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Propolis as a potential alternative for the control of *Dekkera bruxellensis* in bioethanol fermentation

Própolis como alternativa potencial para o controle de *Dekkera bruxellensis* em fermentação alcoólica

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Abstract

Dekkera bruxellensis is one of the most important contaminant yeasts of alcoholic fermentation. The use of propolis, which can selectively target contaminating yeasts without affecting the starter one, *Saccharomyces cerevisiae*, could be a useful nonconventional strategy for controlling the growth of contaminant yeasts. The objective of this research was to evaluate four samples of propolis produced by *Apis mellifera* honeybees from different regions of Argentina as antimicrobial agents against the growth of *D. bruxellensis* and *S. cerevisiae*. Hydroalcoholic extracts of propolis were prepared with ethanol:water (70:30 v/v), and the specific absorbance and final concentration of the samples were evaluated. A qualitative *in vitro* assay in solid medium was performed with different propolis concentrations, and the evaluation of yeast growth was based on a qualitative scale. A quantitative *in vitro* assay in liquid medium was also performed to assess the yeast cell number, using two different propolis concentrations. The cell number of *D. bruxellensis* decreased 1.52 and 1.85 log cycles with the two propolis extracts utilised at a concentration of 4.5 mg mL^{-1} while the cell number of *S. cerevisiae* decreased 0.48 and 0.76 log cycles with the same samples of propolis. The results from both assays demonstrated the selectivity of propolis use on the yeast species, leading to a higher inhibition of *D. bruxellensis* growth. This indicates a good potential for using propolis at the concentration of 4.5 mg mL^{-1}, as a nonconventional strategy to control the growth of *D. bruxellensis* without significantly affecting *S. cerevisiae*, the yeast starter of ethanol fermentation.

Key words: *Apis mellifera* honeybees. Antimicrobial activity. Hydroalcoholic extracts. Argentina. *Saccharomyces cerevisiae.*

Resumo

Dekkera bruxellensis é uma das mais importantes leveduras contaminantes da fermentação alcoólica. O uso de própolis, que pode seletivamente afetar a levedura contaminante mas não a levedura do processo,

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Saccharomyces cerevisiae, pode ser uma estratégia não convencional útil para o controle de leveduras contaminantes. O objetivo deste trabalho foi avaliar quatro amostras de própolis produzidas por *Apis mellifera* de diferentes regiões da Argentina como agentes antimicrobianos no controle do crescimento de *D. bruxellensis* e *S. cerevisiae*. Foram preparados extratos hidroalcoólicos de própolis com etanol: água (70:30 v/v) e avaliadas a absorbância específica e concentração final das amostras. Um ensaio qualitativo *in vitro* foi realizado em meio sólido com diferentes concentrações de própolis e a avaliação do crescimento da levedura foi baseada em uma escala qualitativa. Um ensaio quantitativo *in vitro* em meio líquido foi realizado com duas concentrações de própolis, avaliando-se o número de leveduras. O número de células de *D. bruxellensis* diminuiu 1,52 e 1,85 ciclos log com dois extratos de própolis na concentração de 4,5 mg mL^{-1} enquanto para *S. cerevisiae,* a diminuição no número de células foi de 0,48 e 0,76 ciclos log com as mesmas amostras de própolis. Os resultados de ambos os ensaios demonstraram claramente a seletividade do efeito do emprego de própolis nas leveduras estudadas, resultando em maior inibição no crescimento da levedura *D. bruxellensis*. Isso indica a boa perspectiva do uso de própolis, na concentração de 4,5 mg mL^{-1}, como uma estratégia não convencional para controlar o crescimento de *D. bruxellensis* sem afetar substancialmente *S. cerevisiae*, a levedura agente da fermentação etanólica.
Palavras-chave: Abelhas *Apis mellifera.* Atividade antimicrobiana. Extratos hidroalcoólicos. Argentina. *Saccharomyces cerevisiae.*

Dekkera bruxellensis is commonly cited as one of the most important contaminant yeasts of alcoholic fermentations, especially in Brazilian industrial setups. This yeast has been responsible for several episodes of contamination in fermentation processes in distilleries in the United States, Canada, Europe and in the Northeast region of Brazil. The species *D. bruxellensis* replaced the initial *Saccharomyces cerevisiae* strains during one harvesting season and reached levels of almost 50% of the total yeast population. The ethanol productivity of *D. bruxellensis* isolates are much lower than that of *S. cerevisiae* and impaired the global ethanol yield of the distillery (SOUZA-LIBERAL et al., 2007). Low sugar consumption, low alcohol production and expressive growth were found in bioethanol fermentation contaminated with *D. bruxellensis* in batch systems (MENEGHIN et al., 2013).

