

Application of Heated Water to Reduce Populations of *Brettanomyces bruxellensis* Present in Oak Barrel Staves

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Submitted for publication: June 2018

Accepted for publication: October 2018

Key words: *Brettanomyces bruxellensis*, oak staves, heated water, spoilage

New 16 L and three-year-old commercial 225 L barrels representing French and American oaks of different toasting levels, contaminated with *Brettanomyces bruxellensis*, were obtained. Center sections of individual staves were sawn into 3 x 3 cm cubes and submerged 2 mm into heated water at 50°C, 60°C, 70°C, or 80°C. Following heat treatment, cross sections and/or shavings were collected and transferred into a yeast recovery medium for incubation for ≥ 60 days. Culturable cells were not recovered from cubes heated in water at 70°C for 20 minutes, or 80°C for 15 minutes, when the yeast was present in oak at depths of ≤ 4 mm. However, longer heating times (70°C for 30 min or 80°C for 20 min) were required if *B. bruxellensis* was present at depths of 5 to 9 mm within cubes made from staves. Based on these results, heating water to at least 70°C for a minimum of 30 minutes is recommended to reduce risk of wine spoilage by barrels contaminated with *B. bruxellensis*.

INTRODUCTION

Contaminated oak barrels are well-known to contribute to the spread of the yeast *Brettanomyces bruxellensis* within wineries (Chatonnet *et al.*, 1992; Fugelsang & Edwards 2007). To minimise the potential for spoilage, various sanitation methods for barrels have been evaluated, including microwaves (González-Arenzana *et al.*, 2013), ozone (Guzzon *et al.*, 2011; 2013) and high power ultrasonic waves (Yap *et al.*, 2008; Schmid *et al.*, 2011). However, culturable yeasts have been recovered from 8 mm deep within staves (Malfeito-Ferreira *et al.*, 2004; Barata *et al.*, 2013; Cartwright *et al.*, 2018), a depth beyond penetration by these methods.

As such, alternative approaches to these methods, such as using heat (*e.g.* steam or hot water) have been more widely adopted by the wine industry. In fact, *B. bruxellensis* can be inactivated even at mildly elevated temperatures, beginning at approximately 50°C (Couto *et al.*, 2005; Fabrizio *et al.*, 2015). More recently, Cartwright *et al.* (2018) reported that steaming staves for 9 to 12 minutes eliminated yeast populations, depending on oak species and toasting level. Even though evidence does not currently exist, application of commercial steaming units could result in uneven heating or “cold spots” within barrels, thereby not completely inactivating viable cells.

Compared to steam, filling and/or submersion with heated water may, in fact, more reliably reduce microbial populations by attaining uniform temperatures throughout the barrel. Of the few studies available, Fabrizio *et al.* (2015) noted that immersing barrels for extended periods of time in

heated water significantly reduced resident *B. bruxellensis* populations. However, this study analysed rinse water from barrels to evaluate efficacy of heated water treatments. Even though *B. bruxellensis* can deeply penetrate staves, there are areas where cells will not be removed through rinsing. In fact, the yeast can survive application of steam to the surface of wood if present at depths of 5 to 9 mm (Cartwright *et al.*, 2018).

The objective of this research was to determine the impact of heated water on the recovery of *B. bruxellensis* from oak staves.

MATERIAL AND METHODS

Cultures and barrels

Strains of *B. bruxellensis* I1a and E1 (Jensen *et al.*, 2009), media used for starter culture preparation and recovery (WRM and EBB), as well as sources of barrels were described by Cartwright *et al.* (2018). Briefly, a commercially prepared Cabernet Sauvignon wine (pH 3.39, 6.09 g/L titratable acidity as tartaric acid, 13.5% v/v alcohol, and 18 mg/L free SO₂) was added to 16 L new American or French oak barrels with light or heavy toast levels prior to inoculation, with *B. bruxellensis* strain I1a or E1, with an incubation period of several weeks. Additionally, four 225 L three-year-old commercial barrels infected with an unidentified *B. bruxellensis* strain(s) were obtained from a regional winery.

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Acknowledgments: The authors gratefully acknowledge the Washington State Grape and Wine Research Program, the Ivory Tower Scholarship Fund, regional and national wineries and the School of Food Science at Washington State University for financial and material support

Heated water or wine treatments

Center portions of side staves from disassembled barrels were sawn into 3 x 3 cm cubes. A stainless steel plate (0.3 cm x 28 cm x 43 cm), fitted with bottomless circular chambers (4 cm diameter x 2 cm depth), was manufactured locally and used to hold 24 individual cubes immersed approximately 2 mm into heated water. The loaded plate was placed into 4 L of circulating distilled water held at 50°C, 60°C, 70°C, or 80°C. During heat application, a steel cover was overlaid to prevent movement of the stave cubes. Selected cubes were fitted with thermocouples (Omega Engineering, Stamford, CT) installed into 1.0 mm diameter holes and were centrally located at varying depths 1.5 cm into each block. Each experiment was conducted twice, with three cubes removed at each designated time and the water changed each time. After heating, cubes were sawn into 4 mm thick horizontal cross-sections and individually placed into sterile 100 mL sampling bags (Nasco, Salina, CA). Sterilized wine recovery medium (WRM, 40 mL) was added to all bags, which were then incubated at 27°C for at least 60 days.

