Effect of malolactic fermentation and ageing on the concentration of ρ-coumaric acid of Pinot Noir wine and the consequence for volatile phenol production by *Brettanomyces*

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Abstract

Background and Aims: This study compared changes in the concentration of ρ-coumaric acid in wine because of enzymatic hydrolysis of ρ-coutaric acid by *Oenococcus oeni* with that of chemical hydrolysis during ageing.
Methods and Results: Malolactic fermentation of a Pinot Noir wine with a cinnamoyl esterase-positive (CE+) *O. oeni* strain resulted in a significant increase in ρ-coumaric acid concentration. No significant change in ρ-coumaric acid concentration occurred during ageing regardless of wine pH, ethanol concentration or storage temperature. *Brettanomyces bruxellensis* produced a significantly higher concentration of volatile phenols in wines that underwent malolactic fermentation with the CE (+) *O. oeni* strain than with the CE (−) strain.
Conclusions: The *O. oeni* strain had significantly more impact on wine ρ-coumaric acid concentration than chemical hydrolysis during ageing.
Significance of the Study: The use of CE (+) *O. oeni* strains should be avoided in wines at risk of *Brettanomyces* spoilage because of an increase in the concentration of the volatile phenol precursor, ρ-coumaric acid.

Keywords: Brettanomyces bruxellensis, *cinnamoyl esterase,* Oenococcus oeni, *wine ageing,* ρ-*coumaric acid*

Introduction

Brettanomyces bruxellensis is a significant wine spoilage yeast encountered during winemaking and it can cause significant financial losses (Loureiro and Malfeito-Ferreira 2003, Puig et al. 2011). Wine spoilage by *B. bruxellensis* is primarily caused by production of ethylphenols that impart medicinal [4-ethylphenol (4-EP)] or smoky/clove [4-ethylguaiacol (4-EG)] characters. 4-Ethylphenol and 4-EG are produced from the hydroxycinnamic acids, ρ-coumaric acid and ferulic acid, respectively, through a two-step enzymatic process (Chatonnet et al. 1992). ρ-Coumaric acid and ferulic acid are present naturally in grapes and wine, and are typically found as esters of tartaric acid (ρ-coutaric and fetaric acids, respectively) (Nagel and Wulf 1979). While *B. bruxellensis* can metabolise ρ-coumaric and ferulic acids, it cannot metabolise the esterified forms, ρ-coutaric and fetaric acids (Schopp et al. 2013). Because ρ-coumaric and ferulic acids are often present in wine in an esterified form, hydrolysis of ρ-coutaric and fetaric acids to release ρ-coumaric and ferulic acids could result in higher 4-EP and 4-EG production by *B. bruxellensis*.

During winemaking, ρ-coutaric and fetaric acids can be hydrolysed because of enzymatic action or chemical hydrolysis (Nagel et al. 1979, Chescheir et al. 2015). Chemical hydrolysis typically occurs slowly during ageing (Nagel et al. 1979), while enzymatic hydrolysis occurs more rapidly (Chescheir et al. 2015). Enzymatic hydrolysis can occur because of the addition of cinnamoyl esterase (CE) used in some enzyme products or through the action of an *Oenococcus oeni* strain that possesses CE activity (Chescheir et al. 2015). While commercial enzyme preparations containing CE can be avoided, MLF with *O. oeni* is a key process during red winemaking.

