JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Metabolism of Nonesterified and Esterified Hydroxycinnamic Acids in Red Wines by *Brettanomyces bruxellensis*

Lauren M. Schopp,[†] Jungmin Lee,[‡] James P. Osborne,[§] Stuart C. Chescheir,[§] and Charles G. Edwards*[,†]

[†]School of Food Science, Washington State University, Pullman, Washington 99164−6376, United States

[‡]USDA−ARS−HCRU worksite, Parma, Idaho 83660, United States

[§]Department of Food Science and Technology, Oregon State University, Corvallis, Oregon 97331, United States

ABSTRACT: While *Brettanomyces* can metabolize nonesterified hydroxycinnamic acids found in grape musts/wines (caffeic, *p*-coumaric, and ferulic acids), it was not known whether this yeast could utilize the corresponding tartaric acid esters (caftaric, *p*-coutaric, and fertaric acids, respectively). Red wines from Washington and Oregon were inoculated with *B. bruxellensis*, while hydroxycinnamic acids were monitored by HPLC. Besides consuming *p*-coumaric and ferulic acids, strains I1a, B1b, and E1 isolated from Washington wines metabolized 40−50% of caffeic acid, a finding in contrast to strains obtained from California wines. Higher molar recoveries of 4-ethylphenol and 4-ethylguaiacol synthesized from *p*-coumaric and ferulic acids, respectively, were observed in Washington Cabernet Sauvignon and Syrah but not Merlot. This finding suggested that *Brettanomyces* either (a) utilized vinylphenols formed during processing of some wines or (b) metabolized other unidentified phenolic precursors. None of the strains of *Brettanomyces* studied metabolized caftaric or *p*-coutaric acids present in wines from Washington or Oregon.

KEYWORDS: *Brettanomyces, hydroxycinnamic acids, phenolic acids, cinnamates, volatile phenols*

INTRODUCTION

Brettanomyces has been regarded by some as the most severe microbiological threat to wine quality[1] causing serious financial losses each year.[2] As part of its metabolism, *Brettanomyces* utilizes hydroxycinnamic acids to produce ethylphenols (i.e., volatile phenols) which have been sensorily described as "barnyard," "leather," "horse sweat," and others.[3−5] Biochemically, the hydroxycinnamic acids are first decarboxylated into vinylphenols, which are subsequently reduced to ethylphenols.[3,6] Biosynthesis is dependent on many factors including the strain and energy source such as glucose or ethanol.[7−13]

As reviewed by Conde et al.,[14] hydroxycinnamic acids (caffeic, *p*-coumaric, or ferulic acids) are located in the vacuoles of the skin and pulp cells of grapes, primarily as esters of tartaric acid (caftaric, *p*-coutaric, or fertaric acids, respectively). Once a must is prepared and during the course of fermentation, tartaric acid can be hydrolyzed to form the nonesterified acids with concentrations varying according to cultivar, vintage, and winemaking conditions.[15−18] Analyzing Merlot grapes from Washington, Nagel et al.[16] reported that the average concentrations of caftaric, *p*-coutaric, and fertaric acids were 59.2, 16.9, and 3.2 mg/L, respectively. Furthermore, concentrations of caftaric and *p*-coutaric acids decreased 34% to 61% during fermentation and subsequent storage of Cabernet Sauvignon and Merlot wines.[15] In a recent study, Ginjom et al.[18] calculated *p*-coutaric/*p*-coumaric acids molar ratios to vary from 0.6 to 2.1 depending on cultivar and fermentation temperature.

While *Brettanomyces* possess enzymatic activity to act on a number of esters[19] including the ethyl esters of hydroxycinnamic acids,[20] it is not known whether tartaric acid esters can serve as substrates. If *Brettanomyces* are able to metabolize tartaric acid esters, these compounds could represent a large pool of precursors available for conversion to volatile phenols. In fact, Nikfardjam et al.[21] postulated that those grape cultivars with high amounts of hydroxycinnamic acids could be more prone to *Brettanomyces* infections, although specific precursors were not studied. As such, the objective of this study was to determine utilization of red wine hydroxycinnamic acids by strains of *B. bruxellensis* isolated from Washington and California.

MATERIALS AND METHODS

Strains and Starter Cultures. For the first experiment, *B. bruxellensis* strains B1b, B5, E1, and I1a were originally isolated from Washington wines as described by Jensen et al.[22] All strains were maintained in glycerol at −70 °C as well as being streaked on WL medium (Oxoid, Hampshire, England). Starter cultures were prepared by transferring a single colony into 100 mL of YM broth (Difco, Sparks, MD) containing 5% v/v ethanol and adjusted to pH 3.8. After a week of growth, 1 mL of culture was transferred to additional YM broth containing 10% v/v ethanol to improve acclimation to wine conditions. Cells were harvested by centrifugation (4200*g*, 15 min) after an additional week of incubation, washed in 0.1% w/v peptone, and resuspended in additional peptone. Wines were inoculated, in triplicate, at initial populations of 10^5 to 10^6 cfu/mL and monitored over a nine-week period by spiral plating with an Autoplate 4000 (Spiral Biotech, Bethesda, MD) on WL agar. All plates were incubated at 26 °C prior to counting.

As part of a second experiment, additional strains of *B. bruxellensis* (493, 495, 496, 497, 607, 613, 614, 615, 616, 635, and 643) were provided by E.&J. Gallo Winery (Modesto, CA) and maintained in glycerol stored at −80 °C. Starter cultures were prepared as previously

Received: August 5, 2013
Revised: November 6, 2013
Accepted: November 12, 2013
Published: November 12, 2013

