZ 13772	1	0.5	0.125	143±22.2[a]	42±7.2[a]	30±6.9[b]	23±1.2[b]
S. aureus DMSZ 20231	1	0.5	0.125	365±17.0[a]	92±6.1[a]	64±2.9[b]	25±1.1[b]
L. rhamnosus ATCC 7469	2	1	0.25	208±21.6[a]	99±6.8[a]	61±3.7[b]	61±8.9[b]
L. plantarum ATCC 8014	1	0.5	0.125	207±13.0[a]	87±12.5[a]	132±21.0[b]	58±7.4[b]
L. plantarum UNISS pb5	1	0.5	0.0625	207±28.4[a]	65±4.8[a]	86±7.9[b]	60±2.5[a]

748 MIC: minimal inhibitory concentration; MMC: minimal microbicidal concentration; MCAG: minimal
749 concentration affecting growth; λ: lag phase; g: generation time; NC: culture medium with NCLrKpkt
750 (lyophilized supernatant of rc#24, utilized as negative control). UNISS: Microbial Collection,
751 Department of Agriculture, University of Sassari, Sassari, Italy; DSMZ-German Collection of
752 Microorganisms and Cell Cultures, Leibnitz Institute, Braunschweig, Germany; ATCC American Type
753 Culture Collection, Manassas, Virginia, USA. Same superscript letters indicate results not
754 significantly different from NC ($p<0.05$).

Table 2. Evaluation of rKpkt toxicity on yeasts

Species	MMC (AU/mL)	MIC (AU/mL)	MCAG (AU/mL)	λ MCAG (min)	g MCAG (min)	λ NC (min)	g NC (min)
S. cerevisiae EC1118	2	1	0.5	627±17.0[a]	128±4.4[a]	467±25.0[b]	115±5.6[b]
S. cerevisiae Okay	2	1	0.5	513±22.0[a]	144±3.3[a]	422±12.0[b]	121±1.2[b]
S. cerevisiae K1	2	1	0.5	569±19.0[a]	174±6.7[a]	429±21.0[b]	103±7.1[b]
S. cerevisiae UNISS JD31	2	1	0.25	470±29.3[a]	163±23.4[a]	417±17.7[a]	107±0.8[b]
S. cerevisiae UNISS JC12	2	1	0.25	696±31.0[a]	191±1.9[a]	450±38.0[b]	134±5.5[b]
S. cerevisiae UNISS 99	2	1	0.5	401±15.5[a]	94±2.5[a]	266±19.8[b]	111±10.2[a]
S. cerevisiae UNISS 118	1	0.5	0.25	237±22.3[a]	92±7.5[a]	180±36.3[a]	76±6.1[b]
S. cerevisiae UNISS 182	1	0.5	0.25	780±26.0[a]	117±1.4[a]	89±34.0[b]	70±7.5[b]
S. cerevisiae UNISS 178	1	0.5	0.25	750±48.2[a]	106±10.0[a]	155±49.0[b]	72±2.3[b]
S. cerevisiae UNISS 179	1	0.5	0.25	792±25.8[a]	325±18.1[b]	362±21.4[a]	88±2.1[b]
S. cerevisiae UNISS236	1	0.5	0.5	826±51.0[a]	337±15.0[a]	169±6.6[b]	83±4.3[b]
S. cerevisiae UNISS 274	2	1	0.5	331±32.1[a]	99±9.8[a]	149±13.6[b]	70±3.3[b]
L. thermotolerans UNISS 84	1	0.5	0.25	542±45.3[a]	328±14.5[a]	241±21.5[b]	74±5.8[b]
L. thermotolerans UNISS 165	1	0.5	0.25	833±28.7[a]	122±31[a]	276±0.6[b]	77±0.1[a]
L. thermotolerans UNISS 175	1	0.5	0.25	674±27.7[a]	375±12.3[a]	238±12.5[b]	78±2.0[b]

