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An *in vitro* and *in vivo* evaluation of peroxyacetic acid as an alternative sanitizer for wine barrels

Avaliação *in vitro* e *in vivo* do ácido peroxiacético como desinfetante alternativo para barricas

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SUMMARY

Peroxyacetic acid is a common sanitizer used in the food and wine industry, but its use as a sanitizer for wine barrels has not been reported. We are reporting the findings for *in vitro* studies using three different concentrations of peroxyacetic acid (0, 60, and 120 mg/L) as sanitization challenges against seven strains of wine spoilage yeast representing three different species: *Dekkera*/*Brettanomyces bruxellensis* (three strains), *Saccharomyces cerevisiae* (three strains) and *Zygosaccharomyces bailii* (one strain). *In vitro* sensitivity to peroxyacetic acid concentration varied within and between species. A *post hoc* study (*in vivo*) using the highest concentration from the *in vitro* studies (120 mg/L) as well as 200 mg/L was performed to validate a sanitization method for wine barrels. Exposure of barrels to 200 mg/L of peroxyacetic acid for one week resulted in no detectable levels of wine spoilage microorganisms after treatment. These findings are crucial for establishing protocols to assure the maximum reduction of microbial contaminants.

RESUMO

O ácido peroxiacético é um desinfetante comum usado na indústria alimentar e vinícola, mas o seu uso como desinfetante de barricas não foi reportado. Neste trabalho apresentamos os resultados de estudos *in vitro* utilizando três concentrações diferentes de ácido peroxiacético (0, 60 e 120 mg/L) em sete estirpes de leveduras contaminantes do vinho de três espécies diferentes: *Dekkera*/*Brettanomyces bruxellensis* (três estirpes), *Saccharomyces cerevisiae* (três estirpes) e *Zygosaccharomyces bailii* (uma estirpe). A sensibilidade *in vitro* à concentração de ácido peroxiacético variou dentro e entre espécies. No estudo *post hoc* (in vivo), foi utilizada a concentração mais elevada dos estudos *in vitro* (120 mg/L), bem como 200 mg/L para validar um método de higienização de barricas usadas para vinho. A exposição das barricas a 200 mg/L de ácido peroxiacético durante uma semana resultou em níveis não detetáveis de microrganismos contaminantes de vinho após o tratamento. Estes resultados são cruciais para o estabelecimento de protocolos para assegurar o máximo de redução de contaminantes microbianos.

Key words: peroxyacetic acid, barrel, sanitation, validation, yeasts.
Palavras-chave: ácido peroxiacético, barrica, higienização, validação, leveduras.

INTRODUCTION

The food and beverage industry has used a variety of different sanitizers that have been scientifically shown to be effective against targeted microorganisms. However, comparatively little research has focused upon scientific evaluation of sanitizers specifically relevant to the wine industry. Wine spoilage microorganisms interfere with the winemaking process, causing stuck fermentations or degraded wine quality, caused by off flavors that develop during the aging process, as a consequence of poor sanitation practices. Peroxyacetic acid (PAA) is a sanitizer approved for fresh produce by the United States (US) Food and Drug Administration (Neo *et al.*, 2013). PAA has been used for food contact surface sanitizing and aseptic packaging. Its efficacy is a function of concentration, exposure time, and treatment surface (Gonzalez Aguilar *et al.*, 2012). The potential application of PAA to reduce populations of wine spoilage microorganisms has not been

comprehensively studied on the variety of different surfaces used in the vinification process.

PAA is a strong oxidant due to its chemical composition. In its commercially available form, it is a quaternary equilibrium mixture containing acetic acid, hydrogen peroxide, PAA and water. PAA solutions are produced from the reaction of acetic acid or acetic anhydride with hydrogen peroxide in the presence of sulfuric acid, which functions as a catalyzing agent (Vandekinderen *et al.*, 2009). In spontaneous decomposition, peracetic acid is decomposed to form acetic acid and oxygen, thus representing a loss of oxidation power (Yuan *et al.* 1997). It acts primarily on lipoproteins in the cell membrane, and it may be equally effective against outer membrane lipoproteins (Silveira *et al.*, 2008).

The PAA efficacy against yeasts is reduced at lower temperatures. This is not surprising, as disinfection, like other chemical processes, almost invariably takes place at a slower rate as the temperature falls (Baldry, 1983). Microbial spoilage of wine can occur at any stage of the vinification process due to non-*Saccharomyces* yeasts and lactic and acetic acid bacteria. Of these potential contaminants, wild species of non-*Saccharomyces* yeasts in the genera *Dekkera/Brettanomyces*, *Candida*, *Hanseniaspora/Kloeckera*, *Pichia*, *Metschnikowia*, *Saccharomycodes*, *Schizosaccharomyces* and *Zygosaccharomyces* have been involved in wine spoilage (Enrique *et al.*, 2007). Moreover, if the aging conditions are not perfectly controlled, barrel-aged wines may be more easily exposed to several types of microbiological contamination likely to have a negative impact on their composition (Chatonnet *et al.*, 2010).