dx.doi.org/10.1021/jf403440k | *J. Agric. Food Chem.* 2013, 61, 11610−11617

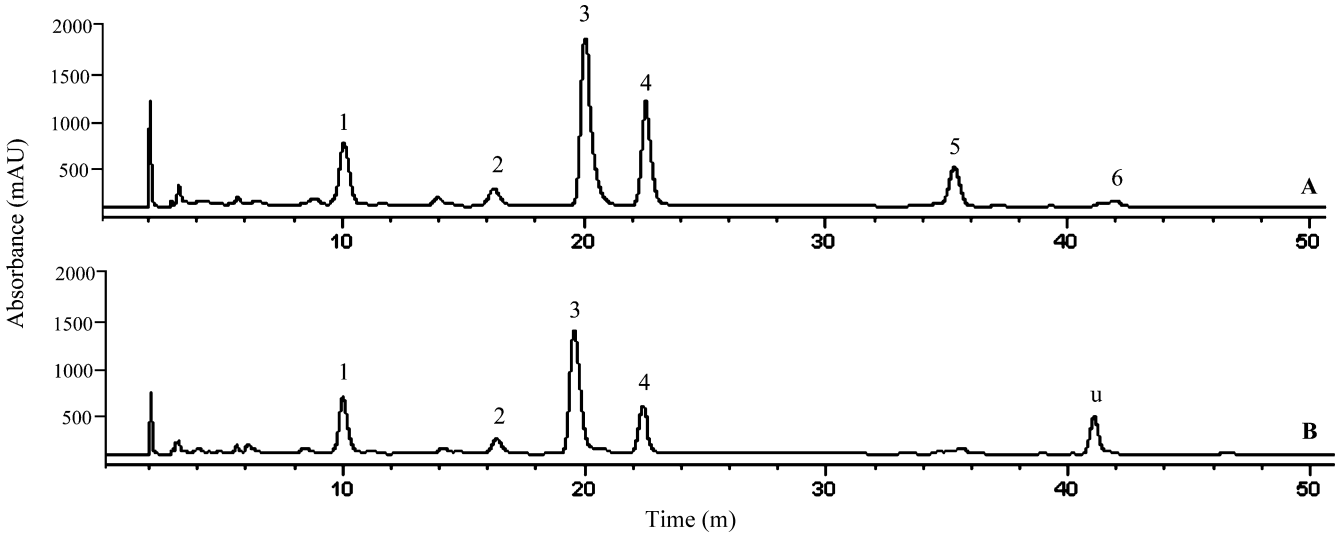

Figure 1. HPLC chromatogram of commercially prepared Pinot noir wine 63 days after no inoculation (A) or inoculation with *B. bruxellensis* strain E1 (B). Peak identifications: (1) caftaric acid, (2) *p*-coutaric acid, (3) internal standard chlorogenic acid, (4) caffeic acid, (5) *p*-coumaric acid, (6) ferulic acid, and (u) unknown.

described except harvested cells were resuspended in 0.1% w/v peptone for inoculation into wine at 10^3 to 10^4 cfu/mL.

Wines. In a first experiment, commercially prepared wines of Cabernet Sauvignon (pH 3.79; 13.7% v/v alcohol), Merlot (pH 3.71; 13.4% v/v alcohol), and Syrah (pH 3.69; 13.1% v/v alcohol) were obtained from a Washington winery, while Pinot noir (pH 3.57; 12.2% v/v alcohol) originated from a winery located in Oregon. Residual SO_2 was removed by the addition of equimolar amounts of H_2O_2 (J.T. Baker, Phillipsburg, NJ), while ethanol concentrations were standardized to 12.8% v/v by the addition of Milli-Q water and/or 200-proof ethanol. Additionally, 0.5% w/v glucose (J.T. Baker, Phillipsburg, NJ), 0.5% w/v fructose (Spectrum, Gardena, CA), and 0.1% w/v yeast extract (Difco, Sparks, MD) were added to all wines to limit potential differences in nutrient composition. Finally, the pH of all four wines was adjusted to 3.94 through the addition of 5 N NaOH. After additions, wines were sterile-filtered through 0.22 μm PVDF filters (Millipore, Bedford, MA) prior to the addition of 0.1% w/v autoclaved suspension cellulose (Sigma-Aldrich, St. Louis, MO). While wines were stirred, 100 mL aliquots were transferred into sterile milk dilution bottles using a varistaltic dispenser pump before inoculation. Uninoculated wines serves as controls.

In a second experiment, a Pinot noir wine was produced using grapes obtained from the Woodhall Vineyard at Oregon State University (Alpine, OR). Once harvested, the grapes were stored at 4 °C overnight before being crushed/destemmed, pooled, and distributed (60 L) into 100 L stainless steel tanks. No SO_2 additions were made. Each lot was inoculated with an active dry form of *Saccharomyces cerevisiae* strain VQ-15 (Lallemand, Montréal, Canada) rehydrated according to manufacturer's specifications to yield 10^6 cfu/mL. For fermentation, the tanks were placed into a room held at 27 °C with punch downs performed twice daily. Upon completion of alcoholic fermentation (<2 g/L reducing sugar) as confirmed with Clinitest tablets (Bayer, Morristown, NJ), wines were pressed and cold settled at 4 °C for 48 h. After filtration through 3.0 μm nominal filter sheets (Beco K-1, Langenlonsheim, Germany), the wines were sequentially filtered through 1.0 μm nylon and then 0.45 μm polyethersulfone cartridges (G. W. Kent, Ypsilanti, MI) before being dispensed into sterile 4 L glass carboys for storage at 4 °C. When needed, 150 mL were dispensed into sterilized milk dilution bottles using a varistaltic pump before inoculation. The wine pH was 3.89, while the ethanol concentration was 13.4% v/v. Additional nutrients were not added to these wines prior to inoculation with *Brettanomyces*.

Hydroxycinnamic Acid Analyses. For experiments involving strains of *Brettanomyces* obtained from Washington, changes in concentrations of hydroxycinnamic acids were monitored by aseptically collecting 5 mL samples and adding 40 mg/L chlorogenic acid (Acros Organics, Geel, Belgium) as the internal standard. Samples were immediately frozen at −10 °C until phenolic acid purification using neutral Bakerbond SPE C_{18} column (J. T. Baker, Phillipsburg, NJ) and subsequently through a preconditioned acidic SPE column. The columns were preconditioned according to the methods of Jaworski and Lee.[23] Wines were adjusted to pH 7.0 using 5 N NaOH and filtered through a 0.45 μm PVDF syringe filter (Whatman, Piscataway, NJ) before 3 mL was then passed through the preconditioned neutral SPE column. The neutral column was washed with Milli-Q water (4 mL) with the effluent acidified to pH 2.0 using 0.1 N HCl prior to being passed through a preconditioned acidic SPE column. Phenolic acids were eluted using 2 mL of methanol (J.T. Baker, Phillipsburg, NJ) which was concentrated to approximately 0.5 mL using a Rotavapor R-210 equipped with a heating bath at 32 °C (Buchi, Flawil, Switzerland).

A HPLC-DAD unit with detection set at 320 nm (Agilent 1100 series, Wilmington, DE) was used to analyze samples as described by Lee and Finn.[24] Phenolic compounds were identified based on UV−visible spectra and retention times of known standards (caftaric, caffeic, *p*-coumaric, and ferulic acids) obtained from Sigma-Aldrich (St. Louis, MO). When standards were not available (i.e., *p*-coutaric acid), retention times were used from Lee and Scagel[25] and verified with UV−visible spectra.[26] Calibration curves were prepared for caffeic, *p*-coumaric, and ferulic acids in 10% v/v ethanol. Calibration graphs were prepared by plotting the ratios of the standard peak areas to the internal standard peak area versus the ratios of the standard concentrations to the internal standard concentration. The curves (five data points) were linear with R^2 values higher than 0.999. Caftaric acid was quantified using the caffeic acid curve, whereas *p*-coutaric acid was quantified using the *p*-coumaric acid curve.

For experiments involving California strains, hydroxycinnamic acids were quantified by HPLC-DAD as described by Burns and Osborne.[27] Compounds were monitored at 320 nm and identified by UV−visible spectra and retention times of known standards as previously described.

