Evaluation of behaviour of *Lachancea thermotolerans* biocontrol agents on grape fermentations

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**Significance and Impact of the Study:** Generally it is not evaluated if the biofungicide yeasts sprayed on vegetables alter the quality of the fermented products. This work focused on the importance of assessing the possible effects of yeast-based fungicides used in vineyards on grape fermentation, especially on *Saccharomyces cerevisiae* growth. In this context, the competition between biofungicide yeasts and *S. cerevisiae* under winemaking conditions is investigated.

**Keywords**
behaviour, biocontrol, fermentation, *Lachancea thermotolerans*, *Saccharomyces cerevisiae*, substrate competition.

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**Abstract**

Previous researches have showed that *Lachancea thermotolerans* strains RCKT4 and RCKT5 inhibited the growth of *Aspergillus*. However, currently, there are no data on their nutritional preferences, as a possible substrate competitor against *Saccharomyces cerevisiae*, and their effects on fermentation. In this work, we observed that the biocontrol yeasts and *S. cerevisiae* BSc203, based on the utilization of 16 carbonate sources, revealed significant differences in the nutritional profile (biocontrol yeasts NS:0/25, BSc203 NS:0/56). *Lachancea thermotolerans* strains did not occupy the same niche as that of BSc203 (NOI:0/44). The biocontrol agents and BSc203 presented similar competitive attitude in terms of the sugar, ethanol and sulphite tolerances. During fermentation, the biocontrol yeasts were found to tolerate up to 12% v/v ethanol, 250 mg ml⁻¹ of total SO₂ and 30° Brix sugar. In mixed cultures, *L. thermotolerans* strains did not negatively affect the growth of BSc203 and the wine quality, except when RCKT4 was initially inoculated at a high proportion in the mixed culture 1MSK4 (1%BSc203/99%RCKT4), resulting in a lower production of CO₂ and ethanol, in comparison with pure BSc203. RCKT5, at a high proportion, in 1MSK5 (1%BSc203/99%RCKT5) presented promising oenological properties. This fermentation showed lower acetic acid contents and higher total acidity than pure BSc203.

**Introduction**

Grapes are susceptible to fungal diseases, especially grey rot, downy mildew and black rot (Covarelli *et al.* 2012). Conventional approaches to fungal control have focused on chemical applications. However, sole reliance on this approach is not sustainable because of the emergence of fungicide resistance in vineyards (Leroch *et al.* 2011) and the adverse effects of chemical pesticides on the environment and human health (Komárek *et al.* 2010). A biological approach is highly desirable to control fungal growth on grapes, as this helps to reduce the amount of agrochemical residues on grape, wine and its related products (Cabras and Angioni 2000). Among the various potential antagonists, yeasts have been studied as fungal biocontrol agents on grapes (Ponsone *et al.* 2011, 2016; Nally *et al.* 2012, 2013; Calvo-Garrido *et al.* 2013). The major mode of action of these yeasts is the competition...
for nutrient and space (Nally et al. 2015). Few data have been published about the influence of yeast-based biofungicides used in vineyards on grape fermentation (Calvo-Garrido et al. 2013; Guzzon et al. 2014). Generally, it is not evaluated if the antifungal yeasts sprayed on vegetables alter the quality of the fermented products, and if these micro-organisms continue competing for nutrients and space, especially with Saccharomyces cerevisiae in fermentation.

Some investigators have reported that strains belonging to Lachancea thermostolerans species have increased the acidity (Kapsopoulou et al. 2007; Balikci et al. 2016), the aroma complexity (Escribano et al. 2018) or secondary ones as biogenic amines reduction (Benito et al. 2015), aroma complexity (Escribano et al. 2018).

Ponsone et al. (2011, 2016) found that two L. thermostolerans strains, RCKT4 and RCKT5, increased the lag phase, diminished the in vitro growth rate of Aspergillus and decreased ochratoxin (OTA) accumulation in wine grapes. The use of biofungicide Lachancea yeasts in vineyards is to produce mycotoxin-free wine. However, it is unknown if these micro-organisms affect the fermentation. Previous investigations reported that some non-Saccharomyces were capable to survive throughout the fermentation and compete with Saccharomyces for nutrients, causing a fermentative stuck (Fleet and Heard 1993; Bisson 1999). Because there is little information on the oenological behaviour of biofungicide yeasts during grape ferments, the aims of this study were: (1) to evaluate the competition for nutrients between biocontrol yeasts and S. cerevisiae: nutritional size (NS) and niche overlap index (NOI); and (2) to evaluate the behaviour of biocontrol yeasts in fermentation conditions: SO2, ethanol, sugar tolerances, effects on BSc203 growth, persistence time and wine quality in mixed cultures biofungicide/Lachancea.