Decimal reduction times at a given temperature to kill 1 log of yeast (D_T) were estimated by heating 3 x 3 cubes obtained from heavy toasted 16 L barrels (French oak) in a water bath. The heated cubes were immediately placed in sterile bags and allowed to cool to room temperature for at least 24 hours. Wood shavings were then prepared using a sterilized 2.5 cm Forstner drill bit, which bored 2 mm holes in increments. The shavings were collected and transferred into 25 mL EBB medium, to incubation at 20°C with shaking (100 rpm) for 12 hours to remove cells from oak before determination of yeast culturability.

All equipment used (table and band-saws, Forstner drill bits, heating plate, etc.) were regularly sanitized using 70% v/v ethanol.

Analyses

Culturability was determined using both manual and spiral plate methods (Autoplate 4000 spiral plater, Spiral Biotech, Bethesda, MD), and Wallerstein Differential medium (Difco, Detroit, MI). After incubation at 27°C for six days, colonies were counted and tentatively identified based on physical appearance as well as cellular morphologies viewed with phase-contrast microscopy. Identification of randomly selected colonies (three per barrel) was later confirmed using genetic ITS sequencing (MIDI Labs, Newark, DE).

Chemical analyses (pH, titratable acidity and SO_2) were performed by standardized methods (Edwards & Watson, 2013); 4-ethylphenol (4-EP) and 4-ethylguaiacol (4-EG) by gas chromatography-mass spectroscopy (Jensen *et al.*, 2009); ethanol using an ebulliometer (Alla, Chemillé, France).

Analysis of variance (ANOVA) and Tukey's HSD for mean separation was determined at $p \leq 0.05$ using XLSTAT software (Addinsoft, New York, NY).

RESULTS AND DISCUSSION

Heating staves

Increases in temperature of the 3 x 3 cubes at different layers during water heating depended primarily on stave depth, not on oak species or the toasting level. In fact, since American and French oak from 16 L barrels heated almost identically ($p \leq 0.05$), data were pooled to further assess heating profiles within staves (Fig. 1). Typically, innermost surfaces directly in contact with water reached the treatment temperature within ≤ 5 minutes, while furthestmost surfaces (≥ 20 mm) remained below 30°C, similar to the observations regarding 225 L barrels (data not shown). At 9.5 mm, a stave depth slightly beyond where *B. bruxellensis* has been recovered (Malfeito-Ferreira *et al.*, 2004; Barata *et al.*, 2013; Cartwright *et al.*, 2018), heated treatments of 50°C (Fig. 1A) or 60°C (Fig. 1B)