The relative contribution of enzymatic hydrolysis by *O. oeni* during MLF and chemical hydrolysis during ageing to *Brettanomyces* wine spoilage has not been established. Chescheir et al. (2015) reported significantly higher volatile phenol production by *B. bruxellensis* in wines that underwent MLF with a CE-positive (CE+) *O. oeni* strain, compared to a CE-negative (CE−) strain. In contrast, Madsen et al. (2017) noted that during the ageing of Cabernet Sauvignon wine over 180 days, hydrolysis of ρ-coutaric acid to ρ-coumaric acid occurred such that *B. bruxellensis* produced a relatively similar concentration of volatile phenols, whether the wine had undergone MLF with a CE (+) *O. oeni* strain or not. Given the hydrolysis of ρ-coutaric acid to ρ-coumaric acid during ageing, Madsen et al. (2017) concluded that the strain of *B. bruxellensis* was more important than the strain of *O. oeni* inoculated to promote MLF. The Madsen et al. (2017) study, however, was conducted with one wine (pH 3.50, 12.5% v/v ethanol), under one storage condition (20°C). The influence of wine conditions, such as storage temperature, wine pH and ethanol concentration, on the hydrolysis of ρ-coutaric acid during ageing, remains unknown. These factors may explain the contrasting results reported by Chescheir et al. (2015) and Madsen et al. (2017). Therefore, the objective of this study was to determine the relative impact of *O. oeni* strain, wine pH, ethanol

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concentration and storage temperature on the ρ-coumaric and ρ-coutaric acid concentration of Pinot Noir wine during ageing and the effect on volatile phenol production by *B. bruxellensis*.

Materials and methods

Microorganisms

A CE (+) *O. oeni*, Vinoflora oenos (VFO) (Chr. Hansen, Hørsholm, Denmark), and CE (−) *O. oeni*, Enoferm Alpha (Alpha) (Lallemand, Montréal, QC, Canada) were used, based on Chescheir et al. (2015). The *B. bruxellensis* strain AWRI-1499 was obtained from The Australian Wine Research Institute culture collection (Adelaide, SA, Australia). Microorganisms were stored in glycerol (15%) at −80°C until needed and prepared by inoculation into acidic grape juice broth [250 mL/L white grape juice, 5 g/L yeast extract, 0.125 g/L magnesium sulfate, 0.025 g/L manganese sulfate, 1 mL/L 5% (w/v) Tween 80, pH 3.50] and grown at 25°C for 5 (*B. bruxellensis*) or 7 (*O. oeni*) days. Cells were harvested by centrifugation (4000 *g* for 10 min) and resuspended in sterile 0.2 mol/L phosphate buffer (pH 7.0) prior to inoculation.

Winemaking

Pinot Noir wines were produced at the Oregon State University Research Winery from grapes harvested at Woodhall Vineyard (Alpine, OR, USA) in 2016. Approximately 25 kg of destemmed grapes were aliquoted into 3×40 L plastic fermenters. After mixing, samples were taken and assessed for pH, TSS, TA and yeast assimilable nitrogen (YAN). pH was measured by ion-selective electrode (Thermo Scientific, Waltham, MA, USA). Titratable acidity was determined by titration with 0.1 N NaOH, and TSS was measured with an Anton-Paar DMA 35 N densitometer (Graz, Austria). Yeast assimilable nitrogen was measured by assessing the primary-amino nitrogen concentration as described by Dukes and Butzke (1998), and by the enzymatic analysis of ammonia (Boehringer, Mannheim, Germany). Due to the high initial TSS of the grape must (approximately 27°Brix), an addition of distilled water was made to each fermenter to achieve a TSS of approximately 24°Brix. Additional grape parameters were pH 3.40, TA 4.35 g/L and YAN 143 mg/L. The yeast nutrient Fermaid K (Lallemand) was added to each tank at 0.25 g/L. No SO_2 was added. Each tank was inoculated with *Saccharomyces cerevisiae* RC-212 (Lallemand) at 0.25 g/L of must and fermented at 27°C with twice-daily punch-downs. Total soluble solids was measured daily and alcoholic fermentations were completed in less than 14 days [i.e. residual sugar <0.5 g/L as measured by Clinitest (Bayer, Morristown, NJ, USA)].