Results and discussion

Nutritional and oenological behaviour of L. thermostolerans RCKT4 and RCKT5: nutritional size (NS) and niche overlap index (NOI)

The two biocontrol yeasts assimilated the same carbon sources in vitro. From 16 carbon sources tested, four were utilized by RCKT4 and RCKT5 (NS:0.25) and nine were utilized by S. cerevisiae BSc203 (NS:0.56) (Table 1). Glucose, sucrose, raffinose and arginine were used by all the yeasts strains tested. Proline, asparagine, alanine, fructose and melibiose were used only by BSc203. The biocontrol strains did not occupy the same ecology niche as that occupied by BSc203 (NOI:0.44), showing a low level of competence between biocontrol yeasts and BSc203 (Table 1). These results suggested that biocontrol strains were not able to successfully assimilate a wide variety of nutrients of the wine grape, making them available to BSc203. The NOI between yeast–filamentous fungi (La Penna et al. 2004; Nally et al. 2015), bacteria–bacteria (Jaspers and Overmann 2004) and bacteria–filamentous fungi (Neschi et al. 2005) have been previously studied. Until now, there is only one publication that focussed on the NOI between yeast isolates of the same genera (Janisiewicz 1996). This present work provides new data on NOI between yeast isolates from grape musts, which belong to different genera.

Tolerances to SO2, sugar and ethanol

In the present study, it was observed that biocontrol yeasts were able to ferment in 25–250 mg l–1 SO2 media, but they were unable to ferment in 300–400 mg l–1 SO2 media (Table 2). Comitini et al. (2011) showed that L. thermostolerans isolates assayed were less resistant to SO2 than RCKT4 and RCKT5. These strains did not ferment grape musts with 20–30 mg l–1 SO2. The discrepancy between results may be explained by differences in the concentration of extracellular acetaldehyde (Stanley et al. 1993; Nadai et al. 2016).

RCKT4 and RCKT5 were able to tolerate high ethanol (7–11% v/v) and sugar (21 and 30% Brix) levels (Table 2). Levels of ethanol tolerance in the present study exceeded the values reported by Kapsopoulou et al. (2005), who observed that L. thermostolerans strains did not tolerate must with 9% v/v ethanol. Gobbi et al. (2013) mentioned that L. thermostolerans presented a high fermentation power (10-46%). The discrepancy between the results on tolerances may be explained by differences in the plasma membrane fluidity, integrity of strains assayed (Henderson and Block 2014) or by the strain variability in the fermentation power (Comitini et al. 2011).

In general, the biocontrol agents and BSc203 presented similar competitive attitude in terms of the sugar, ethanol and sulphite tolerances. The current study is the first that provides data on tolerance of L. thermostolerans biocontrol agents to ethanol, sugar and SO2 concentrations under winemaking conditions.

Impact of L. thermostolerans strains on S. cerevisiae BSc203 and on the wine quality