Antimicrobials such as sulphur dioxide, chitosan and dimethyl dicarbonate have been utilised to control populations of this undesirable yeast in wine (BARATA et al., 2008; GÓMEZ-RIVAS et al., 2004). However, the continued administration of these products can lead to the creation of resistant strains, raise processing costs and allow the incorporation of residues in the product, lowering the beverage quality. In addition, acid cell washing is occasionally inefficient, and the corrosive acids are a serious safety risk. Strategies to control the growth of *D. bruxellensis* in the bioethanol industry are scarce (BASSI et al., 2013, 2014). Thus, new antimicrobials should be researched and tested. Among them, natural products can be considered as nonconventional strategies to control yeast growth in the bioethanol industry. Propolis is an example of an animal-derived natural product with good potential in controlling bacterial contamination (CECCATO-ANTONINI, 2018).

Propolis is an extremely complex, resinous substance collected by honeybees from buds and tree leaves, mixed with pollen and enzymes. It contains a variety of chemical compounds, such as flavonoid aglycones, phenolic acids and their esters, phenolic aldehydes, alcohols and ketones, sesquiterpenes, quinones, coumarins, steroids, aminoacids, and inorganic compounds (VIUDA-MARTOS et al., 2008). The composition is quite variable, depending on the origin of the samples, which is strongly related to the flora surrounding the hive (SFORCIN; BANKOVA, 2011). The antibacterial and antifungal properties are the most popular and extensively investigated biological activities of propolis, with many applications in medicine, cosmetology, the food industry, agriculture, etc. (VIUDA-MARTOS et al., 2008). Studies have demonstrated the antimicrobial effect of propolis against bacteria in alcoholic fermentation for fuel production, both using hydroalcoholic and oily extracts (BADIN,

2010). However, the effect of propolis on the growth of native (wild) yeasts, such as *D. bruxellensis,* has not yet been reported.

Propolis has been utilised as a biological alternative for the control of phytopathogenic fungi (GALLEZ et al., 2014, 2017). In this work, we proposed to study propolis as a new nonconventional method to control a contaminant yeast in ethanolic fermentations. We hypothesised that it would be possible to use propolis as an antimicrobial agent that can selectively target the contaminating yeast without affecting the starter one, *S. cerevisiae*. The aim of this work was to evaluate four propolis samples as antimicrobial agents on the growth of *D. bruxellensis* and *S. cerevisiae*. Qualitative and quantitative assessments were carried out *in vitro* with propolis samples from Argentinean apiaries.

In this study, four samples of raw propolis produced by *Apis mellifera* honeybees were collected from apiaries located in different regions of Argentina (Figure 1): 1) Río Colorado, Río Negro Province, (36°09′02″S and 70°23′47″W); 2) Luján de Cuyo, Mendoza Province (33°01′S and 68°52′W); 3) Bahía Blanca, Buenos Aires Province (38°43′S and 62°16′W); and 4) Carmen de Patagones, Buenos Aires Province (40°47′S and 62°58′W). Propolis were collected with propolis traps, minimising the contamination with foreign substances, and stored at -20°C. The hydroalcoholic extracts of the propolis samples were prepared as described in Gallez et al. (2014). Briefly, 15 g of propolis were extracted in a flask with 150 mL of ethanol:water (70:30 v/v) for 24 h at room temperature (~30°C) with shaking prior to use. The specific absorbance was determined in an UV spectrophotometer (JASCO V-630 BIO), as well as the final concentration (mg mL^{-1}) of soluble compounds of each propolis sample, which were obtained from the hydroalcoholic extracts according to Bedascarrasbure et al. (2006).

Figure 1. Map of Argentina with the collection sites of propolis and the respective UV-VIS spectra from the hydroalcoholic extracts (range from 240 to 420 nm). 1. Río Colorado, Río Negro Province; 2. Luján de Cuyo, Mendoza Province; 3. Bahía Blanca, Buenos Aires Province and 4. Carmen de Patagones, Buenos Aires Province.