Indeed, the maintenance of sanitary practices during the various steps of wine production is essential to prevent the contamination of wine. The selection of detergents and disinfectants in the food and beverage industry is dependent upon several factors, such as the efficacy in removing a wide range of microorganisms, handling safety, the rinse-ability of the agent, its corrosiveness on contact surfaces, and its impact on the sensory quality of the products manufactured (Tristezza *et al.*, 2010). Wood is one of the surfaces to be sanitized in wineries and has played a historically significant role in the history of wine, and continues to be important in production today. Wooden barrels are used as containers in the wine making process, and wineries often reuse barrels for several cycles of wine production due to the initial high investment costs.

The microporous structure of wood favors the penetration of microorganisms into the internal structure of the wood, increasing the difficulty of cleaning and sanitization, and increasing the risk of wine spoilage due to contamination during the fermentation and aging of wines (Gonzalez Arenzana *et al.*, 2013). Wineries around the world, in an attempt to improve their product and process, have been requesting that the scientific community develop effective, safe, and reliable methods to eliminate the microorganisms responsible for wine spoilage. There are very few reports of the efficacy of PAA against yeasts and more specifically towards wine spoilage yeasts. Moreover, PAA has not been evaluated under controlled conditions to sanitize wine barrels where wine spoilage microorganisms could be harbored. However, it has been evaluated on stainless steel vats surface (Duarte *et al.*, 2011). In this study we assessed three different concentrations of PAA *in vitro* by challenging seven strains yeast commonly found in wine environments and known for causing spoilage of wines. Those results were used to achieve a *post hoc* study in naturally contaminated barrels (*in vivo*) with *Dekkera*/*Brettanomyces* and general yeast populations. This study was designed to explore an alternative method for sanitizing wine barrels using a moderate concentration of PAA.

MATERIAL AND METHODS

Strain selection

Three isolates of *Dekkera/Brettanomyces bruxellensis* (CE261, 2080, CE149) were obtained by donation or from the Department of Food Science collection at Cornell University.

Three isolates of *Saccharomyces cerevisiae* (CE81, CE9 and CE78) were obtained from the Department of Food Science collection at Cornell University.

One isolate of *Zygosaccharomyces bailii* (4A1) was obtained from the Department of Food Science collection at Cornell University.

Preparation of starter culture and inoculation

The yeast cultures were stored at -80 °C in glycerol 15 "%" (w/v), revitalized and maintained on YPD agar (yeast extract 10 g/L, peptone 20 g/L, dextrose 20 g/L, agar 15 g/L; Difco™; Sparks, MD, USA). For the *in vitro* experiments, the strains were grown until stationary phase (growth under agitation at 200 rpm, 30 °C) in YPD broth. Once the cultures reached the stationary phase (10^6 CFU/mL-10^8 CFU/mL), they were verified via a culture count. CE149, CE9, CE78, CE81 and 4A1 reached 10^8, CE261 10^7 and 2080 10^6 CFU/mL. To prepare the target yeast for treatment, culture volumes of 1 mL were centrifuged (4500 rpm, 5 min and ambient temperature), the supernatant

discarded, and the cells re-suspended in 1 mL of sterile deionized water. This washing step was repeated. Concurrently, a flask of the test solution: sterile deionized water and PAA (peroxyacetic acid 15.2 "%", hydrogen peroxide 11.2 "%", inert ingredients 73.6 "%") (Tsunami 100 ECOLAB USA Inc.; St. Paul, MN, USA) at the desired concentrations (0 mg/L, 60 mg/L and 120 mg/L) were prepared. Subsequently, 1 mL of the microbial suspension was added to the flask to yield 100 mL (total volume). Samples were taken from this flask at different times (0, 1, 5 and 15 min).