Other Chemical Analyses. Concentrations of 4-ethylphenol and 4-ethylguaiacol were analyzed using the method described by Jensen et al.[22] Free and total SO_2 was measured by the aeration/oxidation method, alcohol was measured using an ebulliometer (AllaFrance, Chemillé, France), while volatile acidity relied on a R&D 80 Cash still (Research and Development Glass Products, Berkeley, CA) as described by Edwards and Watson.[28]

Statistical Analyses. Significant sources of variation were evaluated using one-way analysis of variance (ANOVA) followed by

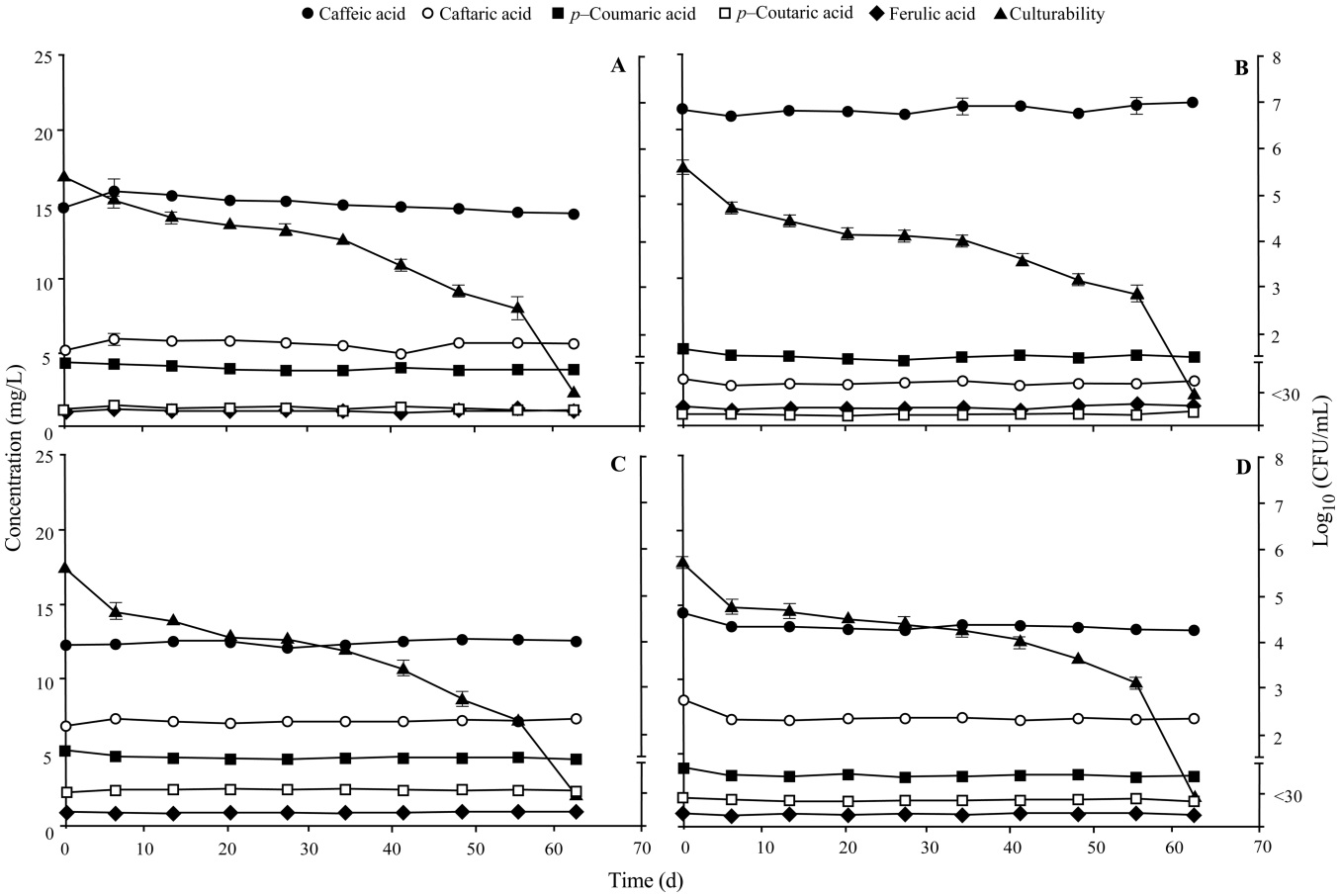

Figure 2. Culturability of *B. bruxellensis* strain B5 and changes in concentration of caffeic, caftaric, *p*-coumaric, *p*-coutaric, or ferulic acids in Cabernet Sauvignon (A), Merlot (B), Syrah (C), or Pinot noir (D) commercial wines.

Tukey's honestly significant difference for mean comparison. Paired comparisons between *p*-coumaric acid/ferulic acid consumed and 4-ethylphenol/4-ethylguaiacol produced were performed using Student's *t* test. All tests of significance were conducted at a probability level of $p \leq 0.05$ using Minitab (State College, PA) and XLSTAT (New York, NY) statistical software.

RESULTS

Metabolism of the red wine hydroxycinnamic acids was monitored as illustrated in chromatograms presented in Figure 1. While Figure 1A is that of an uninoculated Pinot noir wine, Figure 1B is representative of those wines inoculated with *B. bruxellensis*. For all wines, caftaric, *p*-coutaric, caffeic, *p*-coumaric, and ferulic acids were present in varying quantities, while fertaric acid was not detected in any wines. Additional unidentified peaks were also noted in some wines; in particular, a single peak (retention time = 41 min) formed in a few wines. For example, the peak was observed in Pinot noir inoculated with strain E1 but not the same wine with I1a. As this unknown peak was not noted in any of the control wines stored under the same conditions as inoculated wines, it appears to be a unknown byproduct of some *Brettanomyces* strains.

B. bruxellensis strain B5 behaved similarly in the four wines from Washington where populations slowly declined up to day 63 (Figure 2). Even though culturable populations were recovered during this period, B5 exhibited little utilization of the nonesterified hydroxycinnamic acids. For example, <15% (≤0.60 mg/L) of the *p*-coumaric acid was metabolized from each wine. In Pinot noir and Cabernet Sauvignon wines, this strain consumed only 8.4% (1.2 mg/L) and 2.4% (0.36 mg/L) of caffeic acid, respectively, while concentrations of this hydroxycinnamic acid remained unchanged in the Syrah or Merlot wines. However, B5 utilized a small amount of ferulic acid from the Merlot wine only, approximately 4.4% or 0.076 mg/L.

In contrast to B5, strain I1a eventually entered log growth in all four wine cultivars. After approximately seven days, populations peaked around day 30 in excess of 10^7 cfu/mL (Figure 3), while utilization of *p*-coumaric and ferulic acids commenced by day 14 and was completed by day 30. Overall, this strain converted 96−98% *p*-coumaric acid and 37−78% of ferulic acid depending on cultivar. In addition, I1a also metabolized caffeic acid but lagged behind the utilization of *p*-coumaric and ferulic acid. In the Syrah wine, concentrations of caffeic acid declined from 12.4 to 6.84 mg/L within 28 days and then remained relatively unchanged. A similar trend was also observed in the other wines where I1a transformed 43−53% of caffeic acid in Cabernet Sauvignon, Merlot, or Pinot noir. Neither caftaric nor *p*-coutaric acids present in these wines were metabolized by I1a.

