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Uncovering the killer activity of *Lachancea* species against the wine spoilage yeast *Brettanomyces bruxellensis*

Phuti Lekokotla[1,2,3], Cécile Miot-Sertier[1], Rémi Schneider[3], Benoit Divol[2], Warren Albertin[1,*]

[1] Université de Bordeaux, Bordeaux INP, INRAE, BSA, UMR 1366 OENO, ISVV, 33140 Villenave d'Ornon, France
[2] South African Grape and Wine Research Institute, Stellenbosch University, Private Bag X1, Stellenbosch 7599, South Africa
[3] Oenobrands SAS, 34980 Montferrier-sur-Lez, France

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*correspondence:
warren.albertin@u-bordeaux.fr

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ABSTRACT

Wine harbours a wide range of beneficial and spoilage yeasts, including *Brettanomyces bruxellensis*. Its genotypic diversity, persistence during vinification, and tolerance to various chemical preservatives (including sulfur dioxide) make it difficult to control. Killer toxins secreted by other yeast species may represent a promising biological alternative to chemical preservatives. Killer phenotype against *B. bruxellensis* has been described in various yeasts, but their application in industry remains limited. Such killer activity was recently reported in *Lachancea thermotolerans* but not fully investigated. This study aimed to determine and further explore killer phenotypes in the *Lachancea* genus against *B. bruxellensis* (29 strains representative of the six main genetic groups) and other wine spoilage and beneficial yeast species from the *Torulaspora*, *Schizosaccharomyces*, *Candida*, *Hanseniaspora*, *Metschnikowia* and *Zygosaccharomyces* genera. The killer phenotype of 17 *Lachancea* isolates was assessed against the target isolates, using a solid medium-based killer assay at pH 3.5 and a temperature of 24 °C. Ten of the 17 *Lachancea* isolates screened displayed variable growth inhibition phenotypes against spoilage yeasts. Of these, *L. thermotolerans* CTL 302, *L. thermotolerans* CTL 277 and *L. waltii* NRRL Y-8285 consistently displayed high permanent growth inhibitory effect against *B. bruxellensis* strains across the six genetic groups. Remarkably, these strains had no killer effect against all the commercial wine yeasts tested, highlighting them as promising biocontrol yeasts. The impact of lactic acid (3 g/L to 18 g/L) on the growth of spoilage yeasts was also assessed, and lactic acid was deemed ineffective at inhibiting the growth of target isolates. It also showed that lactic acid was not involved in the killer phenotype. Additionally, the killer compound was identified as a killer toxin through both heat and protease treatments. Altogether, these results highlighted the three killer strains as potential biocontrol yeasts, even though further investigations are yet to be carried out under real winemaking conditions.

KEYWORDS: killer phenotype, *Brettanomyces bruxellensis*, *Lachancea* species, wine, bioprotection

INTRODUCTION

Wine is home to a diversity of microorganisms including yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) (Aires *et al.*, 2025). Amongst these, the yeast *Saccharomyces cerevisiae* is the main microorganism conducting alcoholic fermentation. While spontaneous fermentation by indigenous strains remains an option for winemakers, hundreds of strains have been carefully selected and are available as active dry yeasts for inoculated fermentations. Additionally, some non-conventional yeast species like *Lachancea thermotolerans, Metschnikowia pulcherrima* and *Torulaspora delbrueckii* are also commercialised for various benefits, *e.g.*, managing acidity in warm winemaking regions, improving aroma complexity and mouthfeel of wines, and bioprotection. These non-*Saccharomyces* species are used in mixed or sequential cultures with *S. cerevisiae* to ensure the completion of alcoholic fermentation. Nevertheless, while some yeast species are recognised as beneficial, others mainly contribute negatively to wine quality through the overproduction of undesirable sensory compounds. These include *Hanseniaspora uvarum*, *Zygosaccharomyces* spp., *Schizosaccharomyces pombe* and *Brettanomyces bruxellensis*. Amongst all wine spoilage microorganisms, the latter is the main culprit of microbial spoilage in dry red wine, due to its ability to utilise a wide range of nitrogen and carbon sources, offering it a better survival advantage (Crauwels *et al.*, 2015; Crauwels *et al.*, 2017; Rodrigues *et al.*, 2001; Smith & Divol, 2018). *B. bruxellensis* strains can enter a viable but non-culturable (VBNC) state during fermentation, which may be followed by resuscitation when the environmental conditions become more favourable, typically during ageing (Millet & Lonvaud-Funel, 2000; Serpaggi *et al.*, 2012). They contribute negatively to the sensory profile of wine through the production of undesirable compounds such as acetic acid (vinegar aroma), isovaleric acid (cheesy and pungent aromas) and volatile phenols (VPs; horse sweat, leathery, smoky, band-aid and medicinal aromas) (Kheir *et al.*, 2013). Although these metabolic traits are conserved within the *B. bruxellensis* species, strains are highly diverse from a genetic viewpoint. Recent studies have identified and sorted *B. bruxellensis* strains into genetic groups based on both substrate origin (groups mostly associated with wine, beer, tequila and kombucha) and ploidy levels (Avramova *et al.*, 2018; Loegler *et al.*, 2025). Indeed, most *B. bruxellensis* strains display a primary diploid genome, while 40 % possess an additional haploid genome, making them triploid. At least six genetic groupings are currently recognised: two diploid populations (D1 and D2), three allotriploid populations (A1, A2, A3), and one autotriploid admixed population (D1/D2). These populations display different characteristics, particularly regarding sensitivity to chemical or physical treatments (Avramova *et al.*, 2018; Paulin *et al.*, 2020; Pilard *et al.*, 2021).