D. bruxellensis strain CCA155 (CCT7784), isolated from a Brazilian industrial alcohol-producing unit, and *S. cerevisiae* strain PE-2, the most common starter yeast utilised in fermentation processes for fuel ethanol production, were both grown in YPD broth (2% glucose, 1% yeast extract and 2% bacteriological peptone in distilled water) until an optical density at 600 nm of 0.45 and 0.75 (approximately 10^7 UFC mL^{-1} each) were obtained for each yeast, respectively. These yeast suspensions were utilised as inocula.

The qualitative *in vitro* assay was performed in Petri dishes, where YPD was mixed with each hydroalcoholic extract of propolis to obtain final concentrations of 0.1125, 0.225, 0.45 and 0.9 mg mL^{-1} of propolis in a final volume of 20 mL of medium per Petri dish. The yeast cell suspensions were diluted (10^{-1}, 10^{-3} and 10^{-5}), and 3 drops (10 µL/drop) were plated employing a modification of the drop plate method described by Herigstad et al. (2001). Four plates per propolis sample and concentration were performed. Plates with only YPD or with YPD without propolis but containing ethanol:water (70:30 v/v) were used as control. All Petri dishes were incubated at 30°C for 48 h. The evaluation of yeast growth was based on a qualitative scale.

Two hydroalcoholic extracts of propolis were selected for the quantitative *in vitro* assay, which was performed according to Bassi et al. (2014) with some modifications. Briefly, 50 mL Falcon tubes containing a final volume of 10 mL were prepared with 7.5 mL of YPD broth, 1 mL of yeast suspension (as described above) and 0.75 or 1.5 mL of each hydroalcoholic extract of propolis (propolis final concentration of 4.5 and 9 mg mL^{-1}). When 0.75 mL of propolis was added, the volume was brought up to10 mL with sterile distilled water. Falcon tubes with only YPD or with YPD plus ethanol:water (70:30 v/v) were used as control. The Falcon tubes were incubated at 30°C for 48 h at 160 rpm. The yeast viability was assessed by staining the samples with sodium citrate-methylene blue solution (LEE et al., 1981) and counting the cells in a Neubauer chamber. The assay was carried out in a completely randomised design with three replicates per treatment. For each yeast strain, the logarithmic reduction in growth was calculated considering the log of the cell number at 48 h of cultivation for each propolis type and concentration in relation to the log of the cell number at 48 h of cultivation in the control treatment without propolis (only YPD).

The results showed that the absorption spectra of hydroalcoholic extracts from Argentinean propolis were in the wavelength range expected: from 200 to 600 nm (Figure 1). UV spectrograms showed that all the samples displayed a maximum absorbance range between 250 and 300 nm, with a main absorption peak at 295 nm. The existence of an absorption maximum at 295 nm is indicative of an important biological activity, due to the content of flavonoid compounds (SFORCIN; BANKOVA, 2011). The UV-VIS absorbance spectra were also consistent with data recorded from other propolis samples from Argentina (BEDASCARRASBURE et al., 2006).

The final concentrations of the soluble compounds of the propolis samples in the hydroalcoholic solutions were as follows: 63 mg mL^{-1} for Río Colorado (Río Negro Province), 35 mg mL^{-1} for Luján de Cuyo (Mendoza Province), 60 mg mL^{-1} for Bahía Blanca (Buenos Aires Province) and 90 mg mL^{-1} for Carmen de Patagones (Buenos Aires Province). Talero et al. (2012) described that 70% (v/v) ethanol extractions had average values of dry extracts lower than those obtained with 96% (v/v) ethanol. However, the choice of hydroalcoholic solution is supported by the studies of Silva Frozza et al. (2013), which indicated that this extractant is able to solubilize phenols and different bioactive compounds in considerable quantities, and that it is less toxic than other solvents. Moreover, Sforcin and Bankova (2011) encouraged the idea that the most often utilised solvent is a diluted solution of ethanol in water because it was found to extract most of the active components from propolis, but not from waxes.

In the qualitative *in vitro* assay, the yeasts showed strong growth in both control samples in all dilutions tested (10^{-1}, 10^{-3} and 10^{-5}, data not shown). The results of this qualitative screening clearly demonstrated that the alcoholic solution utilised as an extractant in the propolis solutions, ethanol:water (70:30, v/v), did not inhibit the yeast growth. These results are in agreement with studies from other authors (CIGUT et al., 2011; GALLEZ et al., 2014).