Following fermentation, wines were pressed (0.1 MPa for 5 min), combined, and cold settled at 4°C for 72 h before being filtered through a plate-and-frame filter fitted with Beco K-1 2.0 μm nominal filter sheets (E. Begerow, Langenlonsheim, Germany), followed by filtration through a 1.0 μm nylon cartridge and a 0.45 μm sterile polyethersulfone cartridge (G.W. Kent, Ypsilanti, MI, USA) in succession. Filtered wine was dispensed into sterilised 12 L carboys. Three carboys were inoculated with the CE (+) *O. oeni* VFO, while an additional three carboys were inoculated with the CE (−) *O. oeni* Alpha, at approximately 10^6 colony-forming unit (CFU)/mL. Malolactic fermentation was conducted at 25°C and when completed [i.e. malic acid <50 mg/L, measured by enzymatic test kit (Vintessential, Dromana, Vic., Australia)], the wines were assessed for pH and ethanol (ebullliometer, Laboratoires Dujardin Salleron, Noizay, France). The ethanol concentration of the Pinot Noir wine was 13.1 ± 0.2% (v/v) and pH 3.80 ± 0.04. Portions (4 L) of the wines were then adjusted to either pH 3.80 or 3.60 using 25% (v/v) phosphoric acid, and ethanol was adjusted to either 13 or 15% (v/v) using 95% (v/v) ethanol (Luxco, St Louis, MO, USA). After pH and ethanol values were checked, wines were sterile-filtered with a 0.45 μm sterile polyethersulfone cartridge (G.W. Kent) and dispensed into 375 mL screw-capped (Stelvin, Amcor, Zurich, Switzerland) wine bottles. A set of each of the wine treatments [CE (+) or CE (−), pH 3.60 or 3.80, 13 or 15% ethanol] was then stored at 13 and 21°C. After 0, 30, 100 and 180 days storage, samples were taken from three bottles of each treatment, and frozen at −80°C until needed for HPLC analysis of ρ-coutaric and ρ-coumaric acids.

Analysis of ρ-coutaric and ρ-coumaric acids by HPLC

ρ-Coutaric and ρ-coumaric acids were determined by HPLC with a diode array detector (DAD) as described by Burns and Osborne (2013). ρ-Coutaric and ρ-coumaric acids were identified and quantified at 320 nm based on UV–Vis spectra and retention times of known standards obtained from Sigma Aldrich (St Louis, MO, USA). Calibration curves were prepared for ρ-coumaric acid quantification. Because of the lack of available standards, ρ-coutaric acid is reported as ρ-coumaric acid equivalents.

Brettanomyces *growth and volatile phenol production*

On completion of the ageing study (i.e. after 180 days storage at either 13 or 21°C), wines were inoculated with *B. bruxellensis* AWRI-1499, prepared as described earlier. Three bottles of each treatment were inoculated at approximately 10^5 CFU/mL and incubated at 25°C. Wine samples from each treatment were plated on de Man, Rogosa and Sharpe (MRS) plates (pH 4.5) prior to *B. bruxellensis* inoculation to check for microbial sterility. No microbial growth was observed on any plates after 10 days incubation at 25°C (data not shown). The population of *B. bruxellensis* was monitored for 6 weeks by plating on yeast extract peptone dextrose (YPD) media (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose, 20 g/L agar, pH 6.50) after suitable dilution in sterile peptone (0.1%). Wines were assessed for volatile phenol concentration 8 weeks post-inoculation.

Volatile phenol analysis

A headspace solid phase microextraction (SPME)–GC/MS method was used to quantify volatile phenol compounds in the wine samples. Wine (2 mL) was diluted with saturated sodium chloride buffer (8 mL) in a 20 mL glass SPME vial. Then 20 μL of internal standard (50 mg/L of 3,4-dimethyl phenol) was added into the diluted sample and the vial was tightly capped with a Teflon-faced silicone septa. Samples were first equilibrated at 50°C in a thermostatic bath for 30 min. A preconditioned 2 cm 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane coated SPME fibre (Supelco, Bellefonte, PA, USA) was then inserted into the sample headspace using an autosampler (Gerstel, Linthicum, MD, USA) at 50°C for 30 min. The sample was stirred at 500 rpm during extraction. On completion of extraction, the fibre was removed from the sample vial and inserted into the injection port of the GC to desorb the analytes. The injection was in splitless mode and the injector temperature held at 250°C. The sample was analysed on an Agilent 6890 gas

Table 1. Concentration of ρ-coutaric and ρ-coumaric acids in Pinot Noir wine after 180 days of ageing at either 21 or 13°C.