Similar to I1a, B1b (Figure 4) and E1 (Figure 5) entered exponential growth and reached populations of $>10^7$ cfu/mL. Unlike I1a, these strains began converting all three non-esterified hydroxycinnamic acids relatively quickly. Decreases in the concentrations of the caffeic, *p*-coumaric, and ferulic acids were observed by day 7 and continued until populations reached stationary phase. While most of the *p*-coumaric and

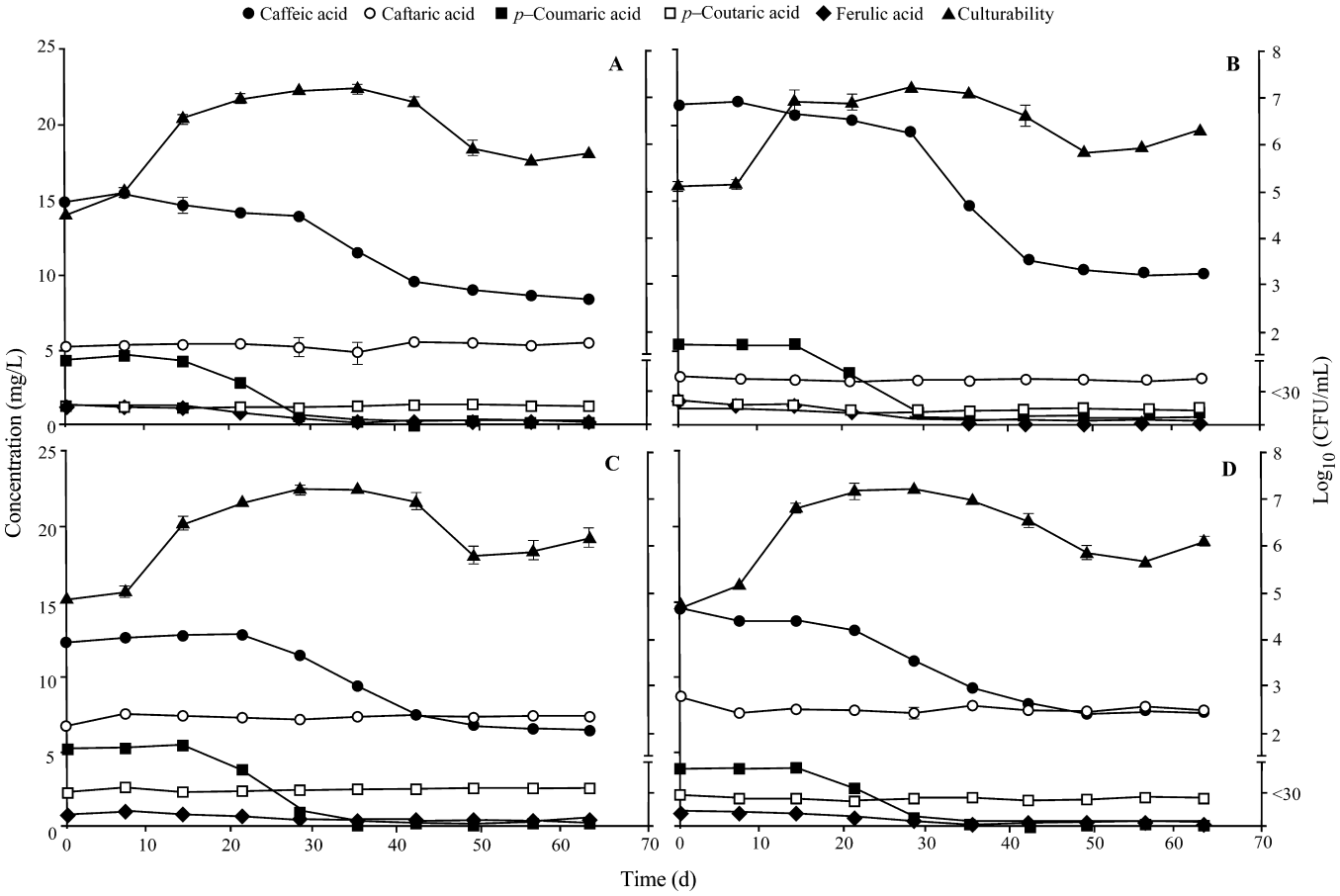

Figure 3. Culturability of *B. bruxellensis* strain l1a and changes in concentration of caffeic, caftaric, *p*-coumaric, *p*-coutaric, or ferulic acids in Cabernet Sauvignon (A), Merlot (B), Syrah (C), or Pinot noir (D) commercial wines.

ferulic acids were metabolized (94–98%), both strains only transformed 52–71% of the caffeic acid. One exception is strain B1b inoculated into the Cabernet Sauvignon wine where 87% of the *p*-coumaric acid was consumed. Although B1b and E1 utilized a large portion of the nonesterified hydroxycinnamic acids, conversion of caftaric or *p*-coutaric acid was not observed.

An additional 11 strains of *B. bruxellensis* originally isolated from California wines were assessed for their abilities to metabolize hydroxycinnamic acids during growth in the noncommercial Oregon Pinot noir wine. All strains grew well and reached populations in excess of 5×10^6 cfu/mL within 36 days (data not shown). However, no changes were observed in the concentrations of caftaric, *p*-coutaric, or caffeic acids after 56 days compared to the uninoculated control (Table 1). In addition, although this wine contained a relatively low concentration of *p*-coumaric acid (≈1 mg/L), only 24– 40% was metabolized by these strains. This finding was in contrast to three of the Washington strains (I1a, B1b, and E1) where >94% of this compound was metabolized regardless of the wine (Figures 3–5).

Strains B5, I1a, B1b, and E1 produced 4-ethylphenol and 4-ethylguaiacol in the commercially prepared wines (Table 2) with concentrations dependent on yeast strain and cultivar. Overall, the greatest amounts of volatile phenols were produced by I1a, B1b, and E1 with far lesser amounts by B5. Volatile acidity was also produced following the same trend, with the highest concentration being 0.99 g/L present in Cabernet Sauvignon wine inoculated with B1b.

Using data presented in Table 2 and Figures 2–4, concentrations of nonesterified hydroxycinnamic acids and the respective product of being metabolized (i.e., volatile phenols) were expressed on a micromolar basis. Here, individual strain values pooled in order to compare nonesterified hydroxycinnamic acid consumed against the respective volatile phenol produced (Figure 6). For both Cabernet Sauvignon and Syrah, the concentrations of *p*-coumaric and ferulic acids consumed were significantly lower than the corresponding volatile phenol, 4-ethylphenol or 4-ethylguaiacol, respectively. While significantly lower amounts of ferulic acid compared to 4-ethylguaiacol were observed for the Pinot noir, this was not noted for *p*-coumaric acid/4-ethylphenol. No significant differences were observed between *p*-coumaric acid/ferulic acid consumed and 4-ethylphenol/4-ethylguaiacol synthesized in the Merlot wine.