Microbiological enumeration

Yeasts were enumerated by serially diluting samples in BPW (buffered peptone water) (0.1 "%") (Hardy Diagnostics; Santa Maria CA, USA) and 0.1 "%" sodium thiosulfate to neutralize the reaction of PAA (Fisher Scientific; Fair Lawn, NJ, USA). At the concentrations of PAA used (60 and 120 mg/L) sodium thiosulfate at 0.1 "%" ensured that any remaining reaction would be neutralized (Davenport, 2016). Then, 100 µL were immediately spread plated from the corresponding dilutions in duplicate on YPD agar. When necessary, direct plating of the sample (10^0 dilution) was performed to enumerate low concentrations of cells after treatment. The plates were incubated at 30 °C for 48 to 72 h for *S. cerevisiae* and *Z. bailii*. Genera such as *Dekkera/Brettanomyces bruxellensis* grew slowly, and required 3-4 weeks of incubation. *D./B. bruxellensis* has visible growth only after 72 hrs with very tiny colonies. For the control experiments, the same experimental procedures were performed but deionized water was added in place of PAA. The counts were averaged and expressed on a Log_{10} scale. The reduction due to treatment was likewise calculated and expressed on a Log_{10} scale for each strain. Every experiment was performed in triplicate with duplicate plating.

***In vivo* decontamination experiments using naturally contaminated barrels**

The wineries that donated the barrels used for this study, had already identified *Dekkera/Brettanomyces* in the donated barrels via VINEO™ Brettanomytest PCR Kit-Bio-Rad. Twenty barrels naturally contaminated with both *Dekkera/Brettanomyces bruxellensis* and general yeast populations, were split in two groups of ten barrels each and treated with two different concentrations of PAA (peroxyacetic acid 5.1 "%", hydrogen peroxide 21.7 "%", inert ingredients 73.2 "%") (VigorOx®LS&D FMC Corporation; Philadelphia PA, USA): 120 mg/L and 200 mg/L. Briefly, the 20 barrels were added with 7 L of distilled water before PAA treatment. The barrels were rolled from time to time in order to enhance the contact of water with the inner surface of the barrel, and then stored bung side up for 24 hrs and then sampled in order to know the initial microbial load. Afterwards, 120 mg/L concentration was applied for 15 min and 200 mg/L concentration was applied for 1 week. The reason to use different contact times is because we were mimicking the i*n vitro* conditions as they occurred. However, since we know that other factors can hinder the antimicrobial activity of the sanitizer (i.e. smooth surface *vs* porous surface), the second concentration and contact time used (200 mg/L and one week) were to ensure that PAA could counteract the effect that debris and porosity in the barrels can do.

Each barrel had a code number to be identifiable in the cellar and those numbers were also used to present our results in this article. The PAA solutions were prepared immediately prior to usage in distilled water and added quickly to the barrels. The barrels were fully filled with the PAA solution and were stored bung side up for their respective treatment time. 500 mL liquid samples were taken manually before and after treatment. Before treatment what was sampled was the distilled water that was put inside of the barrel for 24 hrs, after treatment what was sampled was the PAA solution that was in contact with the barrel for 15 min or 1 week. For this purpose, the barrels were put in a rack with the aid of a forklift and then the bung hole was directed towards the floor in order to facilitate the sampling. Then the first portion of the sample was discarded in order to "rinse" the bung hole which was additionally sprayed with 70 "%" ethanol. The 500 mL liquid samples were taken in sterile bottles before treatment to count the initial microbial load in each barrel and after PAA treatment to see the log reduction. Samples were stored at 4 °C until analysis. The samples were analyzed for microbial population by filtration (EZ-Fit™ Manifold for universal laboratory filtration; Concord Road Billerica, MA USA). Some filtered samples required pertinent dilutions due to a high microbial load was found after incubation. If samples needed to be diluted a 0.1 "%" (wt/vol) solution of BPW was used.

For the filtration, 0.22 µm nitrocellulose membrane filters (GE* Nitrocellulose-Mixed Esters of Cellulose Membrane Filters; Pittsburg, PA, USA) were used and the samples were filtered twice, and the results were averaged. The maximum volume filtered was 100 mL and the results were calculated as CFU/100mL and then transformed to Log_{10}. After filtration, the membrane filters were transferred with sterile forceps to both WL and YPD agar. WL agar (yeast extract 4 g/L, tryptone 5g/L, glucose 50 g/L,