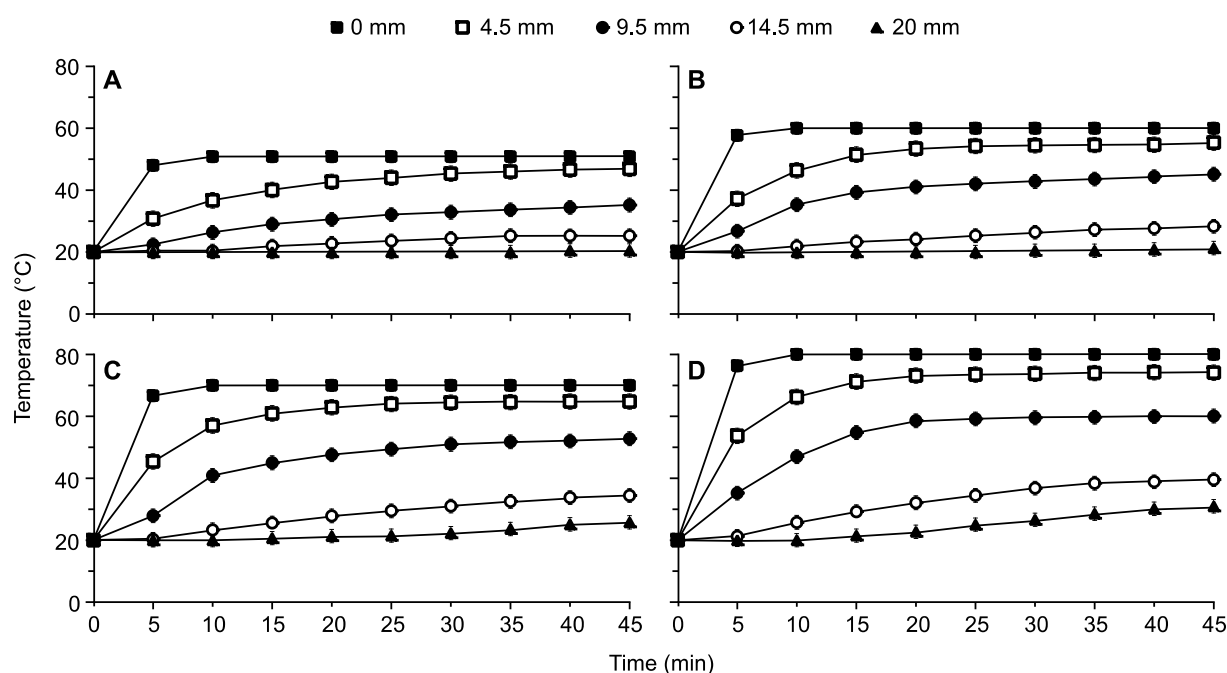


FIGURE 1

Heat penetration at different layers into French/American oak staves from 16 L barrels (pooled data), held at a depth of 2 mm in circulating water at 50°C (A), 60°C (B), 70°C (C), or 80°C (D). Data represent means \pm standard error (n=8).

resulted in maximum temperatures of 35° or 47°C after 45 minutes, respectively. However, shorter times of 25 minutes or 15 minutes were required at 70° (Fig. 1C) or 80°C (Fig. 1D), respectively, in order to reach $\geq 50^\circ\text{C}$. Cubes never attained $\geq 60^\circ\text{C}$ at 9.5 mm depths within 45 minutes. Temperatures higher than those have been reported by Cartwright *et al.* (2018) for the same oak staves, but were heated by steam.

Yeast recoveries from heated staves

B. bruxellensis strain I1a was found in the 5 mm to 9 mm layer of heavy toasted 16 L barrels made from French oak, but not in the same layer within the light or heavy toasted American oak (Fig. 2 to 5). These analyses were in agreement with observations of 16 L barrels as reported by Cartwright *et al.* (2018), where the two types of oak had different permeabilities to *B. bruxellensis*, yet similar thermal conductivities.

In general, as the temperature of heated water increased, shorter treatment periods were required to eliminate culturable cells. Application of 50°C (Fig. 2) resulted in elimination of the yeast after 60 minutes from the 0 mm to 4 mm layer of American oak, but required 120 minutes for the French oak due to the presence of *B. bruxellensis* in the 5 mm to 9 mm layer. After 30 minutes of heating American oak, 15 days of incubation in WRM was required to detect *Brettanomyces* populations. An incubation period of 15 days implied that the populations were low but still culturable after water heating. In comparison, 30 days of incubation was needed to observe viable cells from staves treated for 60 minutes for French oak. For French oak treated with water at 60°C (Fig. 3), 70°C (Fig. 4) or 80°C (Fig. 5), the times

needed to eradicate the yeast decreased from 45, 30, to 20 minutes, respectively. At these same temperatures, yeast recovery was not observed from the American oak after 30, 20, or 15 minutes, respectively. While these data were based on heavy toasted staves, similar trends were observed for the light toasted French and American oak (data not shown).

D_T values for *B. bruxellensis* strains I1a (Fig. 6A) or E1 (Fig. 6B) were determined using cubes prepared from 16 L heavy toasted French oak staves. From initial populations of approximately 6×10^3 cfu/g, culturabilities were monitored over time within the 0 mm to 2 mm layer immersed in the heated water. Based on slopes of best fit lines ($r^2 \geq 0.88$), $D_{50^\circ\text{C}}$, $D_{60^\circ\text{C}}$, $D_{70^\circ\text{C}}$ and $D_{80^\circ\text{C}}$ were estimated to be 223 sec, 50 sec, 20 sec, and 11 sec for strain I1a (Fig. 6A), values similar to that of strain E1 (Fig. 6B).

To date, the limited studies available regarding thermal inactivation of *B. bruxellensis* relied upon liquid cultures, not on oak staves as described in the current research. Couto *et al.* (2005) noted $D_{50^\circ\text{C}}$ to be 204 to 228 sec for strain PYCC 4801, in agreement with strains I1a and E1 (Fig. 6). Using liquid cultures, Couto *et al.* (2005) observed $D_{55^\circ\text{C}}$ of a single strain of *B. bruxellensis* to vary between 18 to 24 sec whereas Fabrizio *et al.* (2015) found $D_{60^\circ\text{C}}$ values to range from 19.5 to 20.7 sec. Furthermore, Fabrizio *et al.* (2015) noted that three strains behaved similarly, while the fourth exhibited a slightly greater heat resistance. In general, the D_T values reported by Couto *et al.* (2005) and Fabrizio *et al.* (2015) at temperatures $>50^\circ\text{C}$ were lower than those obtained with staves, possibly due to matrix effects, as oak exhibits a lower thermal conductivity (Lagüela *et al.*, 2015) compared to