Oenococcus oeni strain	Storage temperature (°C)	Ethanol (% v/v)	pH	ρ-Coutaric acid (mg/L)[†,‡]	ρ-Coumaric acid (mg/L)[†]
Alpha CE (−)	21	13	3.60	7.40 ± 0.03	1.34 ± 0.04
			3.80	7.42 ± 0.02	1.43 ± 0.06
		15	3.60	7.38 ± 0.03	1.33 ± 0.04
			3.80	7.34 ± 0.07	1.34 ± 0.06
	13	13	3.60	8.43 ± 1.54	1.17 ± 0.07
			3.80	7.55 ± 0.03	1.11 ± 0.04
		15	3.60	7.50 ± 0.06	1.20 ± 1.06
			3.80	9.20 ± 1.53	1.27 ± 0.08
Vinoflora oenos CE (+)	21	13	3.60	0.07 ± 0.02	7.61 ± 0.08
			3.80	0.27 ± 0.29	6.32 ± 0.69
		15	3.60	0.07 ± 0.01	7.86 ± 0.03
			3.80	0.06 ± 0.01	7.82 ± 0.08
	13	13	3.60	0.09 ± 0.03	7.77 ± 0.77
			3.80	0.07 ± 0.01	7.20 ± 0.22
		15	3.60	0.07 ± 0.01	7.81 ± 0.03
			3.80	0.06 ± 0.02	7.58 ± 0.1

Wines completed malolactic fermentation with either a CE (−) or CE (+) *Oenococcus oeni* strain and were adjusted to pH 3.60 or 3.80, and to 13 or 15% v/v ethanol, prior to ageing. [†]Expressed as ρ-coumaric acid equivalents. [‡]Values are means from biological triplicates. CE, cinnamoyl esterase.

chromatograph equipped with an Agilent 5973 mass selective detector (Agilent Technologies, Santa Clara, CA, USA), with separation achieved with a ZB-WAX-PLUS capillary column (30 m × 0.25 mm i.d., 0.5 μm film thickness (Phenomenex, Torrance, CA, USA). The initial oven temperature was 35°C, which was held for 4 min and then ramped up to 230°C at a rate of 4°C/min, and held at 230°C for 10 min. A constant helium flow of 1.5 mL/min was applied. The temperature of the MS transfer line and ion source was 280 and 230°C, respectively. Electron ionisation mass spectrometric data were collected with a select ion monitoring (SIM) mode with an ionisation voltage of 70 eV. Compounds were identified by comparing mass spectral data from the Wiley 275.L (G1035) database (Agilent Technologies). The unique quantification mass ion and qualifying mass ions were carefully selected to give the highest response and lowest interference for each compound.

Statistical analysis

Statistical analysis was conducted using JMP Pro version 13.0 (SAS, NC, USA) and a significance value (α) of 0.05. Statistical differences among treatments were determined by ANOVA followed by Tukey's honestly significant difference (HSD) if appropriate.

Results and discussion

Malolactic fermentation induced in Pinot Noir wine by inoculation of commercial strains of either CE (+) or CE (−) *O. oeni* proceeded quickly and was completed (<0.05 g/L malic acid) for both strains in <21 days (data not shown). Prior to MLF, the concentration of ρ-coumaric and ρ-coutaric acids of wines was 1.84 ± 0.08 and 9.21 ± 1.56 mg/L, respectively. No significant change in the concentration of ρ-coumaric and ρ-coutaric acids was measured in wines that underwent MLF with the CE (−) *O. oeni* strain. In contrast, a significant difference ($P \leq 0.05$) was noted in the concentration of ρ-coumaric and ρ-coutaric acids when wine underwent MLF with the CE (+) *O. oeni* strain. These wines contained 0.05 ± 0.01 mg/L of ρ-coutaric acid and 7.47 ± 0.08 mg/L of ρ-coumaric acid. This is in agreement with Chescheir et al. (2015) and Madsen et al. (2017) who both reported a significant increase in ρ-coumaric acid and a concurrent decrease in ρ-coutaric acid after MLF with a CE (+) *O. oeni* strain.