In pure cultures, viable cells of RCKT4, RCKT5 and BSc203 were present until the end of the fermentations (22 days) (Fig. 1a,b). In biocontrol/BSc203 co-cultures, the survival time of RCKT4 and RCKT5 depended on the biocontrol strain used, and the initial ratio of the yeasts assayed. In general, RCKT4 persisted more time than
RCKT5. In the RCKT4/BSc203 co-cultures, RCKT4 was detected until day 5 (3MSK4), 11 (2MSK4) and 12 (1MSK4) (Fig. 1a). RCKT5 was detected until day 2 (3MSK5), 8 (2MSK5) and 10 (1MSK5) (Fig. 1b). By analysing investigations, cell viability of \textit{L. thermotolerans} in mixed cultures with \textit{S. cerevisiae} was different. In 50% \textit{L. thermotolerans}–50% \textit{S. cerevisiae} mixed culture, \textit{L. thermotolerans} disappeared on day 7 (Kapsopoulou et al. 2006). On day 22, the BSc203 cell concentration in all mixed cultures assayed was not significantly different in pure BSc203 (\(P \leq 0.068\)). On day 22, the BSc203 cell concentration in all mixed cultures assayed was not significantly different when compared to pure BSc203, except for co-culture 1MSK4 (Fig. 2a). These values were not significantly different in pure BSc203 (7–69 log_{10} CFU per ml on day 3), except for 1MSK4. In BSc203/RCKT5 mixed fermentations, BSc203 reached a maximum cell density on day 4 in 3MSK5 (7–68 log_{10} CFU per ml) and 2MSK5 (7–76 log_{10} CFU per ml), and on day 6 in 1MSK4 (6–44 log_{10} CFU per ml) (Fig. 2a). These values were similar to a pure BSc203 (7–69 log_{10} CFU per ml on day 3), except for 1MSK4. In BSc203/RCKT5 mixed fermentations, BSc203 reached a maximum cell density on day 4 in 3MSK5 (7–68 log_{10} CFU per ml) and 2MSK5 (7–76 log_{10} CFU per ml), and on day 6 in 1MSK4 (7–65 log_{10} CFU per ml), and on day 6 in 1MSK4 (6–44 log_{10} CFU per ml). In pure cultures, RCKT4 and RCKT5 produced significantly lower ethanol concentrations (7–91 and

### Table 1 Nutritional profile analysis of \textit{Lachancea thermotolerans} yeasts and BSc203

| Nutritional sources | RCKT4 | RCKT5 | BSc203 |
|---------------------|-------|-------|--------|
| **Amino acids**     |       |       |        |
| Proline             | –     | –     | +      |
| Lysine              | –     | –     | –      |
| Arginine            | +     | +     | +      |
| Asparagine          | –     | –     | +      |
| Alanine             | –     | –     | –      |
| Glycine             | –     | –     | –      |
| Methionine          | –     | –     | –      |
| Tyrosine            | –     | –     | –      |
| **Organic acids**   |       |       |        |
| Glumatic acid       | –     | –     | –      |
| Malic acid          | –     | –     | –      |
| Tartaric acid       | –     | –     | –      |
| **Carbohydrates**   |       |       |        |
| Fructose            | –     | –     | +      |
| Glucose             | +     | +     | +      |
| Sucrose             | +     | +     | +      |
| Raffinose           | +     | +     | +      |
| Rhamnose            | –     | –     | –      |
| Melibiose           | –     | –     | +      |
| **Nutritional Ecology** |     |       |        |
| NOI                 | 4/9 = 0.44 | 4/9 = 0.44 | 4/9 = 0.44 |
| NOI                 | 4/9 = 0.44 | 4/9 = 0.44 | 4/9 = 0.44 |

\[=\text{Not assimilate carbonate source, +, Assimilate carbonate source.}\]

### Table 2 Tolerance of biocontrol yeasts to different concentrations of the ethanol (7–12% v/v), sulphur dioxide (25–400 mg l\(^{-1}\)) and sugar (21 and 30° Brix)

| Treatments | Yeast strains |
|------------|--------------|
|            | RCKT4 | RCKT5 | BSc203 |
| SO\(_2\)   |       |       |        |
| 25 mg l\(^{-1}\)* | +     | +     | +      |
| 50 mg l\(^{-1}\)* | +     | +     | +      |
| 75 mg l\(^{-1}\)* | +     | +     | +      |
| 100 mg l\(^{-1}\)* | +     | +     | +      |
| 150 mg l\(^{-1}\)* | +     | +     | +      |
| 200 mg l\(^{-1}\)* | +     | +     | +      |
| 250 mg l\(^{-1}\)* | +     | +     | +      |
| 300 mg l\(^{-1}\)* | –     | –     | +      |
| 400 mg l\(^{-1}\)* | –     | –     | +      |
| **Ethanol** |       |       |        |
| 7% v/v*    | +     | +     | +      |
| 8% v/v*    | +     | +     | +      |
| 9% v/v*    | +     | +     | +      |
| 10% v/v*   | +     | +     | +      |
| 11% v/v*   | +     | +     | +      |
| 12% v/v*   | –     | –     | +      |
| **Grape must** |       |       |        |
| 21° Brix   | +     | +     | +      |
| 30° Brix   | +     | +     | +      |