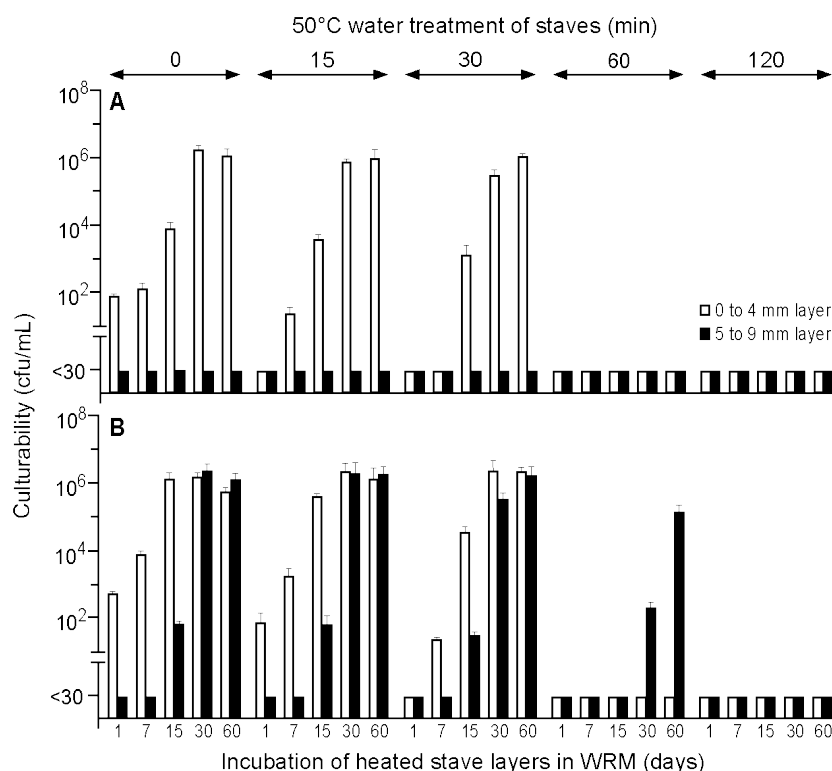


FIGURE 2

Culturabilities of *B. bruxellensis* strain I1a recovered from heavy toasted American (A) or French (B) 16 L oak barrels following 50°C water treatments. Data represent means \pm standard error ($n=6$).

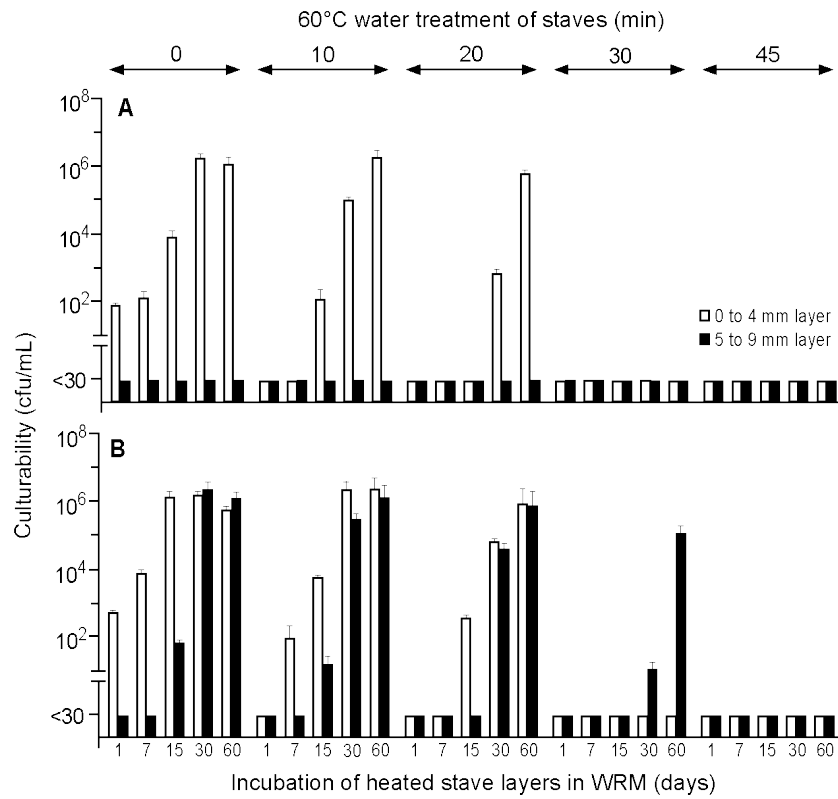


FIGURE 3

Culturabilities of *B. bruxellensis* strain 11a recovered from heavy toasted American (A) or French (B) 16 L oak barrels following 60°C water treatments. Data represent means \pm standard error (n=6).