potassium dihydrogen phosphate 0.55 g/L, potassium chloride 0.425 g/L, calcium chloride 0.125 g/L, magnesium sulphate 0.125 g/L, ferric chloride 0.0025, manganese sulphate 0.0025 mg/L, bromocresol green 0.022 g/L, agar 15 g/L) (Oxoid, LTD; Basingstoke Hampshire, England) was used for detection *Dekkera/Brettanomyces bruxellensis* and was incubated at 30 °C for up to 3-4 weeks and colonies that grew before three days were discarded. Incubation time was another criterion to demonstrate the growth of *Dekkera/Brettanomyces bruxellensis* strains, due to nothing that grows before 3 days in WL with cycloheximide is *Dekkera/Brettanomyces bruxellensis*. WL agar contained 10 mg/L of cycloheximide (Sigma Aldrich; St. Louis, MO, USA), to make it selective for *D./B. bruxellensis* (dissolved in 50 "%" ethanol and filter sterilized), 150 mg/L of biphenyl (Acros Organics; Fair Lawn, New Jersey, USA) (dissolved in ethanol and filter sterilized), to avoid the growth of mold, 30 mg/L of chloramphenicol (MP Biomedicals LLC; Solon, OH, USA) (dissolved in 100 "%" ethanol), to prevent the growth of lactic acid bacteria and 25 mg/L of kanamycin sulfate (AMRESCO; Solon, OH, USA) (dissolved in sterile distilled H_2O), to prevent the growth of acetic acid bacteria. YPD agar was used to enumerate the general yeast population and was incubated at 30 °C for 48-72 h. YPD agar was supplemented with 150 mg/L of biphenyl (Acros Organics; Fair Lawn, New Jersey, USA; dissolved in ethanol and filter sterilized), to prevent the growth of mold, 30 mg/L of chloramphenicol (MP Biomedicals LLC; Solon, OH, USA; dissolved in 100 "%" ethanol), to prevent the growth of lactic acid bacteria, and 25 mg/L of kanamycin sulfate (AMRESCO; Solon, OH, USA; dissolved in sterile distilled H_2O), to prevent the growth of acetic acid bacteria.

Statistical analysis

For the *in vitro* experiments, all CFU/mL data were transformed to Log_{10}. The log reductions in yeast were calculated from the initial concentration of yeast cells (target inoculum) at time zero minus the last concentration of yeast after fifteen min of treatment time. The analysis currently used was a two-way ANOVA where all pairwise multiple comparison procedures were done with a Holm-Sidak method at an alpha level of 0.05. For the reduction of *Dekkera/Brettanomyces* and general yeast populations in naturally contaminated barrels using PAA solutions (*in vivo* experiments), a Fisher's exact test was performed in order to see if the two study groups (120 mg/L or 200 mg/L) differ in the proportions of presence or absence of microorganisms. Statistical analyses were conducted using SigmaPlot 12.0; Systat Software Inc., San Jose CA.

RESULTS AND DISCUSSION

***In vitro* reduction of yeast**

We first evaluated the efficacy of PAA under *in vitro* conditions where three concentrations (120 mg/L, 60 mg/L and 0 mg/L) were used to challenge seven strains of yeast commonly found in wine environments and that are known for causing spoilage of wine. The results showed that at a concentration of 120 mg/L, the strain *Zygosaccharomyces bailii* 4A1 (Figure 1, Table I) was the only one resistant. Hilgren and Salverda (2000) performed a study using *Z. bailii* and peroxyacetic acid at a concentration of 80 mg/L using different exposure times. They used 30 s, 2 min, and 5 min, and an initial concentration of yeast cells of 5.98 Log_{10} CFU/mL, whereas our initial concentration was 6.59 Log_{10} CFU/mL (log mean of three replicates). Additionally, we used longer exposure times (0,1, 5, and 15 min) and none of our exposure times decreased the population of *Z. bailii* to below detectable levels even though a higher concentration of peroxyacetic acid was used. Hilgren and Salverda (2000) who used 80 mg/L claimed to have log reductions that ranged between 0.16 and 0.94 Log_{10} CFU/mL, whereas our log reduction at our longest exposure time (15 min exposure), with a higher concentration of peroxyacetic acid (120 mg/L) and with higher initial number of yeast cells (6.59 Log_{10} CFU/mL) was 4.36 Log_{10} CFU/mL, thus leaving 2.23 Log_{10} alive population (Fig. 1, Table I). This is interesting because the initial concentration of cells that Hilgren and Salverda (2000) used is similar to ours, yet they obtained a considerable reduction. However, other factors such as strain variability and associated resistance could be taken into account for these differing results. Regarding, *S. cerevisiae* strains, the log reduction was immediate using 120 mg/L of PAA for *S. cerevisiae* CE9 and CE81 with initial number of yeast cells of 6.09 and 6.15 Log_{10} CFU/mL respectively (Figure 2, Table II) and after 1 minute of exposure had no detectable levels of the strains. However, *S. cerevisiae* CE78 (Figure 2, Table II) with initial number of yeast cells of 6.64 Log_{10} CFU/mL did not show any reduction until 5 min of exposure. Baldry (1983) exposed *S. cerevisiae* to different concentrations of PAA, however different temperatures and pH were used (5.0, 6.5 and 8.0), *versus* our experiment, where pH was not a factor to control since only deionized water was used to prepare the PAA solutions that were added to the flasks (*in vitro* experiments) and where temperature was stable during the whole time of our experiment. Baldry (1983) found that the efficacy of PAA against two strains of *S. cerevisiae* decreases with increasing

pH, since when alkalinity of the solution increases, peracetic acid is hydrolyzed to form acetic acid and hydrogen peroxide (Yuan *et al.*, 1997). Accordingly, Baldry (1983) found that resistance among genera (particularly between *Z. bailii* and *S. cerevisiae*) is variable, this latter aspect also found in our experiments. However, as we found resistance is also variable among strains.