Table 2. Calculated *F*-value and significant interactions between *Oenococcus oeni* strain, pH, ethanol concentration and storage temperature that affected the concentration of ρ-coutaric and ρ-coumaric acids in Pinot Noir wine.

Source of variation	ρ-Coutaric acid	ρ-Coumaric acid
Main effects		
O. oeni strain (S)	346.69***	888.36***
pH	2.0204	2.7457
Ethanol (E)	2.2993	0.0506
Storage temperature (T)	2.7516	0.0034
Interactive effects		
S × pH	1.8428	3.9948
S × E	2.0772	0.0003
S × T	2.5438	1.1871
pH × E	8.3386**	0.6514
pH × T	1.5515	2.4148
E × T	2.5438	0.1413
S × pH × E	8.2542**	0.5244
S × pH × T	0.5874	2.9019
S × E × T	0.9949	0.3470
T × E × pH	5.0632*	1.6819
S × pH × E × T	3.6928	2.6098

*, $P \leq 0.05$; **, $P \leq 0.001$; ***, $P \leq 0.0001$.

While there is clear evidence that the *O. oeni* strain can change the concentration of ρ-coumaric acid in wine, there is debate over the significance of this formation pathway compared to formation via acid hydrolysis during ageing (Madsen et al. 2017). To determine this, the concentration of ρ-coumaric and ρ-coutaric acids of Pinot Noir wine was measured during 180 days of storage at either 13 or 21°C. The pH, ethanol concentration, and storage temperature was varied as these parameters can affect acid hydrolysis of esters (Ramey and Ough 1980, Scirmgeour et al. 2015). The concentration of ρ-coumaric and ρ-coutaric acid before and after 180 days ageing is shown in Table 1. No significant change in the concentration of ρ-coumaric and ρ-coutaric acids was noted after 180 days of ageing, regardless of pH, ethanol concentration or storage temperature (Table 2). The concentration of ρ-coumaric and ρ-coutaric acids was also measured after 30 and 100 days of ageing, and no

significant change in concentration was noted (data not shown). The lack of effect of ethanol, pH and storage temperature on the concentration of ρ-coumaric and ρ-coutaric acids demonstrates the relative stability of these compounds under these wine conditions. The only factor that significantly altered the concentration of ρ-coumaric and ρ-coutaric acids in the wine was the strain of *O. oeni* used to conduct MLF ($P \leq 0.0001$) (Table 2). While some interactive effects for ρ-coutaric acid concentration were noted (pH × ethanol, strain × pH × ethanol and temperature × ethanol × pH), no interactions were noted for ρ-coumaric acid concentration (Table 2).

In the present study, enzymatic hydrolysis via the action of a CE (+) *O. oeni* strain during MLF had a significant impact on ρ-coumaric acid concentration, whereas acid hydrolysis during ageing did not. While others have noted that ρ-coutaric acid can be converted to ρ-coumaric acid through acid hydrolysis (Kallithraka et al. 2009), the degree and rate of conversion to ρ-coumaric acid by the CE (+) *O. oeni* strain was greater than other studies have indicated can occur over time because of acid hydrolysis alone. For example, while Kallithraka et al. (2009) noted a decrease in the tartaric esters of hydroxycinnamic acids 9 months after bottling, over 65% of the caftaric acid and 60% of the initial ρ-coutaric acid remained. In contrast, nearly 100% of the ρ-coutaric acid was hydrolysed by the CE (+) *O. oeni* during MLF in the present study. Madsen et al. (2017) reported an increase in ρ-coumaric acid concentration during ageing followed by a decrease that coincided with *B. bruxellensis* growth and volatile phenol production. A decrease in ρ-coumaric acid concentration also occurred in wines where *B. bruxellensis* was not inoculated, which the authors attributed to the loss of ρ-coumaric acid because of other chemical reactions involving this compound, such as co-pigmentation with anthocyanins (Hernández et al. 2006). The decrease in ρ-coumaric acid independent of *B. bruxellensis* growth was not observed in the present study and may be because of the low level of co-pigmentation observed in Pinot Noir wine (Burns and Osborne 2013).