■ DISCUSSION

B. bruxellensis strains B5, I1a, B1b, and E1 inoculated into selected cultivars of wine exhibited a range of growth characteristics. While B5 slowly declined over 60 days to populations approaching 30 cfu/mL, strains I1a, B1b, and E1, achieved culturability in excess of 10^7 cfu/mL. Even though all strains were inoculated at similar initial populations, a longer lag period was exhibited by I1a in comparison to B1b or E1. Similarly, Fugelsang and Zoecklein[29] reported differences between the growth rates and stationary phase populations among the studied strains of *B. bruxellensis*. While Medawar et

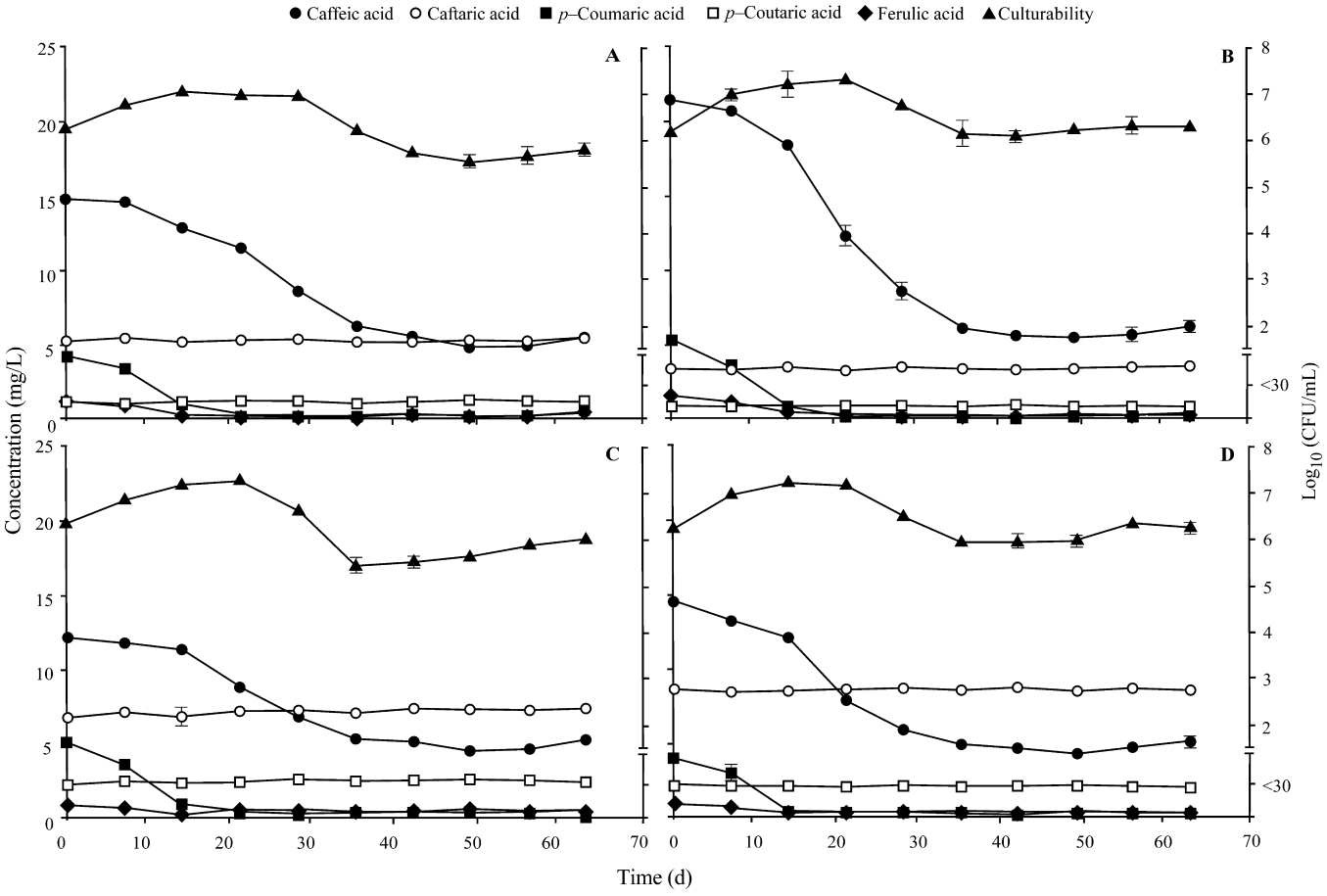

Figure 4. Culturability of *B. bruxellensis* strain B1b and changes in concentration of caffeic, caftaric, *p*-coumaric, *p*-coutaric, or ferulic acids in Cabernet Sauvignon (A), Merlot (B), Syrah (C), or Pinot noir (D) commercial wines.

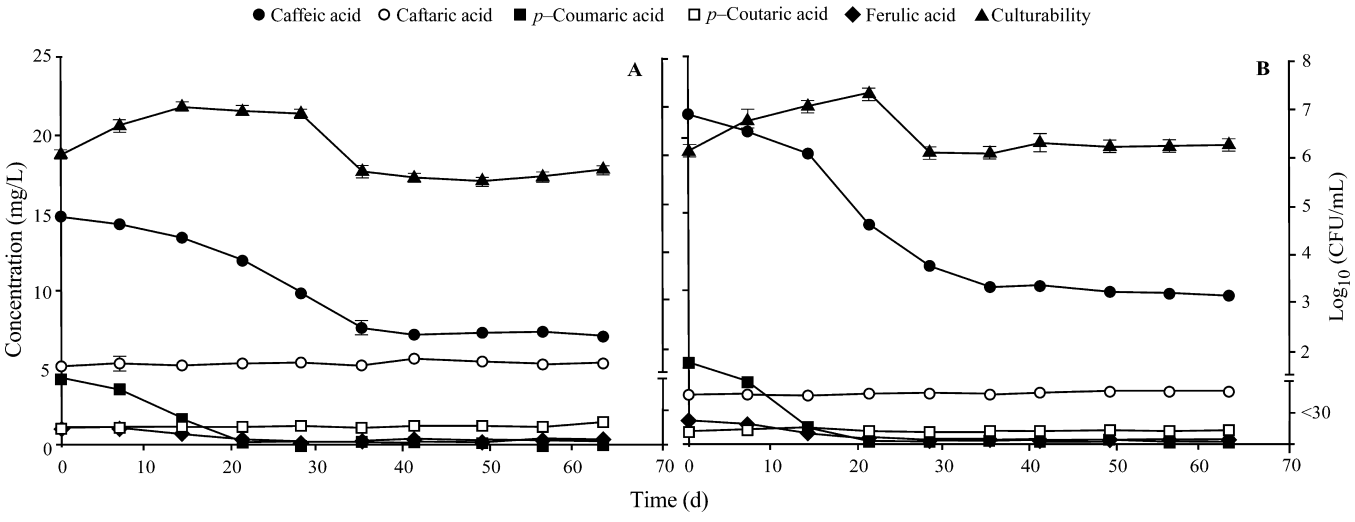

Figure 5. Culturability of *B. bruxellensis* strain E1 and changes in concentration of caffeic, caftaric, *p*-coumaric, *p*-coutaric, or ferulic acids in Cabernet Sauvignon (A) or Merlot (B) commercial wines.

al.[30] reported an increasing lag in growth with increasing ethanol concentrations, ethanol concentrations in all wines had been standardized to 12.8% v/v and SO_2 removed so differences in growth in the present study were not due to these factors.