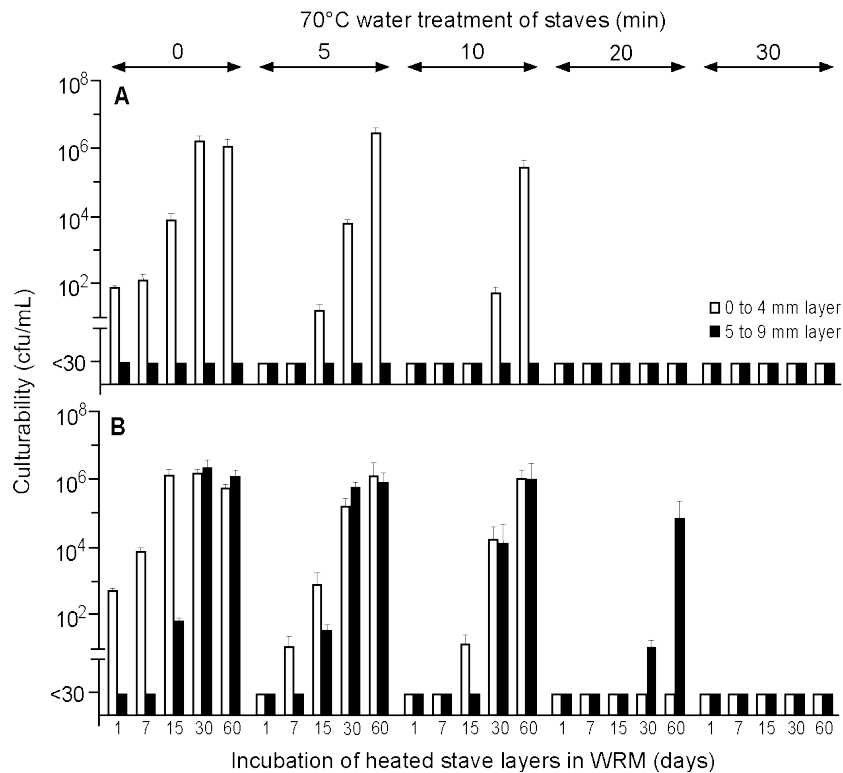


FIGURE 4

Culturabilities of *B. bruxellensis* strain 11a recovered from heavy toasted American (A) or French (B) 16 L oak barrels following 70°C water treatments. Data represent means \pm standard error (n=6).

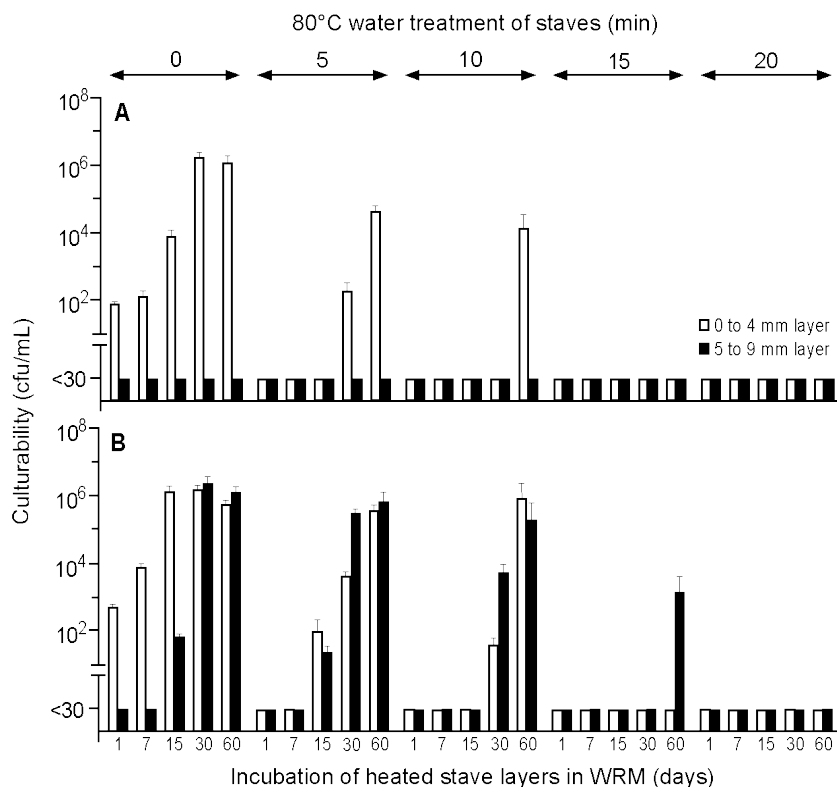


FIGURE 5

Culturabilities of *B. bruxellensis* strain I1a recovered from heavy toasted American (A) or French (B) 16 L oak barrels following 80°C water treatments. Data represent means \pm standard error (n=6).

heating liquid cultures. In addition, heat resistance may vary between different strains of *B. bruxellensis* as suggested by Fabrizio *et al.* (2015).