\[\text{+, ability to ferment, –, not ability to ferment.}\]

\[\text{*In YNB+glucose.}\]
6.05% v/v, respectively) than BSc203 (12.81% v/v) (Table 3). RCKT4 and RCKT5 presented a residual sugar concentration of 22.59 g l\(^{-1}\) and 29.97 g l\(^{-1}\), respectively, whereas BSc203 completed the fermentation (residual sugar 1.91 g l\(^{-1}\)). The sugar consumption rate of BSc203 during the first 3 days (16 g l\(^{-1}\) of sugar consumed) was significantly higher than that of RCKT4 and RCKT5 (6.5 and 6 g l\(^{-1}\), respectively) \((P \leq 0.05)\). As expected, all multistarter cultures with RCKT4 and RCKT5 (Table 3) showed ethanol values that were not significantly different to those produced by pure BSc203, ranging from 12.81 to 12.92% v/v, except for 1MSK4. In the latter, the amounts of ethanol and CO\(_2\) produced were significantly lower when compared to pure BSc203 and other mixed cultures \((P \leq 0.05)\). Similarly, Gobbi \textit{et al.} (2013) reported that ethanol production in a mixed fermentation, 90% \textit{L. thermotolerans}–10% \textit{S. cerevisiae}, was significantly lower than in \textit{S. cerevisiae} used as control. Fermentations in all mixed cultures assayed were completed (residual sugar ≤ 1.96 g l\(^{-1}\)), except for 1MSK4 that presented 8.94 g l\(^{-1}\) of residual sugar.

Mixed cultures with \textit{L. thermotolerans} presented values for total acidity, volatile acidity and pH that were not significantly different to those in a pure culture of BSc203 \((P \leq 0.05)\), except for 1MSK5. In the latter culture, total acidity in wines increased 27.65% and acetic acid reduced 28.57% compared with pure BSc203 culture (Table 3). In agreement with these results, other studies showed that \textit{L. thermotolerans}/\textit{S. cerevisiae} associations significantly affected the final wine composition positively by enhancing total acidity and reducing the pH (Kapsopoulou \textit{et al.} 2007) and volatile acidity (Comitini \textit{et al.} 2011). The oenological industry shows great interest in correcting insufficient acidity (Kapsopoulou \textit{et al.} 2007) and high volatile acidity (Schutz and Gafner 1993) of some grape musts from warm regions such as San Juan and Mendoza (Argentina).

These data suggests that at high initial concentrations RCKT5 is a good candidate for use as a biofungicide in wine grapes, because this strain did not affect the \textit{S. cerevisiae} growth and the wine quality. With respect to RCKT4, it interfered negatively on fermentation, especially on the \textit{S. cerevisiae} growth and on the ethanol production.

Materials and methods

Yeast strains

Biocontrol yeasts \textit{L. thermotolerans} strains RCKT4 and RCKT5 were isolated from the grape surfaces from vineyards in Mendoza province, Argentina (Ponsone \textit{et al.} 2011, 2016).
Table 3 Influence of *Lachancea* thermotolerans strains on the analytical profile of wines in pure and mixed cultures with BSc203. Values followed by the same letter in the same column were not significantly different at *P* ≤ 0.05.