Several mitigation strategies have been employed to combat *B. bruxellensis* spoilage in wine, which include physical, chemical, biochemical and biological approaches (Escott *et al.*, 2017; Pinto *et al.*, 2020). Physical methods such as filtration and cleaning of fermentation equipment are still employed in the wine industry but only transiently control *B. bruxellensis*. Some innovative physical treatments (UV-C, pulsed light) are efficient but not yet authorised for winemaking (Pilard *et al.*, 2021). Most chemical methods inhibit *B. bruxellensis* growth more effectively at concentrations higher than those prescribed by the Organisation Internationale de la Vigne et du Vin (2022) (Benito *et al.*, 2009; Tedesco *et al.*, 2022). Indeed, the OIV recommends the application of 0.4-0.6 mg/L molecular SO_2 to prevent spoilage by *B. bruxellensis* (resolution OIV-OENO 686-2022). However, this concentration range is difficult to reach in wines with pH > 3.5. Furthermore, sulfur dioxide (SO_2) addition has for many years been broadly employed to control the prevalence of wine spoilage microorganisms chemically (Henick-Kling & Park, 1994; Vaquero *et al.*, 2021). Moreover, the over-reliance on SO_2 in wine over the years is believed to have driven the selection of SO_2-tolerant *B. bruxellensis* strains associated with two groups (allotriploid A2 and autotriploid D1/D2) (Avramova *et al.*, 2018). Chitosan is an effective biochemical additive used in the wine industry, but again here, its efficacy depends not only on the strain of *B. bruxellensis* but also on the wine pH (Miot-Sertier *et al.*, 2022; Paulin *et al.*, 2020; Picariello *et al.*, 2023; Tedesco *et al.*, 2025). These emphasise the need for more efficient non-chemical alternatives to combat wine spoilage. Some yeast species, including *Kluyveromyces wickerhamii*, *Pichia anomala*, *Pichia membranifaciens*, and *Ustilago maydis*, have previously been identified as producing killer toxins active against *B. bruxellensis* strains (Table S1). Nevertheless, although effective in laboratory settings, several factors make the use of these yeasts in the wine industry difficult. Indeed, they were either not isolated from the broader wine environment (*e.g.*, *K. wickerhamii*) or classified as potential spoilage yeasts themselves (*e.g.*, *Pichia anomala*). The filamentous fungus *U. maydis* is a plant pathogen, further ruling out its potential future application in wine. Interestingly, a recently discovered killer toxin (Sc54Kt) produced by *S. cerevisiae* is active against *B. bruxellensis* (El Dana *et al.*, 2025a). This contrasts with previously identified killer toxins of *S. cerevisiae*, which typically only harbour a narrow spectrum of activity against other strains of *S. cerevisiae*. However, its efficacy against a wide range of *B. bruxellensis* strains has not been investigated. To date, only one yeast, a strain of *Suhomyces pyralidae*, is commercialised under the name Level2 SALVA™ (Lallemand Inc., Montreal, QC, Canada), for its inhibitory phenotype against *B. bruxellensis* (Lallemand Oenology, 2025; Mehlomakulu *et al.*, 2014). This non-fermentative yeast offers an efficient biocontrol solution in grape juice at the early phase of alcoholic fermentation, but does not prevent later contamination during wine ageing, since its CpKT1 killer toxin is sensitive to high ethanol and sulfur dioxide concentrations. Finally, novel anti-*B. bruxellensis* killer toxins were recently identified in *L. thermotolerans* (strains Lt29Kt & Lt54Kt) and *M. pulcherrima* (El Dana *et al.*, 2025b). However,

although these strains were successfully tested in wine, only four *B. bruxellensis* strains were investigated, thus trivialising the genetic variability of *B. bruxellensis*. Interestingly, the two *Lachancea* strains did not inhibit the growth of the *S. cerevisiae* strains investigated in their study, but only those of *B. bruxellensis* and *H. uvarum* in synthetic grape must.

In this context, this study aimed to explore the inhibitory phenotype of *Lachancea* species against genetically diverse *B. bruxellensis* strains, including additional wine spoilage yeasts.

MATERIALS AND METHODS

1. Yeast strains investigated

All yeast strains used in the study are summarised in Table 1. Precultures were conducted in Yeast Peptone Dextrose (YPD) broth and preserved at –80 °C in 20 % (*v/v*) glycerol solutions.

2. Screening for the killer phenotype of several *Lachancea* species

Precultures were conducted in Yeast Peptone Dextrose medium (YPD) for all strains investigated in this study. 250 mL Erlenmeyer flasks covered with cotton wool (250 rpm agitation on a magnetic stirrer) and 13 mL plastic test tubes (175 rpm agitation on an orbital shaker) were used for both target and killer (*Lachancea*) isolates, respectively. All investigated isolates were incubated at 24 °C for 48 h. Optical density was measured using a spectrophotometer (Lovibond, Nordrhein-Westfalen, Germany) at a wavelength of 600 nm. A 4 × concentrated solution of 5 g/L agar was sterilised in an autoclave for 15 min at 121 °C. The pH of a commercial preservative-free red grape juice was adjusted to 3.5 using 5 M KOH and subsequently filter-sterilised using 0.45 µm pore-sized (ACN) cellulose acetate filter membranes (Sartorius Stedim Biotech, Goettingen, Germany) and maintained in a water bath (JB Aqua 12 plus, Shepreth, UK) at 55 °C for 15 min. This was then mixed

TABLE 1. Collection of wine-associated yeasts investigated in this study.