Sterile filtration of the wine prior to ageing allowed changes to be determined in ρ-coutaric and ρ-coumaric acids due only to acid hydrolysis, as microorganisms were not present. In contrast, Madsen et al. (2017) inoculated non-sterile wine with both *O. oeni* and *B. bruxellensis* prior to ageing to mimic real-life conditions in a winery. As wines were not sterile filtered prior to inoculation and ageing, background *S. cerevisiae* and LAB were also present in some of their wine treatments. This makes it difficult to determine whether changes in ρ-coumaric acid concentration were due strictly to acid hydrolysis or could be attributed to the action of microorganisms, as some *S. cerevisiae* strains and certain LAB that are often present in wine (Berbegal et al. 2019) can degrade ρ-coumaric acid (Chatonnet et al. 1992, Strickland et al. 2016). Furthermore, dominance of the inoculated *O. oeni* strain was not mentioned in previous studies, so it is possible that the presence of a 'native' CE (+) *O. oeni* may also have affected the concentration of ρ-coutaric and ρ-coumaric acids. In the same manner, Kallithraka et al. (2009) did not note whether sterile-filtered wine was used or if any microbial analysis was performed to determine if change to the concentration of ρ-coutaric and ρ-coumaric acids may have been impacted by microbial activity. This may explain some of the differences observed between the results of previous studies and the present study.

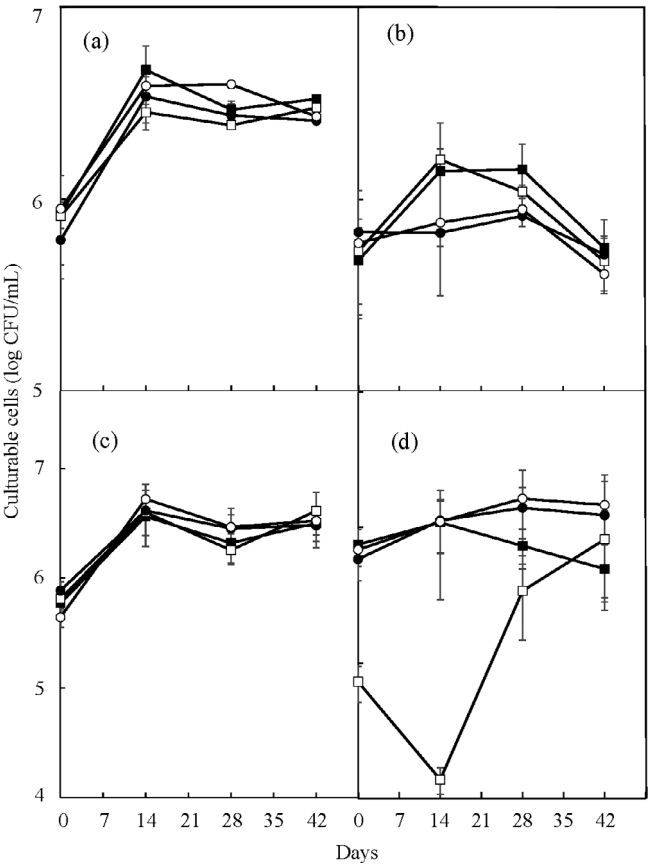

Figure 1. Growth of *Brettanomyces bruxellensis* AWRI-1499 in Pinot Noir wine following MLF with (a,b) cinnamoyl esterase (CE) (–) or (c,d) CE (+) *Oenococcus oeni* strains, and adjustment to (a,c) 13% or (b,d) 15% ethanol (v/v) concentration, and pH 3.6 (■, □) or 3.8 (●, ○). Wine had previously been stored for 180 days, at either 13°C (■, ●) or 21°C (□, ○). Error bars represent SD from the mean.