L. thermotolerans UNISS 226	2	1	0.5	325±11.5[a]	104±1.1[a]	270±37.0[a]	115±14.2[a]
M. pulcherrima UNISS 18	2	1	0.5	1124±84.4[a]	358±31.0[a]	357±7.7[b]	111±2.2[b]
M. pulcherrima UNISS 19	2	1	1	534±113.2[a]	164±27.4[a]	234±50.0[b]	114±4.2[b]
M. pulcherrima UNISS 29	2	1	0.5	851±44.4[a]	352±31.3[a]	274±38.1[b]	126±0.9[b]
M. pulcherrima UNISS 37	2	1	0.5	490±18.2[a]	321±16[a]	258±6.6[b]	126±1.2[b]
M. pulcherrima UNISS 86	2	1	0.5	796±38.2[a]	424±26.8[a]	268±41.2[b]	89±8.1[b]
M. pulcherrima UNISS 92	2	1	0.5	364±20.1[a]	105±8.5[a]	250±12.1[b]	98±8.7[a]
M. pulcherrima UNISS 225	2	1	0.5	552±59.5[a]	115±4.6[a]	460±35.6[a]	92±0.2[b]
S. bacillaris UNISS 267	1	0.5	0.5	403±66.8[a]	110±1.6[a]	134±27[b]	75±3.5[b]
S. bacillaris UNISS 276	2	1	0.5	318±33.2[a]	141±8.5[a]	156±5.9[b]	67±8.1[b]
S. paradoxus UNISS 96	1	0.5	0.25	584±44.4[a]	213±9.3[a]	172±49.9[b]	99±8.2[b]
S. paradoxus UNISS 133	1	0.5	0.5	287±9.0[a]	142±9.3[a]	143±24.0[b]	94±4.9[b]
S. paradoxus UNISS 137	1	0.5	0.25	970±95.0[a]	470±5.4[a]	616+±10.0 [b]	148±4.1[b]
D. bruxellensis DiSVA 638	2	1	0.5	641±36.2[a]	325±22.4[a]	453±21.1[b]	163±8.8[b]
D. bruxellensis DiSVA 640	1	0.5	0.5	478±21.2[a]	183±19.6[a]	313±13.5[b]	127±8.9[b]
D. bruxellensis DiSVA 692	1	0.5	0.25	802±13.1[a]	195±29.9[a]	445±55.8[b]	162±19.0[a]
H. uvarum UNISS 158	2	1	0.5	681±33.1[a]	190±21.5[a]	141±1.4[b]	149±10.8[b]
Z. bailii UNISS 38	0.5	0.25	0.25	674±14.4[a]	424±22.0[a]	156±11.2[b]	75±5.7[b]
W. californica DiSVA 366	nd	2	0.5	545±41.1[a]	276±11.0[a]	406±26.2[b]	236±14.2[b]

MIC: minimal inhibitory concentration; MMC: minimal microbicidal concentration; MCAG: minimal concentration affecting growth; λ: lag phase; g: generation time. NC: culture medium with NCLrKpkt (lyophilized supernatant of rc#24, utilized as negative control). UNISS: Microbial Collection, Department of Agriculture, University of Sassari, Sassari, Italy; DiSVA: Culture Collection of Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona, Italy. *S. cerevisiae* EC1118 and Lalvin ICV Okay are commercial starter strains (Lallemand Inc., Montreal,

768 Canada). Same superscript letters indicate results not significantly different from NC ($p<0.05$).

769 Highlights

770 • Recombinant Kpkt (rKpkt) was produced in bioreactor

771 • Lyophilization increases long term stability of rKpkt

772 • Lyophilized rKpkt (LrKpkt) exerts killer activity on wild yeasts in grape must

773 • LrKpkt is active on lactic acid bacteria and food-borne pathogens

774 • Lyophilization is an interesting option for the management of killer toxins

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