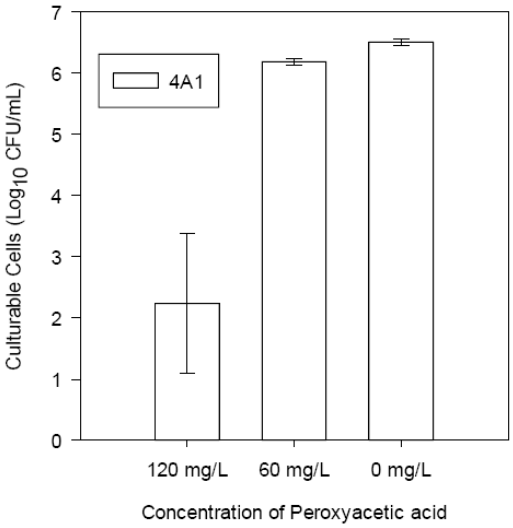

Figure 1. Efficacy of PAA on the reduction of suspended cells of *Z. bailii* at three different concentrations. Bars represent the standard error of the mean from triplicate experiments at the longest exposure time (15 min).

Eficácia de três concentrações diferentes de PAA na redução de células de Z. bailii em suspensão. As barras representam o erro padrão da média de ensaios em triplicado para o maior tempo de exposição (15 min).

Table I

Log Reduction (Log_{10} CFU/mL) of yeast by PAA at different times

Redução logarítmica (Log_{10} CFU/mL) da levedura pelo PAA nos diferentes tempos

Strain 4A1		
Time (min)	120 mg/L	60 mg/L
0	0	0
1	0.60	0.02
5	2.41	0.10
15	1.35	0.33

Average (n=3)

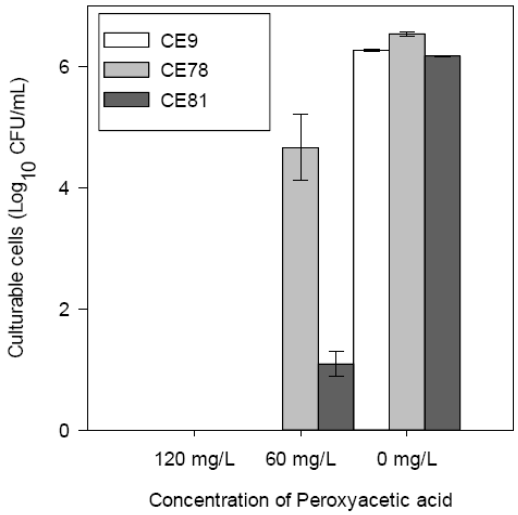

Figure 2. Efficacy of PAA on the reduction of suspended cells of *S. cerevisiae* strains at three different concentrations. Bars represent the standard error of the mean from triplicate experiments at the longest exposure time (15 min).

Eficácia de três concentrações diferentes de PAA na redução de células em suspensão de estirpes de S. cerevisiae. As barras representam o erro padrão da média de ensaios em triplicado para o maior tempo de exposição (15 min).

TABLE II

Log Reduction (Log_{10} CFU/mL) of yeast by PAA at different times

Redução logarítmica (Log_{10} CFU/mL) de leveduras pelo PAA nos diferentes tempos

Strain	CE9		CE81		CE78	
Time (min)	120 mg/L	60 mg/L	120 mg/L	60 mg/L	120 mg/L	60 mg/L
0	0	0	0	0	0	0
1	6.09	6.64	6.15	0.33	0.95	0.02
5	6.09	6.64	6.15	2.67	2.76	0.23
15	6.09	6.64	6.15	2.09	2.93	1.63

Average (n=3)

With regards *D./B. bruxellensis* strains (Figure 3, Table III) CE149, 2080 and CE261 the initial number of yeast cells were 6.73, 4.20 and 5.67 Log_{10} CFU/mL respectively, and the reduction was immediate when 120 mg/L was used. After 1 min exposure, no detectable levels of any of the three strains were found. Duarte *et al.* (2011) also studied the efficacy of PAA to sanitize stainless tanks that were in contact with naturally contaminated wine with populations of yeasts (including *Dekkera/Brettanomyces* yeasts), lactic acid and acetic acid bacteria (> 3000 CFU/mL). However, the PAA concentration used was 1000 mg/L, three times higher than the lower PAA

concentration recommended by the manufacturer in Portugal where this study was performed. PAA used at 1000 mg/L was highly effective in reducing levels of yeast to non-detectable. In the US, by contrast, according to EPA (www.epa.gov/fedrgstr/), up to 500 mg/L of PAA can be used in wineries. However, there are many different surfaces that need to be evaluated separately, and wood should be one of them.