Lachancea spp.	*B. bruxellensis* strains	Other wine-associated yeast spp.	Commercial starter cultures[+]
1. *Lachancea dobzhansky* CTL 276	Diploid 2 (D2):	1. *Torulaspora delbrueckii* CLIB 230	1. *Saccharomyces cerevisiae* Fermivin® MT 48
2. *L. waltii* × *S. cerevisiae* hybrid CTL 443	1. CRBO L14165	2. *Torulaspora delbrueckii* CRBO L0705	2. *Saccharomyces cerevisiae* Fermivin® MT XL
3. *Lachancea lanzarotensis* NRRL Y-63768	2. NCYC 2818	3. *Schizosaccharomyces pombe* CRBO L0422	3. *Saccharomyces cerevisiae* Fermivin® P21
4. *Lachancea mirantina* NRRL Y-63769	3. NCYC 3050	4. *Schizosaccharomyces pombe* NRRL Y-12796	4. *Saccharomyces cerevisiae* Anchor WE 372
5. *Lachancea cidri* NRRL Y-17052	4. YJS 5334	5. *Candida zemplinina* CRBO L0473	5. *Saccharomyces cerevisiae* NT50
6. *Lachancea fermentati* NRRL YB-925	5. YJS 5340	6. *Candida zemplinina* CBS 9484	6. *H. vineae* Fermivin® VINEAE
7. *Lachancea fermentati* NRRL Y-1559	Diploid 1(D1):	7. *Hanseniaspora uvarum* CRBO L1433	7. *Saccharomyces cerevisiae* Fermivin® PF6
8. *Lachancea kluyveri* NRRL Y-12651	1. CBS 2499	8. *Metschnikowia pulcherrima* NRRL Y-7111	8. *Saccharomyces bayanus* Fermivin® 4f9
9. *Lachancea meyersii* NRRL Y-27269	2. DEN 612_9*	9. *Metschnikowia pulcherrima* CRBO L0675	9. *Saccharomyces cerevisiae* × *S. paradoxus* hybrid Exotics Mosaic
10. *Lachancea nothofagi* NRRL Y-48670	3. CRBO L0611	10. *Zygosaccharomyces bailii* CRBO L0446	
11. *Lachancea thermotolerans* CTL 302	4. CRBO L14163	11. *Zygosaccharomyces bailii* CLIB 213	
12. *Lachancea thermotolerans* CTL 277	5. CRBO L14168	12. *Zygosaccharomyces rouxii* CRBO L0314	
13. *Lachancea thermotolerans* CTL 298	Allotriploid 3 (A3):	13. *Dekkera anomala* NRRL Y-17522T	
14. *Lachancea thermotolerans* CLIB 292	1. CBS 5512	14. *Brettanomyces anomalus* NRRL Y-12670	
15. *Lachancea thermotolerans* Ltyq3*	2. CBS 5513		
16. *Lachancea thermotolerans* VineG18*	3. CRBO L14169		
17. *Lachancea waltii* NRRL Y-8285	4. SJ12-4*		
	5. UWOPS 92-255.4		
	Allotriploid 2 (A2):		
	1. CRBO L0516		
	2. 12-LT-VGC3-c-10*		
	3. 13-LT-VGC55d-1*		
	4. CRBO L14190		
	5. CRBO L0424		
	Allotriploid 1 (A1):		
	1. CRBO L0422		
	2. CRBO L14173		
	3. CRBO L14183		
	4. CRBO L1794		
	5. ISA 1700		
	Autotriploid (Admixed D1/D2):		
	1. CRBO L0308		
	2. Gamay 329 CM 5*		
	3. GB59*		
	4. ISA 2211		

CLIB (Collection de Levures d'Intérêt Biotechnologique, now CIRM-Levures = Centre International de Ressources Microbiennes –Levures, Montpellier, France); ISA (Instituto Superior de Agronomia , University of Lisbon, Portugal); NCYC (National Collection of Yeast Cultures, Norwich, United Kingdom); CBS (Centraalbureau voor Schimmel cultures, Utrecht, Netherlands); UWOPS (Culture collection of the University of Western Ontario, Ontario, Canada); NRRL (Northern Regional Research Laboratory, ARS Culture collection, Peoria, IL, USA); YJS (Laboratory for Molecular Genetics, Genomics and Microbiology, Strasbourg University, France); CRBO (Centre de Ressources Biologiques Oenologie, Bordeaux, France); CTL (Collection de Travail Levure, Institut Français de la Vigne et du Vin, Nantes, France). *Strains without collection ID belong to UMR Oenology (Bordeaux, France). [+] Commercial starter cultures are from Oenobrands SAS (Montpellier, France).

with the agar solution to obtain a grape juice agar medium with a final agar concentration of 5 g/L and maintained at 55 °C until needed. An adequate cell suspension of target strains corresponding to an OD_{600nm} of 0.1 was then inoculated into 50 mL Falcon® tubes (Corning, New York, NY), centrifuged at 8228 *g* for 5 min at 25 °C, where the cell pellets were eventually mixed with 30 mL of grape juice agar medium. Subsequently, 30 mL of the grape juice agar medium mixed with target strains was poured into square Petri dishes and allowed to solidify. The optical density of *Lachancea* isolates was adjusted to OD_{600nm} of 2.0, and 5 µL of cell suspensions from each isolate were spotted onto the plates. Similarly, 5 µL of lactic acid solutions (3 g/L; 6 g/L; 9 g/L; 12 g/L; 15 g/L and 18 g/L) were also spotted onto the plates. The plates were incubated at 24 °C for 3 to 7 days, followed by measurements of zones of inhibition (global diameter) in mm. The plates were then reincubated at 24 °C for over 14 days to monitor possible re-growth of sensitive target strains, particularly *B. bruxellensis* strains.