Differences in the amount of the nonesterified hydroxycinnamic acids metabolized by different strains of *B. bruxellensis* were in agreement with other studies.[9,11,29,31] Overall, the strains studied expressed preferential metabolism of *p*-coumaric acid as opposed to caffeic or ferulic acids. For example, strains I1a, E1, and B1b, utilized 96−98% of the available *p*-coumaric acid, whereas only 37−78% of the caffeic or ferulic acid was converted. In contrast, the 11 strains originally from California only metabolized 24−40% of the *p*-coumaric acid and none of

Table 1. Changes in Concentrations of Caffeic, Caftaric, *p*-Coumaric, or *p*-Coutaric Acids in a Noncommercial Oregon Pinot Noir Wine 56 days after Inoculation with Various Strains of *B. brwcellensis*[a]

strain number	caffeic acid (mg/L)	caftaric acid (mg/L)	*p*-coumaric acid (mg/L)	*p*-coutaric acid (mg/L)
none	2.70[a]	26.3[a]	1.04[a]	7.46[a]
493	2.74[a]	26.0[a]	0.77[b]	7.29[a]
495	2.69[a]	26.3[a]	0.70[b]	7.39[a]
496	2.76[a]	26.1[a]	0.79[b]	7.41[a]
497	2.66[a]	26.1[a]	0.72[b]	7.34[a]
607	2.58[a]	26.1[a]	0.65[b]	7.31[a]
613	2.58[a]	25.7[a]	0.74[b]	7.28[a]
614	2.55[a]	25.8[a]	0.72[b]	7.30[a]
615	2.55[a]	25.9[a]	0.72[b]	7.29[a]
616	2.58[a]	25.9[a]	0.70[b]	7.26[a]
635	2.68[a]	26.1[a]	0.70[b]	7.33[a]
643	2.57[a]	25.9[a]	0.62[b]	7.25[a]

[a]Means within a column with different superscripts are significant at $p \leq 0.05$.

Table 2. Chemical Analysis of Commercially Prepared Wines without and with Inoculation of *B. bruxellensis* Strains B5, I1a, B1b, or E1[a]

wine	strain inoculated	volatile acidity (g/L)	4-ethylphenol (μg/L)	4-ethylguaiacol (μg/L)
Cabernet Sauvignon	none	0.83[cde]	7.1[f]	nd
	B5	0.87[bcd]	53.4[f]	21.1[f]
	I1a	1.1[a]	3570[bcd]	1020[a]
	B1b	0.99[b]	3220[de]	759[bcd]
	E1	0.94[bc]	3420[cd]	734[cd]
Merlot	none	0.39[l]	nd	nd
	B5	0.40[kl]	95.8[f]	31.4[f]
	I1a	0.68[fghi]	3810[abc]	1040[a]
	B1b	0.67[ghi]	3810[abc]	947[ab]
	E1	0.61[hij]	3930[abc]	860[abc]
Syrah	none	0.58[ij]	34.3[f]	7.7[f]
	B5	0.57[ij]	91.7[f]	17.3[f]
	I1a	0.84[cde]	4110[ab]	492[e]
	B1b	0.73[efgh]	4160[a]	454[e]
Pinot noir	none	0.57[ij]	nd	nd
	B5	0.53[jk]	102[f]	21.9[f]
	I1a	0.74[defg]	2790[e]	606[de]
	B1b	0.80[def]	2850[e]	536[e]

[a]Means within a column with different superscripts are significant at $p \leq 0.05$. nd = not detected.

the caffeic acid in the Pinot noir prepared at Oregon State University. Concentrations of *p*-coumaric acid in the Pinot noir from Oregon were low compared to other wines, possibly through partial condensation of vinylphenols with anthocyanins.[32] Using strains from Australia, Harris et al.[33] demonstrated complete uptake of *p*-coumaric acid, 80−100% conversion of ferulic acid, and only 25% utilization of caffeic acid. However, Godoy et al.[34] noted that their strains exhibited a 20% greater specificity for caffeic acid compared to *p*-coumaric acid. While Edlin et al.[6] reported that differences in the specificity of cinnamate decarboxylase between strains of *B. anomalus*, it is not known whether this variation is also valid for

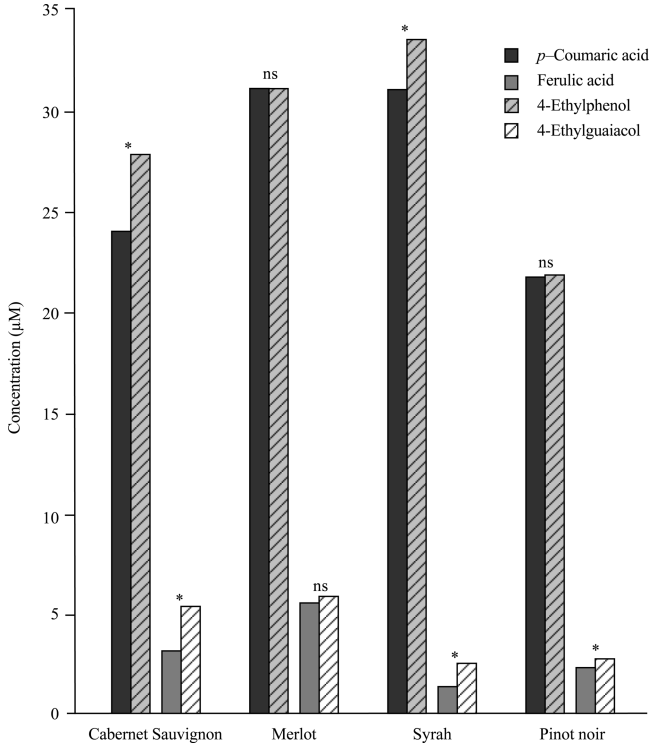

Figure 6. Paired comparisons of *p*-coumaric acid or ferulic acid to their respective products, 4-ethylphenol or 4-ethylguaiacol, based on pooled data from three strains of *Brettanomyces* inoculated into four commercial wines. Significant differences ($p \leq 0.05$) between pairings are indicated by "*", while no significance is designated by "ns".

B. bruxellensis which would provide an explanation for these results.

Concentrations of tartaric acid esters of the hydroxycinnamic acids found in the commercially prepared wines were similar to the concentrations previously reported by Nagel et al.,[16] although fertaric acid was not found in the wines. However, none of the strains were able to metabolize these esters, either the caftaric or *p*-coutaric acids. As Hixson et al.[20] reported that the yeast could utilize ethyl coumarate and ethyl ferulate, it is possible that esterase enzymes may be specific only toward ethyl esters. In any case, it appears that tartaric acid esters are not metabolized by *Brettanomyces* under wine conditions.

Strains I1a, B1b, and E1 generated concentrations of 4-ethylphenol and 4-ethylguaiacol well above the reported sensory thresholds in red wine of 605 μg/L and 110 μg/L, respectively.[3] Here, I1a formed 4,110 μg/L 4-ethylphenol in Syrah and 1040 μg/L 4-ethylguaiacol in Merlot. Although B5 also utilized some *p*-coumaric and ferulic acids, the concentrations of 4-ethylphenol and 4-ethylguaiacol produced were below sensory thresholds, suggesting that declining yet viable populations is not as important as actively expanding populations regarding synthesis of volatile phenols.