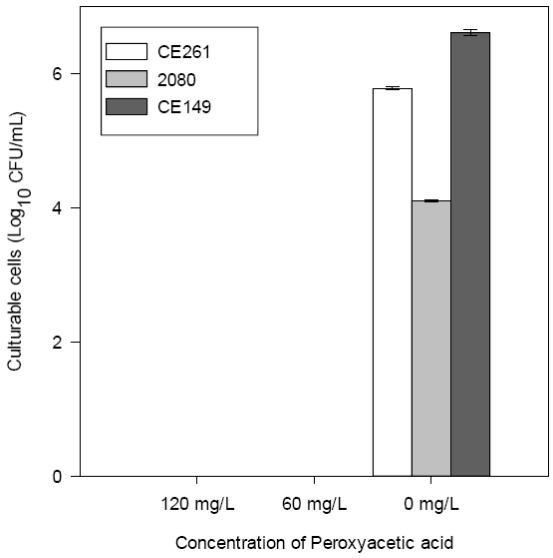

Figure 3. Efficacy of PAA on the reduction of suspended cells of *D./B. bruxellensis* strains at three different concentrations. Bars represent the standard error of the mean from triplicate experiments at the longest exposure time (15 min).

Eficácia de três concentrações diferentes de PAA na redução de células em suspensão de estirpes de D./B. bruxellensis. As barras representam o erro padrão da média de ensaios em triplicado para o maior tempo de exposição (15 min).

TABLE III

Log Reduction (Log_{10} CFU/mL) of yeast by PAA at different times

Redução logarítmica (Log_{10} CFU/mL) de leveduras pelo PAA nos diferentes tempos

Strain	**CE149**		**2080**		**CE261**	
Time (min)	120 mg/L	60 mg/L	120 mg/L	60 mg/L	120 mg/L	60 mg/L
0	0	0	0	0	0	5.68
1	6.73	6.55	4.20	5.21	5.67	2.17
5	6.73	6.55	4.20	5.21	5.67	3.04
15	6.73	6.55	4.20	5.21	5.67	0.47

Average (n=3) Log reduction

When 60 mg/L of PAA were used with *S. cerevisiae* strains (Figure 2, Table II), the initial number of yeast cells for CE9, CE81 and CE78 were 6.64, 6.18 and 6.54 Log_{10} CFU/mL respectively and only CE9 showed a log reduction that was below detectable levels right after the addition of PAA. However, CE81 and CE78 showed a maximum log reduction of 5.08 and 1.88 log10 CFU/mL, respectively (average of three replicates), after 15 min of exposure. These findings suggest that the log reduction for *S. cerevisiae* strains at 60 mg/L is strain and time dependent. In contrast, *Z. bailii* 4A1 (Figure 1, Table I) had an initial concentration of cells of 6.64 Log_{10} CFU/mL and it had a 0.45 log reduction (average of three replicates). Finally, for the *D./B. bruxellensis* strains (Figure 3, Table III), the initial number of yeast cells for CE149, 2080 and CE261 were 6.55, 5.21, 5.68 Log_{10} CFU/mL respectively. The reduction was almost immediate, with both CE149 and 2080 being reduced to non-detectable levels after 1 min exposure. However, *D./B. bruxellensis* CE261 was reduced to non-detectable levels only after 5 min of PAA exposure. This suggests that at this concentration for *D./B. bruxellensis* strains, the reduction is strain and time dependent, since the log reduction varied with time and the strain used.

The statistical analysis was performed doing multiple comparisons where only two factors that included strain and concentration were evaluated.

Z. bailii 4A1 showed significant differences at a concentration of 120 and 60 mg/L of the sanitizer in comparison with all the strains (Table IV). This is, in fact, expected since our results showed that *Z. bailii* 4A1 was highly resistant to the 60 and 120 mg/L PAA concentrations, whereas, the other strains showed more susceptibility to the sanitizer. At 120 mg/L of PAA only *Z. bailii* 4A1 survived. It showed significant differences at all comparison concentrations (120 mg/L *vs* 0 mg/L and 120 *vs* 60 mg/L) except for 60 mg/l *vs* 0 mg/L (Table V). *S. cerevisiae* CE81 and CE78 showed significant differences at all concentration comparisons (120 mg/L *vs* 0 mg/L, 120 mg/L *vs* 60 mg/L and 60 mg/l *vs* 0 mg/L) (Table V). *S. cerevisiae* CE9 also showed significant differences when concentration comparisons were done except for the comparison 120 mg/L *vs* 60 mg/L (Table V). The statistical analysis for all *S. cerevisiae* and *D./B. bruxellensis* strains did not show any significant differences when comparisons where performed between them at a concentration of 120 mg/L (Table IV). However, when concentration comparisons where done in each of the three *D./B. bruxellensis* strains significant differences were found between 120 mg/L *vs* 0 mg/L and 60 mg/l *vs* 0 mg/L except for the comparison 120 mg/L *vs* 60 mg/L (Table V).