3. Determining the nature of the killer compound

Three *Lachancea* isolates (*L. thermotolerans* CTL 302, *L. thermotolerans* CTL 277 and *L. waltii* NRRL Y-8285) exhibiting the strongest inhibitory phenotype were tested against *B. bruxellensis* CBS 2499, which was selected due to its sensitivity and its consistent rapid growth. The precultures were conducted in 5 mL YPD overnight (16 h) inside test tubes. 50 µL of the overnight cultures was then transferred into 5 mL sterile commercial red grape juice medium (pH 3.5) inside test tubes. These were then incubated at 25 °C overnight, after which the cultures were subsequently transferred into 500 mL of the same medium inside 1L Erlenmeyer flasks and incubated for 48 h at 25 °C with 120 rpm agitation on an orbital shaker. The cultures were centrifuged (Multifuge × Pro Series, Thermo Fisher Scientific, Waltham, Massachusetts, United States of America) inside 1 L flasks at 3738 *g* for 5 min. The supernatants were then filter sterilised using 0.22 µm pore-sized filter membranes and ultimately ultrafiltered using PES (Polyethersulfone) Amicon® 30 kDa MWCO (Merck KGaA, Darmstadt, Germany). The 17-fold concentrated recovered retentates were treated as described in paragraphs 3.1 and 3.2 below. *B. bruxellensis* CBS 2499 was precultured only in YPD for 48 h at 25 °C, agitated at 40 rpm on a rotating wheel inside test tubes. Similarly, the grape juice agar medium seeded with *B. bruxellensis* strain was prepared as described in section 2, but only 10 mL of the medium was poured into the 90 mm round Petri dishes. Wells were drilled into the grape juice agar medium using sterile P_{200} micropipette tips, as this method was more robust than spotting on top of the medium when using supernatants. All the plates were incubated at 25 °C for 3 days before analysis of the results.

3.1. Protease treatment

A concentrated stock solution of pepsin (Sigma-Aldrich/Merck, Darmstadt, Germany; CAS 9001-76-6) was prepared using an ice-chilled 10 mM HCl solution at pH 2.0. The ultrafiltered supernatants of the individual killer strains were mixed with a pepsin stock solution to final concentrations of 25 mg/mL in 1.5 mL microcentrifuge tubes. The final volume was 100 µL, and the solutions were then incubated for 4 h at 43 °C in a heating block. The treated supernatants were ultimately spotted (20 µL) into the wells on the grape juice agar medium seeded with *B. bruxellensis* CBS 2499. The non-treated supernatants were used as positive controls.

3.2. Heat treatment

The same ultrafiltered supernatants in section 3.1 (80 µL) from respective killer strains were transferred into 1.5 mL microcentrifuge tubes and 1.8 mL screw-capped microcentrifuge tubes, respectively, and incubated for 2 h at both 100 °C and 120 °C in a heating block. Subsequently, 20 µL of the supernatants were spotted into the drilled wells on the grape juice agar medium and allowed to completely diffuse into the medium before the plates were incubated. The non-treated supernatants were used as positive controls.

4. Statistical analysis

Zones of inhibition ($n = 3$) from the *L. thermotolerans* CTL 302, *L. thermotolerans* CTL 277 and *L. waltii* NRRL Y-8285 tested against all *B. bruxellensis* strains were analysed using two-way analysis of variance (2-way ANOVA) using XLstat (Paris, France). The two factors investigated were killer strains and target strains (*B. bruxellensis*). Means between groups were compared using Tukey's HSD test; $p < 0.05$. Levene's test assessed homoscedasticity, and visual inspection of Q-Q plots showed that the normality assumption was met.

RESULTS

1. Assessing the killer phenotype of *Lachancea* species against wine-associated yeasts

To explore the potential killer phenotype in the *Lachancea* genus, 17 isolates of *Lachancea* spp. were tested on grape juice agar medium against 29 *B. bruxellensis* strains representing the six genetic groups of this species, nine *S. cerevisiae* commercial yeast starter cultures and an additional 14 wine-associated yeasts (Table 1). The latter set of yeasts included potentially beneficial and detrimental yeasts such as *Torulaspora delbrueckii*, *Hanseniaspora uvarum* and *Schizosaccharomyces pombe*. The pH of the commercial red grape juice agar medium used during screening was adjusted to 3.5, *i.e.*, an average wine pH. The inhibitory effects of *Lachancea* spp. against target strains were revealed through the presence of zones of growth inhibition (measured in mm, $n = 3$) on the agar plates after 3 to 7 days of incubation, depending on the growth rate of the target strain seeded in the medium (Figure S1). Subsequently, means for each *Lachancea* strain against target strains were compiled in a heat map (Figure 1).

The plates were also re-incubated for 14 days to determine whether the growth inhibition remained beyond the initial incubation period.