Given differences in dimensions (*i.e.*, stave thickness) and age compared to the 16 L barrels, cubes were sawn from 225 L barrel staves and exposed to heated water using previously described protocols (Fig. 7 to 10). Unlike the 16 L barrels, culturable cells were recovered from 5 mm to 9 mm layers from American oak barrels, most likely because these had been commercially used for three years, allowing slow but deeper penetration of the yeast (Cartwright *et al.*, 2018). Like the 16 L barrels, however, yeast recovery was largely affected by water temperature and time of heat exposure. For example, culturable cells were recovered from the 5 mm to 9 mm layer, but not the 0 to 4 mm for both American and French oaks after 60 minutes of heating at 50°C (Fig. 7). Elimination of *B. bruxellensis* from these staves was noted after 120 minutes of heating. As the temperature of water was increased, the heating time, which resulted in no recoverable cells from the 5 mm to 9 mm stave layer, also decreased from 45 minutes (60°C; Fig. 8) to 30 minutes (70°C; Fig. 9), and then to 20 minutes (80°C; Fig. 10). Overall, inactivation of the unidentified strain(s) that was present in the 225 L barrels followed the same trends as those observed in the 16 L barrels infected with another strain of *B. bruxellensis*.

Even though some studies concluded that heat treatments did not adequately remove *B. bruxellensis* from staves, others disagreed. For instance, Barata *et al.* (2013) concluded that steam treatments of 10 minutes for French

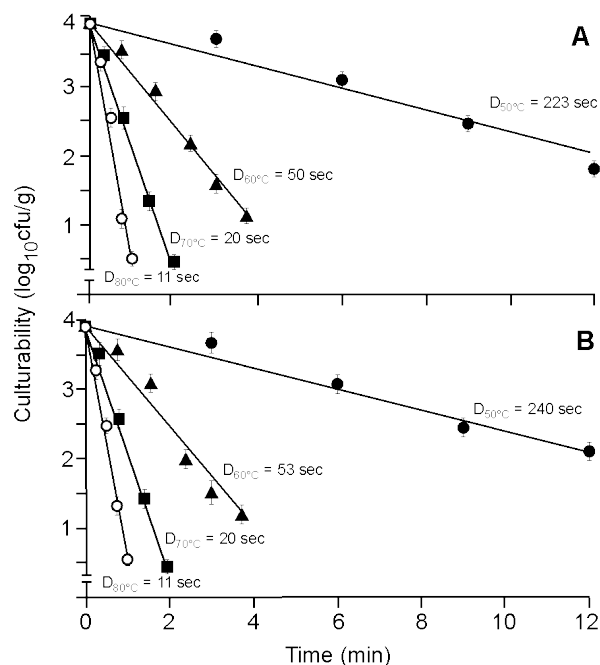


FIGURE 6

D-values for *B. bruxellensis* strain I1a (A) or E1 (B) recovered from the first 2 mm depths of heavy toasted 16 L French oak barrels following heated water treatments. Data represent means \pm standard error (n=3).

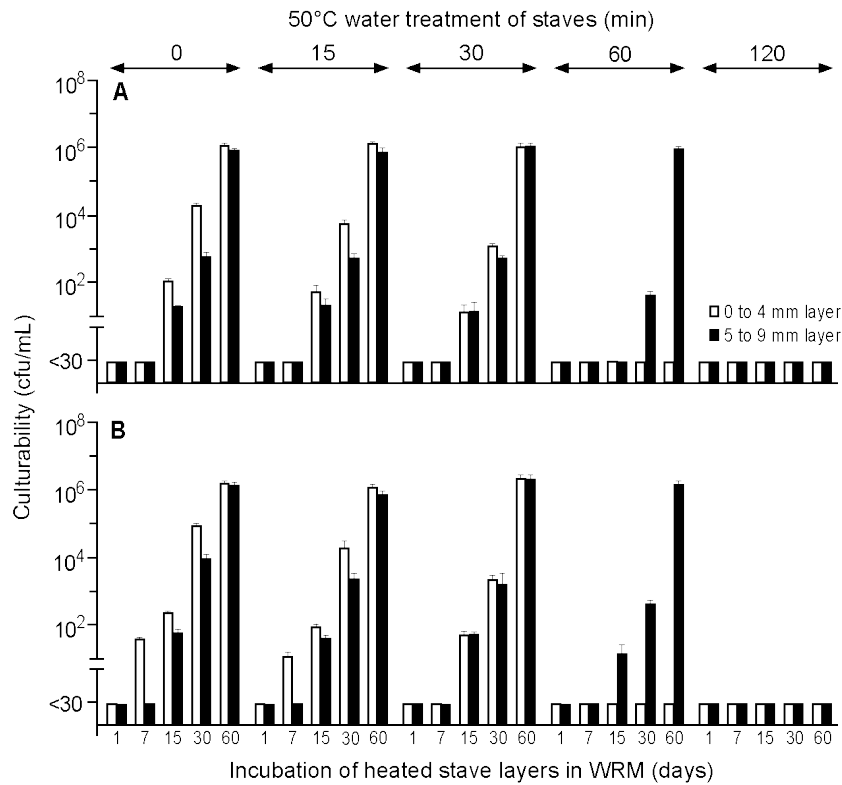


FIGURE 7

Culturabilities of an unidentified strain(s) of *B. bruxellensis* recovered from medium-heavy toasted American (A) or medium-light toasted French (B) 225 L oak barrels following treatment with 50°C water. Data represent means \pm standard error (n=6).