The effect of changes in ρ-coumaric acid during MLF and ageing on volatile phenol production was determined by inoculating wine with *B. bruxellensis* on completion of ageing. *Brettanomyces bruxellensis* AWRI-1499 grew well in all wine treatments (Figure 1) with a higher population being reached in lower ethanol (13%) wines (Figure 1a,c) in agreement with Oswald and Edwards (2017). In wines with 13% v/v ethanol, *B. bruxellensis* growth was similar at pH 3.60 and 3.80. In wines containing 15% v/v ethanol, however, a lower maximum population of *B. bruxellensis* was observed at pH 3.60, compared to that at pH 3.80 (Figure 1b,d). Again, this is in agreement with the findings of others, where *B. bruxellensis* grew better at a higher pH (Strum et al. 2014). The only unusual result was the decrease in *B. bruxellensis* population after inoculation into wine (15% v/v ethanol, pH 3.60) that had undergone MLF with the CE (+) *O. oeni* strain. The decrease in population was likely because of the wine unintentionally being inoculated at a lower population (7.2×10^4 CFU/mL) than the other wine treatments (7×10^5 CFU/mL). The population of *B. bruxellensis* recovered in this treatment after 28 days and had achieved a similar level to that of other treatments by 42 days after inoculation (Figure 1d).

Eight weeks post-inoculation of *B. bruxellensis* the wines were assessed for volatile phenol concentration (Table 3). As was seen with the concentration of ρ-coutaric and ρ-coumaric acids, the only treatment that had a significant impact on the concentration of 4-EP and 4-EG was the

Table 3. Concentration of 4-ethylphenol and 4-ethylguaiacol in Pinot Noir wine 56 days after inoculation with *Brettanomyces bruxellensis* AWRI-1499.

Oenococcus oeni strain	Storage temperature (°C)	Ethanol (% v/v)	pH	4-Ethylphenol (μg/L)†	4-Ethylguaiacol (μg/L)†
Alpha CE (−)	21	13	3.6	418.6 ± 16.3	345.8 ± 17.9
			3.8	436.6 ± 12.5	381.4 ± 13.5
		15	3.6	435.2 ± 46.7	312.4 ± 46.3
			3.8	426.0 ± 8.6	298.5 ± 14.5
	13	13	3.6	410.4 ± 17.4	344.7 ± 14.6
			3.8	426.0 ± 11.2	333.3 ± 13.7
		15	3.6	428.5 ± 14.9	317.4 ± 13.6
			3.8	404.6 ± 6.8	314.8 ± 10.1
Vinoflora oenos CE (+)	21	13	3.6	3597.8 ± 110.7	1421.5 ± 68.3
			3.8	3791.5 ± 129.6	1362.3 ± 59.2
		15	3.6	3827.7 ± 121.3	1364.3 ± 46.1
			3.8	3681.5 ± 215.5	1346.1 ± 64.8
	13	13	3.6	3645.1 ± 295.9	1392.3 ± 127.1
			3.8	3712.1 ± 105.5	1433.1 ± 52.6
		15	3.6	3765.9 ± 257.2	1383.0 ± 118.9
			3.8	3767.9 ± 64.9	1320.8 ± 27.6

Wines had completed malolactic fermentation with a CE (−) or CE (+) *Oenococcus oeni* strain, were adjusted to pH 3.60 or 3.80 and to 13 or 15% v/v ethanol, and were aged for 180 days at either 21 or 13°C prior to *B. bruxellensis* inoculation. †Values are means of duplicate analysis from biological triplicates. CE, cinnamoyl esterase.

Table 4. Calculated *F*-value and significant interactions between *Oenococcus oeni* strain, pH, ethanol concentration and storage temperature that affected production of 4-ethylphenol or 4-ethylguaiacol by *Brettanomyces bruxellensis* AWRI-1499 in Pinot Noir wine.