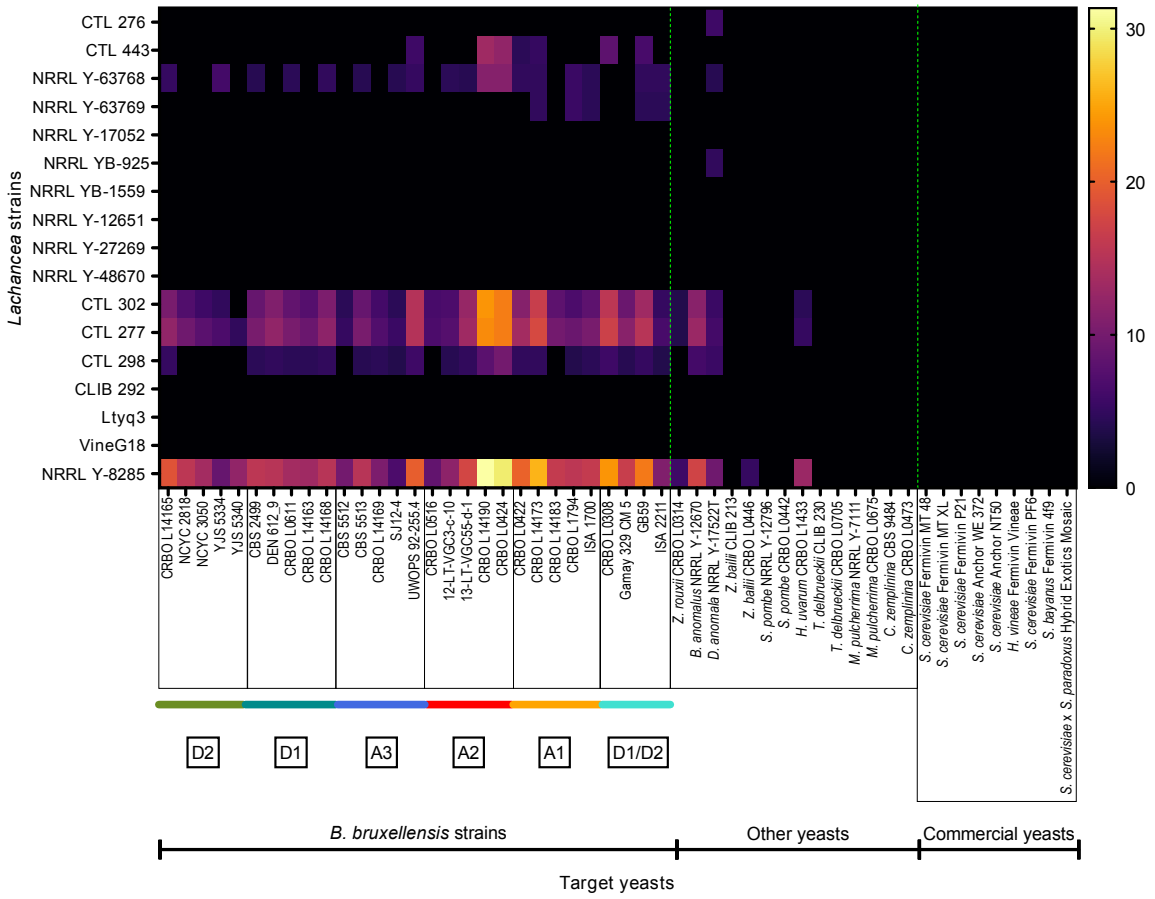


FIGURE 1. The killer effect of several Lachancea species on the growth of targeted wine-associated yeast. The grading scale represents mean values (n = 3) of zones of inhibition measured in mm. Light green: group D2, diploid; dark cyan: group D1; dark blue: group A3, allotriploid; red: group A2, allotriploid; orange: group A1, allotriploid; turquoise: group D1/D2, autotriploid admixed. Distinction of the genetic groups with colours was implemented as described previously (Avramova *et al.*, 2018; Loegler *et al.*, 2025).

Amongst all the *Lachancea* isolates tested, only ten (*i.e.*, *Lachancea dobzhansky* CTL 276, *Lachancea waltii* × *S. cerevisiae* hybrid CTL 443, *Lachancea lanzarotensis* NRRL Y-63768, *Lachancea mirantina* NRRL Y-63769, *Lachancea cidri NRRL* Y-17052, *Lachancea fermentati* NRRL YB-925, *Lachancea thermotolerans* CTL 302, *Lachancea thermotolerans* CTL 277, *Lachancea thermotolerans* CTL 298, and *Lachancea waltii* NRRL Y-8285) displayed growth inhibitory effects specifically against the wine spoilage yeasts. These included *B. bruxellensis* strains from all genetic groups, *Z. rouxii*, *Z. bailii* and *B. anomalus*. However, *B. bruxellensis* strains from different genetic groups showed variable sensitivities. For example, *L. waltii* NRRL Y-8285 exhibited the highest average inhibition halo diameter of 31 mm against the most sensitive *B. bruxellensis* strain CRBO L14190, whereas other identified killer strains such as *L. lanzarotensis* NRRL Y-63768 had no inhibitory effect against some *B. bruxellensis* strains such as CRBO L0516, NCYC 2818 and SJ12-4, amongst others. Interestingly, none of the investigated commercial wine starter culture yeasts and none of the other non-*Saccharomyces* yeasts was inhibited by any of the investigated *Lachancea* strains.