Table 1 presents the results of the qualitative *in vitro* assay of the antimicrobial activity of propolis against *D. bruxellensis* and *S. cerevisiae*. The results clearly revealed that the four hydroalcoholic extracts of propolis inhibited completely the growth of *D. bruxellensis* at concentrations of 0.225, 0.45 and 0.9 mg mL^{-1}. This yeast grew poorly at 0.1125 mg mL^{-1} of propolis and only at 10^{-1} dilution. The results were very different for *S. cerevisiae*: concentrations of 0.45 and 0.9 mg mL^{-1} of propolis were completely inhibitory to the growth of *S. cerevisiae*. However, the lowest concentrations tested, 0.1125 mg mL^{-1} of propolis, did not interfere with the growth of this yeast in all dilutions. Moreover, the observed growth was similar to that of the control without propolis. At 0.225 mg mL^{-1} of propolis, this yeast exhibited a strong growth at 10^{-1} and 10^{-3} dilutions. These results demonstrated the differences in behaviours between the yeasts when they were grown in YPD with different concentrations of propolis, showing that *S. cerevisiae* was inhibited by propolis at higher concentrations than were needed to inhibit *D. bruxellensis* growth. There was no difference in growth inhibition regarding the origin of the propolis for either yeast. Cigut et al. (2011) studied the antioxidative activity of propolis *in vivo* using the yeast *S. cerevisiae*. They demonstrated that propolis did not have a negative effect on this yeast. They also found that yeast cells exposed to 96% ethanolic extracts of propolis showed decreased intracellular oxidation.

Table 1. Antimicrobial activity of propolis at different concentrations against *D. bruxellensis* and *S. cerevisiae* in the qualitative *in vitro* assay. Growth evaluation: 0 (no growth); + (weak growth); ++ (strong growth); +++ (growth similar to the control without propolis).

Yeast	Argentinean propolis															
	Río Colorado (mg mL^{-1})				Carmen de Patagones (mg mL^{-1})				Bahía Blanca (mg mL^{-1})				Luján de Cuyo (mg mL^{-1})			
	0.1125	**0.225**	**0.45**	**0.9**	**0.1125**	**0.225**	**0.45**	**0.9**	**0.1125**	**0.225**	**0.45**	**0.9**	**0.1125**	**0.225**	**0.45**	**0.9**
Dekkera bruxellensis	+[1]	0	0	0	+[1]	0	0	0	+[1]	0	0	0	+[1]	0	0	0
Saccharomyces cerevisiae	+++[2]	++[3]	0	0	+++[2]	++[3]	0	0	+++[2]	++[3]	0	0	+++[2]	+++[3]	0	0

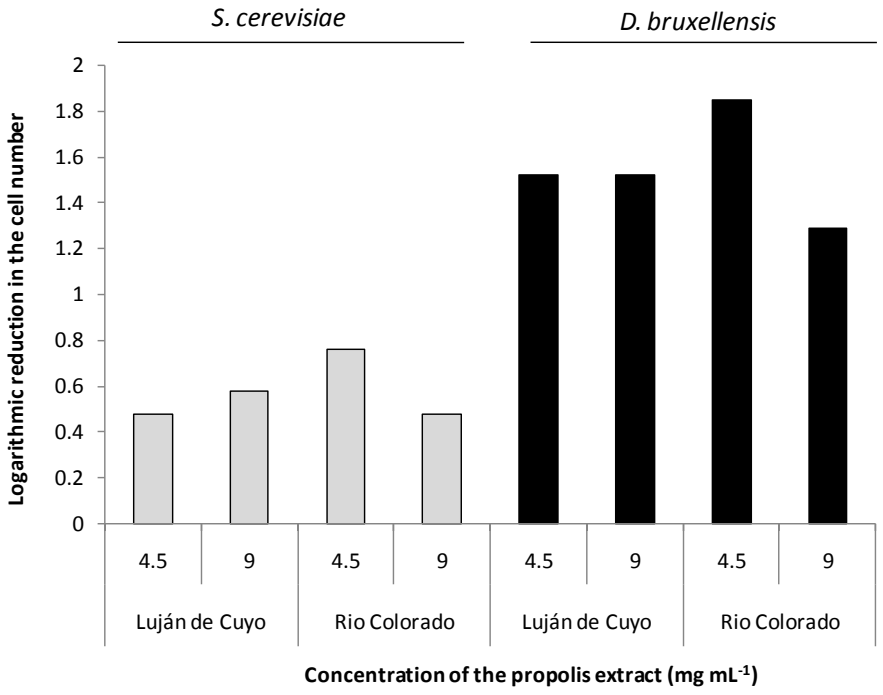
[1]at 10^{-1} dilution only; [2] in all dilutions; [3] at 10^{-1} and 10^{-3} dilutions.