| Cultures     | Sugar consumption rate in 72 h (g l⁻¹) | Ethanol (g l⁻¹) | Total acidity (g l⁻¹) | Volatile acidity (g l⁻¹) | Residual sugar (g l⁻¹) | pH      |
|--------------|---------------------------------------|-----------------|-----------------------|--------------------------|------------------------|---------|
| Pure BSc203  | 16 ± 0.01ab                           | 12.81 ± 0.21a   | 5.17 ± 0.12a          | 0.56 ± 0.02a              | 1.91 ± 0.11a           | 3.39 ± 0.01a |
| 3MSK4        | 15.2 ± 0.4ab                           | 12.75 ± 0.16a   | 5.19 ± 0.13a          | 0.58 ± 0.03a              | 1.9 ± 0.11a            | 3.38 ± 0.01a |
| 2MSK4        | 15.3 ± 0.03ab                          | 12.83 ± 0.05a   | 5.21 ± 0.21a          | 0.53 ± 0.06a              | 1.88 ± 0.03a           | 3.38 ± 0.02a |
| 1MSK4        | 14.7 ± 0.05ab                          | 11.01 ± 0.03a   | 5.28 ± 0.33a          | 0.5 ± 0.09a               | 8.94 ± 0.11b           | 3.37 ± 0.02a |
| 3MSK5        | 15.8 ± 0.03a                           | 12.83 ± 0.08a   | 5.36 ± 0.11a          | 0.55 ± 0.02a              | 1.61 ± 0.14a           | 3.39 ± 0.02a |
| 2MSK5        | 14.5 ± 0.02ab                          | 12.71 ± 0.06a   | 5.45 ± 0.06a          | 0.5 ± 0.02a               | 1.82 ± 0.18a           | 3.37 ± 0.03a |
| 1MSK5        | 15.1 ± 0.03a                           | 12.92 ± 0.04a   | 6.6 ± 0.13b           | 0.4 ± 0.03b               | 1.96 ± 0.02a           | 3.35 ± 0.01b |
| Pure RCKT4   | 6.5 ± 0.04b                           | 7.91 ± 0.41c    | 6.66 ± 0.15b          | 0.39 ± 0.01c              | 22.59 ± 2.01c          | 3.4 ± 0.02b  |
| Pure RCKT5   | 6 ± 0.07c                             | 6.05 ± 0.09d    | 9.43 ± 0.44b          | 0.26 ± 0.05d              | 29.27 ± 2.18d          | 3.32 ± 0.02b |

S.c., *Saccharomyces cerevisiae.*

1MSK4:1% S.c./99% RCKT4; 2MSK4:9% S.c./50% RCKT4; 3MSK4:99% S.c./1% RCKT4.
1MSK5:1% S.c./99% S.c.; 2MSK5:50% S.c./50% RCKT4; 3MSK5:99% S.c./1% RCKT4.

Oenological yeast *S. cerevisiae* BSc203 was isolated from fermentation grape must in San Juan province, Argentina. This yeast has proven good fermentation characteristics (Vazquez et al. 2014).

Both the biocontrol strains and BSc203 were identified by restriction fragment length polymorphism (RFLP) (Ponsone et al. 2011; Nally et al. 2012).

**Media**

The following media were used in this study: YEPD-agar: 10 g l⁻¹ yeast extract, 20 g l⁻¹ peptone, 20 g l⁻¹ dextrose, 20 g l⁻¹ agar; YEPD-MS-phosphate citrate buffer-agar: 10 g l⁻¹ yeast extract, 20 g l⁻¹ peptone, 20 g l⁻¹ dextrose, 0.01% methylene blue, 0.1 mol l⁻¹ phosphate.
citrate buffer, 20 g l⁻¹ agar; CAS-HDTMA-PIPES-YNB-glucose-agar: 60.5 mg l⁻¹ CAS (Chrome Azuroil S), 72.9 mg l⁻¹ HDTMA (hexadecyltrimethylammonium bromide), 30-24 g l⁻¹ PIPES (piperazine-1,4-bis(2-ethanesulphonic acid)), 6-7 g l⁻¹ YNB, 1 mmol l⁻¹ FeCl₃·6H₂O in 10 mmol l⁻¹ HCl, 20 g l⁻¹ glucose, 20 g l⁻¹ agar.

**Characterization of L. thermotolerans-based biocontrol agents**

**Nutritional profiles: NOI and NS**

Biocontrol yeasts and BSc203 aliquots (20 μl, 10⁶ cells per ml) were inoculated on plates. Each plate contained one carbonate source (10 mmol l⁻¹), YNB with 20 g l⁻¹ agar, pH 5-5. The carbonate sources assayed are present in wine grapes and represent the niche size: proline, asparagine, alanine, glutamic acid, tyrosine, arginine, lysine, methionine, glycine, malic acid, tartaric acid, fructose, melibiose, raffinose, rhamnose, sucrose and glucose. Plates were incubated at 25°C for 14 days. NOI were evaluated as the ratio between the number of carbonate sources used in common (biocontrol agent and BSc203) and the total number of carbonate sources utilized only by BSc203. NOI values of >0.9 represent competence between yeasts while scores of <0.9 represent occupation of separate niches. NS values were evaluated as the ratio between number of compounds used by each of the yeasts and number of compounds assayed in total (Collazo et al. 2017).