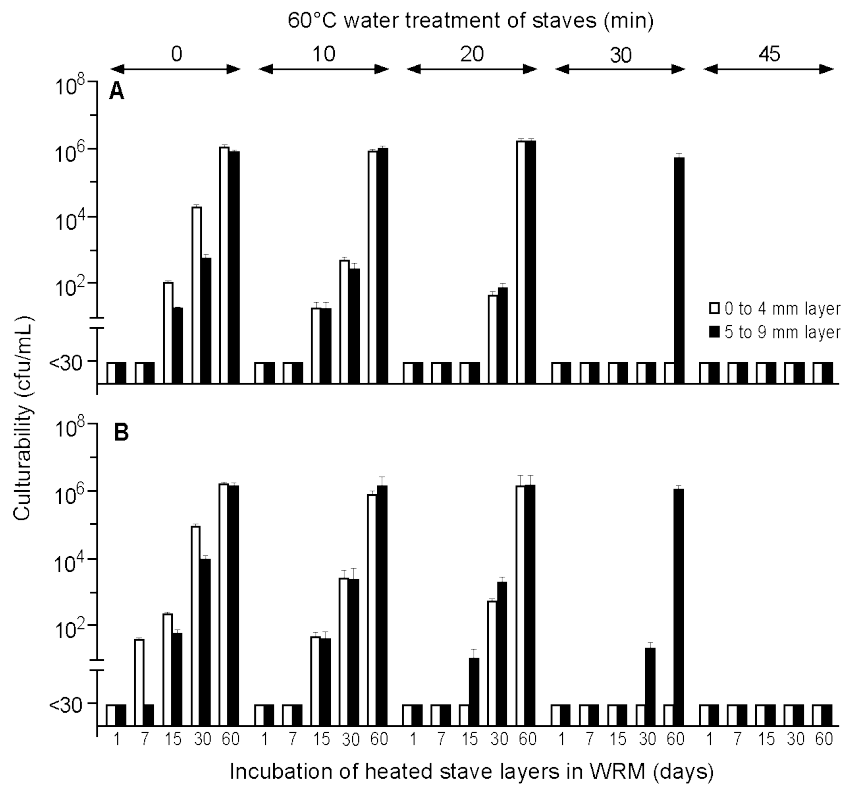


FIGURE 8

Culturabilities of an unidentified strain(s) of *B. bruxellensis* recovered from medium-heavy toasted American (A) or medium-light toasted French (B) 225 L oak barrels following treatment with 60°C water. Data represent means \pm standard error (n=6).

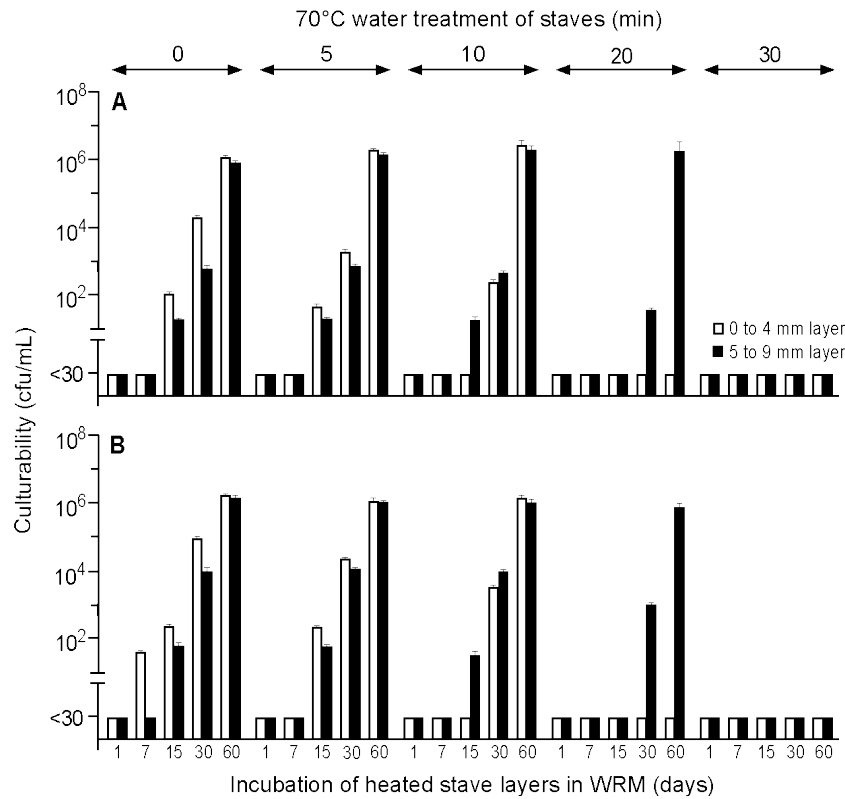


FIGURE 9

Culturabilities of an unidentified strain(s) of *B. bruxellensis* recovered from medium-heavy toasted American (A) or medium-light toasted French (B) 225 L oak barrels following treatments with 70°C water. Data represent means \pm standard error (n=6).