Overall, *L. thermotolerans* CTL 302, *L. thermotolerans* CTL 277 and *L. waltii* NRRL Y-8285 displayed the highest zones of inhibition (24, 23, and 31 mm, respectively), of all *Lachancea* strains. They also inhibited the growth of all *B. bruxellensis* strains from the different genetic groups. To better compare the ability of these three *Lachancea* strains to inhibit *B. bruxellensis* strains, zones of inhibition from only these three strains against all *B. bruxellensis* strains were visualised in box plots (Figure 2).

Evidently, *L. waltii* NRRL Y-8285 was more efficient at inhibiting *B. bruxellensis* strains than both *L. thermotolerans* strains, as its minimum and maximum inhibition zone values were consistently higher on the box plots. The data allowed identification of *B. bruxellensis* strains from the allotriploid 2 genetic group as the most sensitive amongst all the genetic groups investigated, whereas the strains belonging to the diploid 2 genetic group were the least sensitive. Indeed, the A2 genetic group was highly heterogeneous in their tolerance and/or sensitivity to *Lachancea* growth-inhibitory compounds compared to the D1 genetic group strains, which were more homogeneous.

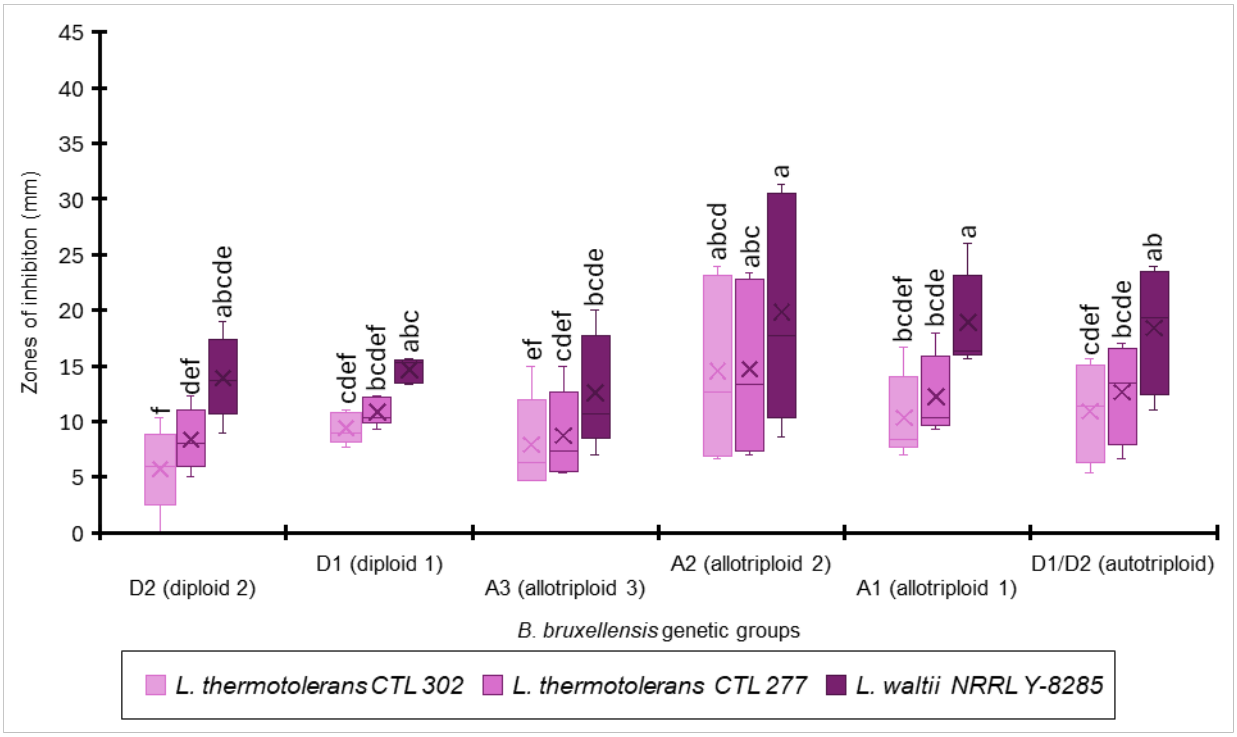


FIGURE 2. The impact of potent killer strains on the growth of several genetically distinct *B. bruxellensis* strains. The figure compares the growth-inhibition potency of killer strains and depicts the overall sensitivity of *B. bruxellensis* strains by genetic group. The letters on top of the bars represent results of a post-hoc statistical test (Tukey's HSD). The same letters mean no statistically significant differences, and different letters mean statistically significant differences ($p < 0.05$).

In a separate experiment, the ten identified growth-inhibitory *Lachancea* strains were tested against each other to determine the possible cross-killer effect (data not shown). This was conducted in a similar manner to the screening described above except that the ten *Lachancea* strains were individually seeded into the grape juice agar medium as target strains. None of the identified killer strains displayed the killer phenotype when tested against each other, *i.e.*, no inhibition zones were observed.

Since strains of *Lachancea* spp. produce varying levels of lactic acid as reviewed by (Porter *et al.*, 2019), different concentrations of lactic acid ranging from 3 g/L, 6 g/L, 9 g/L, 12 g/L, 15 g/L and 18 g/L were also tested on the same grape juice agar medium used during screening to verify whether the killer phenotype observed could be due to this metabolite (Table S2). No growth inhibition was observed for all the tested concentrations against all the target yeasts, indicative of a distinct killer compound apart from lactic acid.