TABLE IV

Comparison among strains with regards the effects of PAA

Comparação entre estirpes relativamente aos efeitos do PAA

	120 mg/L	60 mg/L
Comparison	**p<0.05**	**p<0.05**
CE149 vs. 4A1	yes	yes
CE261 vs. 4A1	yes	yes
CE78 vs. 4A1	yes	yes
CE81 vs. 4A1	yes	yes
CE9 vs. 4A1	yes	yes
2080.000 vs. 4A1	yes	yes
CE78 vs. CE149	no	yes
CE149 vs. 2080.000	no	no
CE81 vs. 2080.000	no	yes
CE261 vs. CE149	no	no
CE261 vs. 2080.000	no	no
CE81 vs. CE149	no	yes
CE78 vs. CE261	no	yes
CE9 vs. CE78	no	yes
CE81 vs. CE9	no	yes
CE78 vs. 2080.000	no	yes
CE81 vs. CE78	no	yes
CE81 vs. CE261	no	no
CE9 vs. CE261	no	no
CE9 vs. CE149	no	no
CE9 vs. 2080.000	no	no

Brettanomyces/*Dekkera bruxellensis* isolates (CE261, 2080, CE149); *Saccharomyces cerevisiae* isolates (CE81, CE9 and CE78); *Zygosaccharomyces bailii* isolate (4A1).

When 60 mg/L of peroxyacetic acid was used, the comparisons among strains showed that only 7 comparisons out of 21 did not show statistical significance ($p < 0.05$) (Table IV). The Control (0 mg/L) had no statistical differences among all the possible comparisons of the strains.

In vivo Reduction of *Dekkera*/*Brettanomyces* and general yeast populations in natural contaminated barrels

The concentration that showed a higher log reduction *in vitro* conditions (120 mg/L), was used in the *in vivo* experiments. Moreover, a higher concentration (200 mg/L) was also used since as it is expected the porosity of the wood can diminish the efficacy of the sanitizer.

PAA at 200 mg/L and a contact time of one week decreased levels of *Dekkera/Brettanomyces* and general yeast populations, below detectable levels (Table VI and VII). However, when the concentration of PAA was reduced to 120 mg/L and contact time of 15 minutes, detectable levels of both yeast populations were present. The statistical analysis was performed using a Fisher's exact test where the response in terms of reduction is recorded as either yes or no. Statistical differences were found between the 15 min and one week treatment for both *Dekkera/Brettanomyces* and general yeast populations, with $p=0.011$ and $p \leq 0.001$ respectively. This means that the proportions of sanitation efficacy is not the same in the 15 min treatment and in the one week treatment. The one week treatment (200 mg/L) is more effective than the 15 min treatment (120 mg/L).

Table V

Concentration comparisons at strain level

Comparação das concentrações ao nível da estirpe

Strain	Comparison	Diff of means	t	p	p<0.05
4A1	120 vs. 0.000	0.65	10.727	<0.001	yes
	60.000 vs. 0.000	0.0558	0.921	0.362	no
	120.000 vs. 60.000	0.594	9.806	<0.001	yes
CE81	120.000 vs. 0.000	0.994	16.414	<0.001	yes
	60.000 vs. 0.000	0.817	13.487	<0.001	yes
	120.000 vs. 60.000	0.177	2.927	0.005	yes
CE9	120.000 vs. 0.000	0.982	16.222	<0.001	yes
	60.000 vs. 0.000	0.982	16.222	<0.001	yes
	120.000 vs. 60.000	0	0	1	no
CE78	120.000 vs. 0.000	0.978	16.152	<0.001	yes
	60.000 vs. 0.000	0.266	4.385	<0.001	yes
	120.000 vs. 60.000	0.712	11.766	<0.001	yes

Brettanomyces/*Dekkera bruxellensis* isolates (CE261, 2080, CE149); *Saccharomyces cerevisiae* isolates (CE81, CE9 and CE78); *Zygosaccharomyces bailii* isolate (4A1).