Comparing precursor to product, the amounts of non-esterified hydroxycinnamic acids consumed were generally significantly less compared to the molar concentration of volatile phenols formed for Cabernet Sauvignon, Syrah, and Pinot noir. While Pinot noir only exhibited significant differences in ferulic acid/4-ethylguaiacol, no differences were observed between precursor/products for Merlot. These data suggest that *Brettanomyces* may have metabolized vinylphenols that were formed during alcoholic and/or malolactic

fermentations. Several microorganisms found in grape musts and/or wines can synthesize 4-vinylphenol or 4-vinylguaiacol from *p*-coumaric or ferulic acids, respectively, including *Acetobacter*, *Oenococcus oeni*, *Lactobacillus hilgardii*, *L. plantarum*, *L. brevis*, *Pediococcus pentosaceus*, *P. damnosus*, and *Saccharomyces*.[3,35,36] While it is possible that some 4-vinylphenol or 4-vinylguaiacol was produced in Cabernet Sauvignon and Syrah wine but not in the Merlot, reasons for differences in the Pinot noir are not understood. Alternatively, there may be additional, unidentified phenolic precursors in some grape cultivars but not others that are involved in volatile phenol synthesis.

Given the much higher concentrations of caffeic acid compared to *p*-coumaric or ferulic acids present in Washington wines and that all regional strains tested could metabolize nonesterified hydroxycinnamic acid, 4-ethylcatechol formed from its metabolism may have more of a sensory impact than previously thought. Furthermore, variability in the ability of *B. bruxellensis* strains to degrade caffeic acid may result in large variability in the concentration of 4-ethylcatechol in wines regardless of the amount of caffeic acid present. Concentrations of caffeic acid have been reported to range worldwide from 0.26 to 26 mg/L.[37-40] Although less volatile than 4-ethylphenol or 4-ethylguaiacol, Botha[41] noted that 4-ethylcatechol suppressed "berry-like" wine characters in Pinotage wine yet elevated "Band-aid" sensory descriptors associated with 4-ethylphenol. Alone, 4-ethylcatechol has been described as having a "spicy", "woody", "smoky", or "horsey" odor.[42,43] Given the range of sensory descriptors used for the different volatile phenols, specific contributions of 4-ethylcatechol coupled to the others require additional study.

AUTHOR INFORMATION

Corresponding Author

*E-mail: edwardsc@wsu.edu. Tel: (509) 335-6612. Fax: (509) 335-4815.

Funding

The authors express their gratitude to the Washington Wine Advisory Committee for financial support and to regional wineries for participation in this study.

Notes

This research was conducted in support of Project No. 0846 at the School of Food Science, Washington State University, Pullman, WA, 99164-6376 as part of the thesis of L. M. Schopp. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

The authors declare no competing financial interest.

ABBREVIATIONS USED

HPLC-DAD, high performance liquid chromatography with diode array detection; PVDF, polyvinylidene difluoride; SPE, solid phase extraction; YM, yeast/mold medium; WL, Wallerstein Laboratories medium

REFERENCES

(1) Loureiro, V.; Malfeito-Ferreira, M. *Dekkera/Brettanomyces* spp. In *Food Spoilage Microorganisms*; Blackburn, C. W., Ed; CRC Press: Boca Raton, FL, 2006; pp 354−398.

(2) Puig, A.; Bertran, E.; Franquet, R.; García, J.; Mínguez, S. *Brettanomyces bruxellensis* prevalence in wines produced and marketed in Spain. *Ann. Microbiol.* **2011**, *61*, 145−151.

(3) Chatonnet, P.; Dubourdieu, D.; Boidron, J.-N.; Pons, M. The origin of ethylphenols in wines. *J. Sci. Food Agric.* **1992**, *60*, 165−178.

(4) Chatonnet, P.; Dubourdieu, D.; Boidron, J.-N.; Lavigne, V. Synthesis of volatile phenols by *Saccharomyces cerevisiae* in wines. *J. Sci. Food Agric.* **1993**, *62*, 191−202.

(5) Licker, J. L.; Acree, T. E.; Henick-Kling, T. What is "Brett" (*Brettanomyces*) flavor?: A preliminary investigation. In *Chemistry of Wine Flavor*; ACS symposium series 714. Waterhouse, A. L.; Ebeler, S. E., Eds.; American Chemical Society: Washington, DC, 1998; pp 96−115.

(6) Edlin, D. A. N.; Narbad, A.; Dickinson, J. R.; Lloyd, D. The biotransformation of simple phenolic compounds by *Brettanomyces anomalus*. *FEMS Microbiol. Lett.* **1995**, *125*, 311−316.

(7) Dias, L.; Pereira-da-Silva, S.; Tavares, M.; Malfeito-Ferreira, M.; Loureiro, V. Factors affecting the production of 4-ethylphenol by the yeast *Dekkera bruxellensis* in enological conditions. *Food Microbiol.* **2003**, *20*, 377−384.

(8) Barata, A.; Pagliara, D.; Piccininno, T.; Tarantino, F.; Ciardulli, W.; Malfeito-Ferreira, M.; Loureiro, V. The effect of sugar concentration and temperature on growth and volatile phenol production by *Dekkera bruxellensis* in wine. *FEMS Yeast Res.* **2008**, *8*, 1097−1102.

(9) Barbin, P.; Cheval, J.-L.; Gilis, J.-F.; Strehaiano, P.; Taillandier, P. Diversity in spoilage yeast *Dekkera/Brettanomyces bruxellensis* isolated from French red wine. Assessment during fermentation of synthetic wine medium. *J. Inst. Brew.* **2008**, *114*, 69−75.

(10) Romano, A.; Perello, M. C.; de Revel, G.; Lonvaud−Funel, A. Growth and volatile compound production by *Brettanomyces/Dekkera bruxellensis* in red wine. *J. Appl. Microbiol.* **2008**, *104*, 1577−1585.

(11) Vigentini, I.; Romano, A.; Compagno, C.; Merico, A.; Molinari, F.; Tirelli, A.; Foschino, R.; Volonterio, G. Physiological and oenological traits of different *Dekkera/Brettanomyces bruxellensis* strains under wine−model conditions. *FEMS Yeast Res.* **2008**, *8*, 1087−1096.

(12) Benito, S.; Palomero, F.; Morata, A.; Calderón, F.; Suárez-Lepe, J. A. Factors affecting the hydroxycinnamate decarboxylase/vinylphenol reductase activity of *Dekkera/Brettanomyces*: Application for *Dekkera/Brettanomyces* control in red wine making. *J. Food Sci.* **2009**, *74*, M15−M22.

(13) Oelofse, A.; Lonvaud-Funel, A.; du Toit, M. Molecular identification of *Brettanomyces bruxellensis* strains isolated from red wines and volatile phenol production. *Food Microbiol.* **2009**, *26*, 377−385.

(14) Conde, C.; Silva, P.; Fontes, N.; Dias, A. C. P.; Tavares, R. M.; Sousa, M. J.; Agasse, A.; Delrot, S.; Gerós, H. Biochemical changes throughout grape berry development and fruit and wine quality. *Food* **2007**, *1*, 1−22.