**Oenological behaviour of the biocontrol yeasts: tolerance to SO₂, ethanol and sugar concentrations**

Yeast tolerance towards SO₂, ethanol and sugar was assayed according to slightly modified methods described by Parish and Carroll (1987). SO₂ concentrations evaluated in the present study were 0, 25, 50, 75, 100, 150, 200, 250, 300 and 400 mg l⁻¹ and added to YNB plus 10 g l⁻¹ of glucose medium (pH 3-5). The ability to start fermentations at 7, 8, 9, 10, 11 and 12% v/v of ethanol was determined similarly. Tubes only containing YNB medium without glucose were used as negative controls.

Strain resistance to osmotic stress was examined by winemaking tests using commercial concentrated grape must from *Vitis vinifera* L. adjusted to 21° Brix and 30° Brix. The grape juice obtained was pasteurized for 30 min at 80°C. This process did not produce caramelization of the grape juice following the Maillard reaction (Bozkurt et al. 1999).

All assays were carried out in 20-ml tubes with 5 ml of medium, and tubes were inoculated with 10⁶ cells per ml. All microfermentations were checked for CO₂ production and considered positive when, after a 3 days incubation period at 25°C, Dühring bells located in the tubes were filled up for at least one-third of their capacity (Ubeda et al. 1995). The results are expressed as + (ability to ferment) and – (not ability to ferment). *S. cerevisiae* BSc203 was used as a positive control.

**Influence of L. thermotolerans strains on S. cerevisiae growth during fermentation**

Commercial must from *V. vinifera* L. was pasteurized as above mentioned. The initial grape must composition was 22° Brix and the pH 3-5. Biocontrol strains and BSc203 were pre-adapted in the same must at 13° Brix and pH 3-5, during 12 h at 22°C. Microviniﬁcations were carried out in 5-l glass flasks with 3 l of pasteurized commercial must, and topped with Müller valves (Ciani and Rosini 1987). The following mixed cultures were assayed: 1MSK4: 1%BSc203/99% RCKT4; 1MSK5: 1%BSc203/99%RCKT5; 2MSK4: 50%BSc203/ 50%RCKT4; 2MSK5: 50%BSc203/50%RCKT5; 3MSK4: 99%BSc203/1%RCKT4; 3MSK5: 99%BSc203/1% RCKT5. Pure and mixed cultures were inoculated at an initial concentration of 10⁶ cells per ml and were inoculated at 18°C. Pasteurized noninoculated must was used as a negative control under the same assay conditions. Fermentations under static conditions were monitored for CO₂ release measuring weight loss every 24 h until the end of the fermentation (constant weight). The sugar consumption rate was calculated as the amount of sugar consumed (g l⁻¹) in 72 h. Fermentation samples were withdrawn every 24 h and spread on Wallerstein Laboratory Nutrient (WLN). This medium allows putative identification of yeasts according to colour of the colonies. On WLN, BSc203 present creamy colonies, whereas RCKT4 and RCKT5 light green colonies (Vazquez et al. 2014). At the end of the assay, fermented products were centrifuged at 11 000 g (10 min, 4°C), filtered and stored at 4°C until further analysis. The most important wine quality parameters (ethanol, volatile acidity, total acidity, pH, residual sugar) were analysed according to the official methods of the OIV (2013) and INV (2015).

**Statistical analysis**

In all the assays, three replicates per treatment were performed and the experiment was repeated twice. To evaluate the effects of *L. thermotolerans* strains on BSc203 growth and on the wine quality, single-factor variance analysis (ANOVA) was carried out after verification of variance homogeneity (Levene test, P ≤ 0.05). Significant differences among treatments were determined by Tukey’s test (P < 0.05) using the computer software SPSS 21.0 (Chicago, IL).
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Conflict of Interest

The authors declare no conflicts of interest.

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