Table V (continuation)

Concentration comparisons at strain level

Comparação das concentrações ao nível da estirpe

Strain	Comparison	Diff of means	t	p	p<0.05
CE261	120.000 vs. 0.000	0.988	16.31	<0.001	yes
	60.000 vs. 0.000	0.988	16.31	<0.001	yes
	120.000 vs. 60.000	0	0	1	no
CE149	120.000 vs. 0.000	0.993	16.397	<0.001	yes
	60.000 vs. 0.000	0.993	16.397	<0.001	yes
	120.000 vs. 60.000	0	0	1	no
2080	120.000 vs. 0.000	0.987	16.294	<0.001	yes
	60.000 vs. 0.000	0.987	16.294	<0.001	yes
	120.000 vs. 60.000	0	0	1	no

Brettanomyces/*Dekkera bruxellensis* isolates (CE261, 2080, CE149); *Saccharomyces cerevisiae* isolates (CE81, CE9 and CE78); *Zygosaccharomyces bailii* isolate (4A1).

Table VI

Dekkera/Brettanomyces populations pre-treatment and post-treatment with PAA

População de Dekkera/Brettanomyces antes e após tratamento com PAA

Barrel	**Initial** (Log_{10})	**Final** (Log_{10})	**Reduction** (Log_{10})	**Time** (min or weeks)	**Concentration** (mg/L)
4A1F125001	3.60	2.13	1.47	15 min	120
4A1F125002	5.59	2.34	3.25	15 min	120
4AD9120915	2.90	1.80	1.10	15 min	120
4AD9120916	ND[a]	ND[a]	ND[a]	15 min	120
4ALL119189	6.37	1.36	5.00	15 min	120
4ALL119188	4.00	1.90	2.09	15 min	120
4A1M124748	4.04	1.90	2.14	15 min	120
4A1M124747	3.60	1.90	1.70	15 min	120
4ALL119242	4.06	0.60	3.46	15 min	120
4ALL119243	3.90	0	3.90	15 min	120
4A1M125066	6.49	0	6.49	1 WEEK	200
4A1M125069	4.06	0	4.06	1 WEEK	200
4AD9120925	4.18	0	4.18	1 WEEK	200
4AD9120926	3.90	0	3.90	1 WEEK	200
4AD9120922	4.44	0	4.44	1 WEEK	200
4AD9120921	4.28	0	4.28	1 WEEK	200
4AD9120919	3.20	0	3.20	1 WEEK	200
4AD9120920	3.60	0	3.60	1 WEEK	200
4AD9120720	1.90	0	1.90	1 WEEK	200
4AD9120721	4.05	0	4.05	1 WEEK	200

[a]ND No detected

Table VII

General yeast populations pre-treatment and post-treatment with PAA

Populações de leveduras genéricas antes e após tratamento com PAA

Barrel	**Initial** (Log_{10})	**Final** (Log_{10})	**Reduction** (Log_{10})	**Time** (min or weeks)	**Concentration** (mg/L)
4A1F125001	3.20	0.60	2.60	15 min	120
4A1F125002	4.57	0.48	4.10	15 min	120
4AD9120915	2.90	1.48	1.43	15 min	120
4AD9120916	1.60	0.70	0.90	15 min	120
4ALL119189	4.57	0.00	4.57	15 min	120
4ALL119188	4.12	1.18	2.94	15 min	120
4A1M124748	7.30	1.76	5.54	15 min	120
4A1M124747	3.50	0.00	3.50	15 min	120
4ALL119242	7.36	1.90	5.46	15 min	120
4ALL119243	3.54	1.90	1.64	15 min	120
4A1M125066	8.30	0	8.30	1 week	200
4A1M125069	3.73	0	3.73	1 week	200
4AD9120925	3.20	0	3.20	1 week	200
4AD9120926	3.85	0	3.85	1 week	200
4AD9120922	4.38	0	4.38	1 week	200
4AD9120921	5.26	0	5.26	1 week	200
4AD9120919	2.73	0	2.73	1 week	200
4AD9120920	3.60	0	3.60	1 week	200
4AD9120720	2.41	0	2.41	1 week	200
4AD9120721	3.04	0	3.04	1 week	200

CONCLUSIONS

Effective methods to sanitize wine barrels and other common surfaces in wineries are required due to the high replacement cost of barrels for the wine industry. Our study has demonstrated that PAA is effective to decontaminate wine cooperage. The use of the appropriate concentration of sanitizers must adhere to food regulations, even though higher concentrations could provide improved efficacy. Different surfaces in wineries must be assessed for the best sanitation protocols, since not all surfaces will be sanitized with the same concentrations and contact time. Moreover, autochthonous microbiota should also be taken into account when protocols of sanitation are validated, since microorganisms may present different levels of sensitivity to the common sanitizers used in wine industry.

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