(15) Nagel, C. W.; Wulf, L. W. Changes in the anthocyanins, flavonoids and hydroxycinnamic acid esters during fermentation and aging of Merlot and Cabernet Sauvignon. *Am. J. Enol. Vitic.* **1979**, *30*, 111−116.

(16) Nagel, C. W.; Baranowski, J. D.; Wulf, L. W.; Powers, J. R. The hydroxycinnamic acid tartaric acid ester content of musts and grape varieties grown in the Pacific Northwest. *Am. J. Enol. Vitic.* **1979**, *30*, 198−201.

(17) García-Falcón, M. S.; Pérez-Lamela, C.; Martínez-Carballo, E.; Simal-Gándara, J. Determination of phenolic compounds in wines: Influence of bottle storage of young red wines on their evolution. *Food Chem.* **2007**, *105*, 248−259.

(18) Ginjom, I.; D'Arcy, B.; Caffin, N.; Gidley, M. Phenolic compound profiles in selected Queensland red wines at all stages of the wine-making process. *Food Chem.* **2011**, *125*, 823−834.

(19) Spaepen, M.; Verachtert, H. Esterase-activity in the genus *Brettanomyces*. *J. Inst. Brew.* **1982**, *88*, 11−17.

(20) Hixson, J. L.; Sleep, N. R.; Capone, D. L.; Elsey, G. M.; Curtin, C. D.; Sefton, M. A.; Taylor, D. K. Hydroxycinnamic acid ethyl esters as precursors to ethylphenols in wine. *J. Agric. Food Chem.* **2012**, *60*, 2293−2298.

(21) Nikfardjam, M. P.; May, B.; Tschiersch, C. 4-Ethylphenol and 4-ethylguaiacol contents in bottled wines from the German 'Württemberg' region. *Eur. Food Res. Tech.* **2009**, *230*, 333−341.
(22) Jensen, S. L.; Umiker, N. L.; Arneborg, N.; Edwards, C. G. Identification and characterization of *Dekkera bruxellensis, Candida pararugosa,* and *Pichia guilliermondii* isolated from commercial red wines. *Food Microbiol.* **2009**, *26*, 915−921.
(23) Jaworski, A. W.; Lee, C. Y. Fractionation and HPLC determination of grape phenolics. *J. Agric. Food Chem.* **1987**, *35*, 257−259.
(24) Lee, J.; Finn, C. E. Anthocyanins and other polyphenolics in American elderberry (*Sambucus canadensis*) and European elderberry (*S. nigra*) cultivars. *J. Sci. Food Agric.* **2007**, *87*, 2665−2675.
(25) Lee, J.; Scagel, C. F. Chicoric acid found in basil (*Ocimum basilicum* L.) leaves. *Food Chem.* **2009**, *115*, 650−656.
(26) Baranowski, J. D.; Nagel, C. W. Isolation and identification of the hydroxycinnamic acid derivatives in White Riesling wine. *Am. J. Enol. Vitic.* **1981**, *32*, 5−13.
(27) Burns, T. R.; Osborne, J. P. Impact of malolactic fermentation on color and color stability of Pinot noir and Merlot wine. *Am. J. Enol. Vitic.* **2013**, *64*, 370−377.
(28) Edwards, C. G.; Watson, B. A. *Basic Microbiological and Chemical Analyses for Wine*; Washington State University Cooperative Extension EM047: Pullman, WA, 2013.
(29) Fugelsang, K. C.; Zoecklein, B. W. Population dynamics and effects of *Brettanomyces bruxellensis* strains on Pinot noir (*Vitis vinifera* L.) wines. *Am. J. Enol. Vitic.* **2003**, *54*, 294−300.
(30) Medawar, W.; Strehaiano, P.; Délia, M.-L. Yeast growth: Lag phase modeling in alcoholic media. *Food Microbiol.* **2003**, *20*, 527−532.
(31) Conterno, L.; Joseph, C. M. L.; Arvik, T. J.; Henick-Kling, T.; Bisson, L. F. Genetic and physiological characterization of *Brettanomyces bruxellensis* strains isolated from wines. *Am. J. Enol. Vitic.* **2006**, *57*, 139−147.
(32) Benito, S.; Palomero, F.; Morata, A.; Uthurry, C.; Suárez-Lepa, J. A. Minimization of ethylphenol precursors in red wines via the formation of pyranoanthocyanins by selected yeasts. *Int. J. Food Microbiol.* **2009**, *132*, 145−152.
(33) Harris, V.; Ford, C. M.; Jiranek, V.; Grbin, P. R. *Dekkera* and *Brettanomyces* growth and utilisation of hydroxycinnamic acids in synthetic media. *Appl. Microbiol. Biotechnol.* **2008**, *78*, 997−1006.
(34) Godoy, L.; Martínez, C.; Carrasco, N.; Ganga, M. A. Purification and characterization of a *p*-coumarate decarboxylase and a vinylphenol reductase from *Brettanomyces bruxellensis*. *Int. J. Food Microbiol.* **2008**, *127*, 6−11.
(35) Chatonnet, P.; Dubourdieu, D.; Boidron, J.-N. The influence of *Brettanomyces/Dekkera* sp. yeasts and lactic acid bacteria on the ethylphenol content on red wines. *Am. J. Enol. Vitic.* **1995**, *46*, 463−468.
(36) Shinohara, T.; Kubodera, S.; Yanagida, F. Distribution of phenolic yeasts and production of phenolic off-flavors in wine fermentation. *J. Biosci. Bioeng.* **2000**, *90*, 90−97.
(37) Soleas, G. J.; Dam, J.; Carey, M.; Goldberg, D. M. Toward the fingerprinting of wines: Cultivar-related patterns of polyphenolic constituents in Ontario wines. *J. Agric. Food Chem.* **1997**, *45*, 3871−3880.
(38) Ritchey, J. G.; Waterhouse, A. L. A standard red wine: Monomeric phenolic analysis of commercial Cabernet Sauvignon wines. *Am. J. Enol. Vitic.* **1999**, *50*, 91−100.
(39) Landrault, N.; Poucheret, P.; Ravel, P.; Gasc, F.; Cros, G.; Teissedre, P.-L. Antioxidant capacities and phenolics levels of French wines from different varieties and vintages. *J. Agric. Food Chem.* **2001**, *49*, 3341−3348.
(40) Monagas, M.; Suárez, R.; Gómez-Cordovés, C.; Bartolomé, B. Simultaneous determination of nonanthocyanin phenolic compounds in red wines by HPLC-DAD/ESI-MS. *Am. J. Enol. Vitic.* **2005**, *56*, 139−147.
(41) Botha, J. J. Sensory chemical and consumer analysis of *Brettanomyces* spoilage in South African wines. M.S. Thesis, Stellenbosch University, South Africa, 2010.
(42) Hesford, F.; Schneider, K.; Porret, N.; Gafner, J. Identification and analysis of 4−ethyl catechol in wines tainted by *Brettanomyces* off-flavor. *Am. J. Enol. Vitic.* **2004**, *55*, 304A.
(43) Larcher, R.; Nicolini, G.; Bertoldi, D.; Nardin, T. Determination of 4-ethylcatechol in wine by high-performance liquid chromatography-coulometric electrochemical array detection. *Anal. Chim. Acta* **2008**, *609*, 235−240.