Two hydroalcoholic extracts of propolis were selected for the quantitative *in vitro* assay, considering the different geographical origin of the propolis sample (Figure 1): Luján de Cuyo (Mendoza Province) and Río Colorado (Río Negro Province). As previously mentioned, propolis properties depend on its chemical nature, which is strongly related to the flora surrounding the hive (SFORCIN; BANKOVA, 2011).

In this assay, higher concentrations of propolis (4.5 and 9 mg mL^{-1}) were utilised due to the usage of liquid medium instead of solid medium. Figure 2 displays the results of the quantitative evaluation assay of propolis as an antimicrobial agent against *D. bruxellensis* and *S. cerevisiae* during 48 h of cultivation. There was less inhibition using both types of propolis on *S. cerevisiae* than on *D. bruxellensis*. After the incubation with propolis,

the cell number of *D. bruxellensis* at the lowest concentration (4.5 mg mL^{-1}) decreased 1.52 and 1.85 log cycles for Luján de Cuyo (Mendoza Province) and Río Colorado (Río Negro province) propolis samples, respectively. For *S. cerevisiae*, the decrease varied from 0.48 to 0.76 log cycles for Río Colorado propolis, and from 0.48 to 0.58 log cycles for Luján de Cuyo propolis, depending on the concentration. These results clearly demonstrated the selectivity of propolis on the yeast species, especially considering that the most sensitive is the contaminant strain, *D. bruxellensis*. No noteworthy differences between the propolis samples regarding the growth inhibition of both yeasts were observed.

Figure 2. Logarithmic reduction in cell number of *Dekkera bruxellensis* and *Saccharomyces cerevisiae* in the quantitative *in vitro* assay with different concentrations of hydroalcoholic extracts of propolis from Argentina sites.



It is interesting to note that the number of viable cells of *Dekkera* and *Saccharomyces* after 48 h of incubation in the control treatment consisting of YPD with ethanol:water was not remarkably different from the control consisting of YPD without ethanol (data not shown). Thus, these data confirmed that the antimicrobial activity was due to the presence of bioactive compounds in the hydroalcoholic extracts from Argentinean propolis and not due to the alcohol inhibition, as it was previously described in the qualitative assay.

Other researchers have found similar results regarding *S. cerevisiae* when studying different substances to control contaminants during ethanolic fermentation. Madaleno et al. (2016) evaluated the initial and final yeast viability when antimicrobials as hop extract, oregano essential oil and chlorine dioxide were utilised during the fermentation process to control bacterial growth. They found that the viability of *S. cerevisiae* did not change significantly with any antimicrobial substance, indicating that treatments used at their recommended doses do not eliminate yeast cells from the fermentation process. On the other hand, Mutton et al. (2014) evaluated the efficiency of brown and green propolis to control bacterial contamination in the production of

sugarcane spirit. They observed a reduction in the number of bacterial contaminants with the natural biocides, which did not affect the viability of yeast cells.

In conclusion, our results from both assays demonstrated the selectivity of propolis on the yeast species, especially considering that the contaminant strain *D. bruxellensis* is the most sensitive. This indicates the good prospect of using propolis as a nonconventional strategy to control the growth of *D. bruxellensis* at a propolis concentration of 4.5 mg mL^{-1} of propolis. In addition, *S. cerevisiae*, the most important starter yeast in industrial alcoholic fermentation, was not substantially affected by propolis. Further studies are necessary to evaluate the utilisation of propolis as a nonconventional strategy in similar conditions to those of industrial production, such as the fermentative process with cell recycling and addition of the product in fermentation tanks or during the cell treatment step.

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