2. Identifying the nature of the killer compound

The nature of the inhibitory compound of the three *Lachancea* isolates, *i.e.*, *L. thermotolerans* CTL 302, *L. thermotolerans* CTL 277 and *L. waltii* NRRL Y-8285, was investigated using both protease and heat treatments (Figure 3).

Pepsin (*i.e.*, an acid protease) was chosen as the ideal protease, as the killer effect of these strains was observed at pH 3.5 above. Following pepsin treatment, the killer effect was lost for all killer strains, as indicated by the disappearance of zones of inhibition around the wells. Interestingly, inhibition zones were still visible after the heat treatment at 100 °C for 2 h, albeit much smaller than those of the untreated controls. Nevertheless, the zones of inhibition were no longer observed following treatments at 120 °C.

DISCUSSION

In this study, several *Lachancea* strains displaying a growth-inhibitory phenotype against several spoilage yeasts, including *B. bruxellensis*, were identified. The strains belong to various species, namely *L. dobzhansky*, *L. lanzarotensis*, *L. mirantina*, *L. cidri*, *L. fermentati*, *L. thermotolerans*, *L. waltii* and a *L. waltii* × *S. cerevisiae* hybrid. Growth inhibitory phenotypes within the *Lachancea* genus have been previously identified in both *L. thermotolerans* and *L. waltii* species (El Dana *et al.*, 2025b; Kono & Himeno, 1997). However, this phenotype in *L. waltii* was only investigated against a single *S. pombe* strain and not *B. bruxellensis*. Contrastingly, the two *S. pombe* strains investigated in this study were resistant to the *L. waltii* strain. This highlighted intraspecific resistant phenotypes in *S pombe*. Nevertheless, this study was the first to report the growth-inhibitory phenotype of *L. waltii* against *B. bruxellensis*, *H. uvarum*, *Z. rouxii* and *Z. bailii*. Although more *L. thermotolerans* strains were investigated in this study compared to strains from other *Lachancea* species, not all of them displayed the growth-inhibitory phenotype.

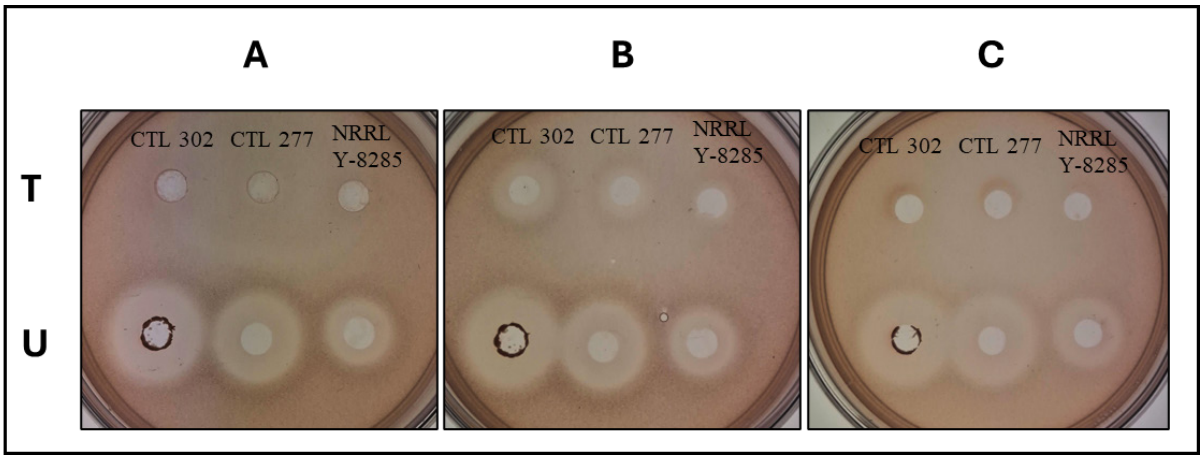


FIGURE 3. Denaturation of the killer compound by both heat and protease (pepsin) treatments. (A): enzyme treatment; (B): heat treatment (100 °C); (C): heat treatment (120 °C); (T): Treated; (U): Untreated control; CTL 302: *L. thermotolerans* CTL 302; CTL 277: *L. thermotolerans* CTL 277; NRRL Y-8285: *L. waltii* NRRL Y-8285.

To unravel the nature of the growth inhibitory compound(s) by the *Lachancea* strains, different concentrations of lactic acid (3 g/L to 18 g/L) were subsequently spotted on the same grape juice agar medium used during screening. All the investigated target strains were resistant to lactic acid. Lactic acid typically acts synergistically with SO_2, pH and ethanol to suppress growth of unwanted microorganisms in wine (Edwards & Oswald, 2018). This could explain why no inhibition at all lactic acid concentrations (3 g/L to 18 g/L) against target strains was observed on solid medium in this study, but this is unlikely considering the very high concentrations tested. The pepsin (a protein-digesting enzyme) and heat treatments, however, allowed us to identify the inhibitory compound as a protein (Figure 3). This confirmed the previous result obtained for *L. waltii* NRRL Y-8285 (Kono & Himeno, 1997), and extended it to the other *Lachancea* species. Amongst many killer toxins previously identified in non-*Saccharomyces* yeasts, some are glucanases that digest the glycan cell-wall structure of target yeasts, as seen in *Tetrapisispora phaffii* (Kpkt), whereas others are small molecular weight proteins of viral origin that can induce different antagonistic mechanisms (K1, K2, K28 and Klus) best described in *S. cerevisiae* strains (Mehlomakulu *et al.*, 2015). The intraspecific variations observed in this study, as well as in that of El Dana and co-workers (2025b), could suggest that dsRNA viruses could also be responsible for the killer phenotype of the *Lachancea* genus, as described in the species *Torulaspora delbrueckii* (Ramírez *et al.*, 2015). This hypothesis is reinforced by the fact that the ratio of killer to non-killer strains was low, like *Saccharomyces* (Pieczynska *et al.*, 2013). Nevertheless, the exact identity of the killer toxins of *L. thermotolerans* CTL 302, *L. thermotolerans* CTL 27 and *L. waltii* NRRL Y-8285 remains to be identified.