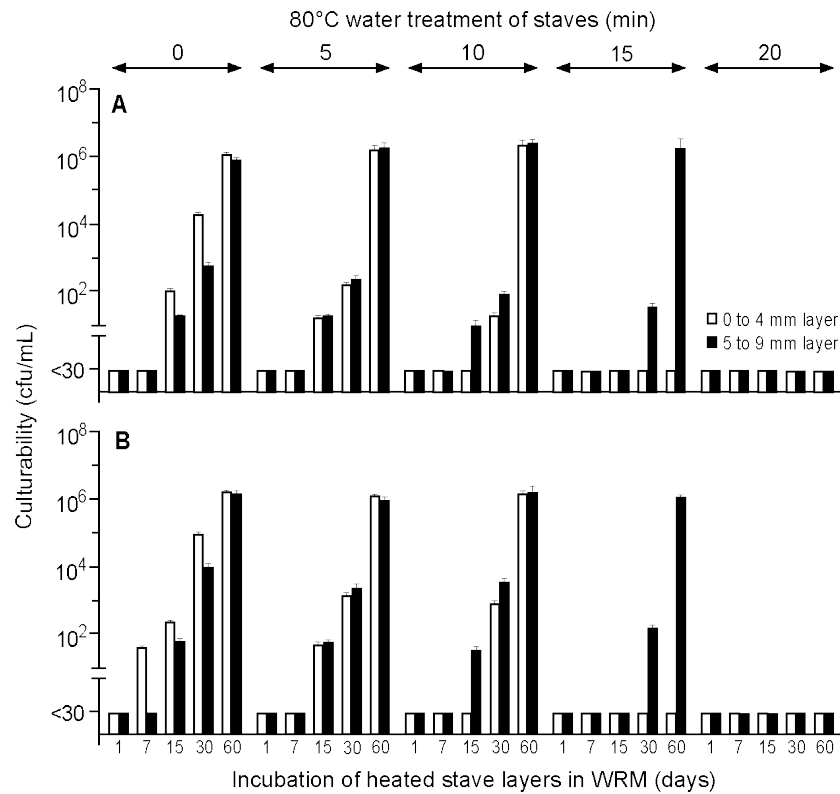


FIGURE 10

Culturabilities of an unidentified strain(s) of *B. bruxellensis* recovered from medium-heavy toasted American (A) or medium-light toasted French (B) 225 L oak barrels following treatments with 80°C water. Data represent means \pm standard error (n=6).

oak were not effective, since the yeast could still be cultured from staves. However, this steam treatment may have been too short in time, as Cartwright *et al.* (2018) noted that 12 minutes were needed to eliminate viable cells up to an 8 mm depth in contaminated French oak barrels. Although hot water rinsing (60°C) had limited impacts on oak flavour extraction, Schmid *et al.* (2011) concluded that this approach would not adequately sanitize barrels due to the underlying porous nature of oak. Rather, soaking barrels in hot water for extended periods of time appear to be effective against *B. bruxellensis*. Even though Fabrizio *et al.* (2015) reported 7-log reductions in *B. bruxellensis* populations present in staves up to 8 mm using 60°C water for 19 minutes, the current study found that higher temperatures and longer treatment times were required to prevent yeast recovery.

Volatile phenol production

Upon incubation of stave layers or shavings in WRM, recovered *B. bruxellensis* continued to produce 4-EP and 4-EG (data not shown). Based on analysis of staves from 16 L barrels, concentrations surpassed 2000 µg/L 4-EP and 300 µg/L 4-EG for strains I1a and E1, where populations exceeded 10⁶ cfu/mL after 60 days. The concentrations produced by these strains were similar to those reported by Oswald & Edwards (2017) using a different wine. Even though similar populations were reached, the unidentified strain(s) originating from 225 L barrels produced lower concentrations of volatile phenols; ≤1300 4-EP and 180 µg/L 4-EG. Neither 4-EP or 4-EG were detected in WRM, where populations remained below the limit of detection; <30 cfu/mL.

CONCLUSIONS

This study evaluated the efficacy of heated water and wine treatments towards the removal of *B. bruxellensis* from a wide range of infected barrels. Water heated to 70°C or 80°C eradicated the yeast if present at depths of 0 mm to 4 mm (20 or 15 minutes, respectively) or 5 mm to 9 mm (30 or 20 minutes, respectively), evidenced by a lack of culturable cells – even after incubation of stave cross-sections in a wine recovery medium for 60 days. Future research should focus on application of heated water on entire barrels, including any potential changes to the sensory impacts of treated barrels.

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