Source of variation	4-Ethylphenol	4-Ethylguaiacol
Main effects		
O. oeni strain (S)	1888.1***	1031.5***
pH	0.5234	0.6146
Ethanol (E)	0.0004	2.2812
Storage temperature (T)	3.4202	0.0873
Interactive effects		
S × pH	0.3758	1.6224
S × E	0.3191	0.1134
S × T	1.8176	0.0604
pH × E	1.5668	0.5955
pH × T	3.4784	0.8636
E × T	6.8953*	0.0128
S × pH × E	1.9398	1.0780
S × pH × T	6.7906*	5.5524*
S × E × T	5.9989*	0.0909
T × E × pH	4.4548*	0.0566
S × pH × E × T	7.1942**	2.2176

*, $P \leq 0.05$; **, $P \leq 0.001$; ***, $P \leq 0.0001$.

strain of *O. oeni* used to conduct MLF (Table 4). Wines that underwent MLF with a CE (−) *O. oeni* strain contained a lower concentration of 4-EP and 4-EG than wines that underwent MLF with the CE (+) strain (Table 3). Chescheir et al. (2015) also reported a significantly higher concentration of 4-EP and 4-EG after growth of *B. bruxellensis* in wine where MLF was conducted by a CE (+) *O. oeni* strain compared to wines where MLF was conducted by a CE (−) strain. This is not surprising given the higher ρ-coumaric acid concentration in wines where MLF was conducted by a CE (+) *O. oeni* strain. Wine pH or ethanol concentration did not impact the production of volatile phenols by *B. bruxellensis* (Table 4), even in treatments where *B. bruxellensis* did not initially grow as well (Figure 1). Instead, the concentration of 4-EP in wines reflected the initial concentration of ρ-coumaric acid at the time of *B. bruxellensis* inoculation and was impacted only by the strain of *O. oeni* used to conduct MLF (Table 2). In agreement with Chescheir et al. (2015), this study demonstrates that the use of CE (+) *O. oeni* strains should be avoided in wines that are at risk of *Brettanomyces* spoilage.

While the most important factor influencing the volatile phenol concentration of wine is growth of *B. bruxellensis*, the action of a CE (+) *O. oeni* strain during MLF can increase the risk of elevated production of volatile phenols. Madsen et al. (2017) concluded that the *B. bruxellensis* strain may have a larger impact on volatile phenol production than the *O. oeni* strain, because of the variable production of volatile phenols by *B. bruxellensis* strains (Conterno et al. 2006, Madsen et al. 2017). Indeed, the growth of a low volatile phenol producing *B. bruxellensis* in a wine may result in a low concentration of volatile phenols no matter what the concentration of ρ-coumaric acid is. As a result using a CE (+) or CE (−) *O. oeni* would be less critical. A winemaker has no control, however, over which *B. bruxellensis* strain infects their wine (high- or low-volatile phenol producer). In contrast, the winemaker has control over the *O. oeni* culture they inoculate to promote MLF and the use of a CE (−) *O. oeni* culture will minimise the amount of volatile precursors in the wine, no matter what strain of *B. bruxellensis* is present. This is a relatively simple step for a winemaker given the availability of CE (−) *O. oeni* cultures from different manufacturers.

Conclusions

While winemakers must continue to use sound winemaking practices to prevent the growth of *Brettanomyces* in their wines, this study has shown that the strain of *O. oeni* used to induce MLF can have a significant impact on volatile phenol production by *Brettanomyces* because of an increase in the concentration of volatile phenol precursors. While a significant change in the concentration of ρ-coutaric and ρ-coumaric acids occurred during MLF by a CE (+) *O. oeni*, no change due to chemical hydrolysis during ageing occurred, regardless of wine pH, ethanol concentration and storage temperature. This finding suggests that the use of CE (+) *O. oeni* strains for MLF should be avoided in wines at risk of *Brettanomyces* spoilage. In addition, results from the present study demonstrate an additional risk of uninoculated MLF, as the CE activity of the LAB performing MLF would be unknown. The CE activity of other LAB that can be used to conduct MLF, such as *Pediococcus* and

Lactobacillus species, must also be considered, as little is known about their ability to hydrolyse *ρ*-coutaric acid.

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