Different strains, particularly within the *B. bruxellensis* species, displayed variable sensitivities to killer toxins by the aforementioned *Lachancea* strains. These variations may be primarily linked to the type and mechanism of action of the growth-inhibitory compounds secreted by *Lachancea* strains, which have not yet been identified. However, the growth rates of different *B. bruxellensis* strains could have also played a role in either accentuating their resistance or susceptibility. Indeed, *B. bruxellensis* strains CRBO L14190 and CRBO L0424 were more sensitive than all the strains (Figure 1), but they are slow-growing strains. Subsequently, the zones of inhibition could only be measured after an extended incubation. Therefore, growth rates of different target strains were acknowledged as a limitation of the assay. Interestingly, the growth of *B. bruxellensis* strains across all genetic groups was inhibited (Figure 1). This was not observed with all other spoilage yeasts (Figure 1). In fact, *L. thermotolerans* CTL 277 and *L. waltii* NRRL Y-8285 inhibited the growth of all the investigated *B. bruxellensis* strains across all the genetic groups. Previously identified killer toxins such as Kwkt, Pikt, PMKT2, KP6, CpKT1, CpKT2, Sc54Kt, Mp2, Lt29Kt and Lt54Kt that could also inhibit the growth of *B. bruxellensis* strains were only tested on a limited number of strains and the impact of *B. bruxellensis* genetic groups was not considered (Comitini *et al*., 2021 ; Comitini *et al*., 2004; Comitini & Ciani, 2011; El Dana *et al*., 2025a; El Dana *et al*., 2025b; Mehlomakulu *et al*., 2014; Santos *et al*., 2009; Santos *et al*., 2011; Marquina *et al*., 1992; Ullivarri *et al*., 2018; Villalba *et al*., 2016). Even then, the identified killer toxins did not inhibit the growth of all the investigated *B. bruxellensis* strains.

In this study, target strains were pre-cultured for 48 h, indicating that all target yeasts except *B. bruxellensis* and *B. anomalus* were already in stationary phase prior to being seeded into the grape juice agar medium. Wood & Bevan (1968) observed enhanced sensitivity and tolerance of target *S. cerevisiae* strains to killer toxins when tested at both exponential and stationary growth phases, respectively. This further suggests that the physiological state of *B. bruxellensis* strains, compared with other target yeasts in this study, could have influenced their sensitivity to *Lachancea* killer toxins. Additionally, Piecyznska *et al.* (2016) observed an acquired resistant phenotype of an initially sensitive *S. cerevisiae* strain that was co-evolved with a killer *S. cerevisiae* strain,

suggesting that resistant phenotypes are more likely to be found in strains that share the same habitat with the killer strains. Although this phenomenon was not explored at a species level, it could still explain why *B. bruxellensis* strains were identified as more sensitive to *Lachancea* killer toxins since they are phylogenetically very distant from *Lachancea* species, indicative of evolution in different niches, as shown by Opulente *et al.* (2024).

Both Avramova *et al.* and Paulin *et al.* respectively investigated the impact of SO_2 and chitosan on the growth of a wide range of *B. bruxellensis* strains across distinct genetic groups (Avramova *et al.*, 2018; Paulin *et al.*, 2020). Indeed, Avramova *et al.* observed that A2 and D2 *B. bruxellensis* populations were more tolerant and sensitive to sulfites, respectively. Moreover, Paulin observed that the sulfite-tolerant A2 *B. bruxellensis* populations were more sensitive to chitosan. Overall, A2 strains were more sensitive, whereas D2 strains were less sensitive to killer toxins (Figure 2). Sulfite-tolerant *B. bruxellensis* strains pose a major spoilage threat to winemakers; thus, the ability of *L. thermotolerans* CTL 302, *L. thermotolerans* CTL 277 and *L. waltii* NRRL Y-8285 to target sulfite-tolerant strains further anchors their strong potential as biocontrol agents in wine.

CONCLUSION

This study identified three *Lachancea* strains from two *Lachancea* species that display a consistent killer effect against spoilage yeasts, with high specificity against *B. bruxellensis* strains. Although the results were obtained from experiments on solid agar medium, the significant potential application of these strains in real winemaking is undeniable, considering the killer toxins function well at wine pH (3.5) used throughout screening. Applications could also extend to cider production, as these strains inhibited the growth of cider spoilage yeast *B. anomalus* at pH 3.5, consistent with pH ranges for both wine and cider production (*i.e.*, 3.0–4.0). With growing interest in trying to mimic spontaneous wine fermentations by utilising different consortia, the addition of these strains in such consortia could be useful as they do not primarily target beneficial yeasts such as *M. pulcherrima*, *T. delbrueckii* and *S. cerevisiae*, which are typically included in such studies.

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