Contribution of Wine Components to Inactivation of Food-Borne Pathogens

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ABSTRACT: Wine is a complex solution containing several components with several likely antimicrobial properties. Low pH (3.0 to 4.0), high organic acid content (titratable acidity ≥6.0 g/L tartaric acid), relatively high ethanol (10% to 15%), and potentially high total sulfur dioxide (0 to 300 ppm) may contribute to inactivation of food-borne pathogens when exposed to wine. The objective was to determine the effect of these 4 parameters on reducing populations of *Escherichia coli* (*E. coli*) O157:H7 and *Staphylococcus aureus*. A factorial design was used to test variables (pH, titratable acidity, sulfur dioxide, ethanol) in combinations of low, medium, and high levels. Suspension tests were performed to compare the efficacy of 81 treatments with controlled exposure time of 20 min. *Staphylococcus aureus* was significantly more resistant to wine treatment than *E. coli* O157:H7. Stepwise regression analysis of *S. aureus* inactivation revealed the ordered impact of pH, molecular sulfur dioxide, titratable acidity, and ethanol concentration. Selected analysis of *E. coli* inactivation revealed the importance of pH and ethanol in predicting inactivation. Total and free sulfur dioxide were not predictive of inactivation of either pathogen. Wine-based solutions may have application as surface disinfectants for food surfaces and food contact equipment. Wine destined to be used as a disinfectant could be enhanced by increasing any of the parameters tested in this study; however, lowering the pH would be the most effective and would likely enhance the efficacy of the other parameters. Additional wine components such as volatile acidity and phenolics were not evaluated but may also contribute to the antimicrobial properties of wine.

Keywords: antibacterial, disinfectant, *Escherichia coli* O157:H7, *Staphylococcus aureus*, wine

Introduction

Microbial contamination of the domestic home environment may be contributing to upwards of 19% of food-borne outbreaks (Zhao and others 1998; Tierney and others 2002). Some percentage of these outbreaks is likely due to poor household hygiene. Targeted disinfectant use may minimize the infection risk in the home (Scott and others 1984; Josephson and others 1997). Household disinfectants typically contain chlorine or ammonium chloride based compounds that may not appeal to all consumers. An alternative, more "consumer friendly" spectrum of household disinfectants has started to appear in the marketplace. These often contain naturally occurring organic acids such as citric and acetic acids. There is another class of products that contain hydrogen peroxide. Earlier observations in our laboratory confirmed that wine possesses significant antibacterial activity, which led us to investigate applications in food safety.

Wine is a complex solution containing a number of antimicrobial parameters. Several studies have demonstrated the possibility for wine consumption to protect individuals from food-borne illness as well as protection against *Helicobacter pylori* infections (Sheth and others 1988; Weisse and others 1995; Luzza and others 1998; Brenner and others 2001; Just and Daeschel 2003). Several studies have investigated the efficacy of wine against food-borne pathogens using suspension tests. Weisse and others (1995) investigated the effectiveness of wine against *Salmonella enteritidis*, *Shigella sonnei*, and *Escherichia coli*, leading to 5- to 6-log inactivation within 20 min of exposure time. Marimon and others (1998b) found red wine to be effective at reducing numbers of *H. pylori* by 8-log CFU/mL with 5 min of exposure time. Moretro and Daeschel (2004) determined the efficacy of a red and white wine against various strains of *S. aureus*, *Listeria monocytogenes*, *E. coli* O157:H, and *Salmonella typhimurium*. Moreover, Friedman and others (2006) demonstrated that wine solutions provide and effective solvent for enhanced antimicrobial activity of several plant essential oils. Properties of wine in itself that are considered to be antimicrobial include ethanol, low pH, high levels of organic acids, and sulfur dioxide.

High concentrations of ethanol are used in laboratories to decontaminate surfaces with short exposure times (Block 1991; Huang and others 2001). Extensive research on ethanol toxicity to yeast and lactic acid bacteria have been studied due to their importance in beer and wine production (Leao and Van Uden 1984; Ingram 1986; Brewer and others 2002).

Sulfur dioxide present in wine may come from 2 different sources. Sulfur dioxide is a natural byproduct of yeast metabolism which may contribute levels between 10 and 40 mg/L (Usseglio-Tomasset 1992; Heinzel 1998). Winemakers may also add sulfur dioxide, in various forms, primarily to control oxidation and prevent microbial spoilage by wild yeast and *Acetobacter* spp. throughout the winemaking process (Dott and others 1976; Usseglio-Tomasset 1992; Carrete and others 2002).

Wine is an acidic environment, primarily due to the presence of tartaric, malic, and lactic acids. Organic acids are known to possess antimicrobial properties, but their effectiveness is dependent on the type of acid, the concentration of the acid, dissociation level, and pH (Uljas and Ingham 1999; Marshall and others 2000). Most wine generally has a pH in the range of 3.0 to 4.0. The pH has considerable influence on the effectiveness of antimicrobial

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compounds. A low pH can cause a loss of enzyme function; however, low pH alone does not ensure sterilization (Uljas and Ingham 1999; Kobayashi and others 2000). Decreasing pH enhances the activity of ethanol against microorganisms, shifts the equilibrium of organic acids towards the undissociated form, and increases the titratable acidity (Jordan and others 1999).

A systematic antimicrobial assessment of combinations of the aforementioned wine components could lead to the optimization a wine-based disinfectant as a useful product to minimize cross contamination in the domestic environment. Waste wine from industry could be utilized as an affordable base to produce an optimized disinfectant. A white wine based disinfectant could provide the necessary combination of antimicrobial compounds to inactivate pathogens in the household without introducing "unfriendly chemicals." The use of red wine would be limited because of possible issues related to staining. This study was designed to look at 4 wine parameters, pH, titratable acidity, sulfur dioxide concentration, and ethanol concentration, in various combinations within a wine background to evaluate antimicrobial activity against the food-borne pathogens *S. aureus* and *E. coli* O157:H7.

Materials and Methods

Culture information

Bacterial strains used for these experiments were from the author's collection and are designated as *S. aureus* (710) and *E. coli* O157:H7 (716). Each strain was cultured in brain-heart infusion broth (BHI, DIFCO, Becton Dickinson, Cockeysville, Md., U.S.A.) at 37 °C and overnight cultures (stationary phase cells) were used for all experiments. All experiments were performed in a class II biological safety cabinet. Following treatment, strains were enumerated on BHI agar for all experiments. *E. coli* plates were enumerated after incubation overnight at 37 °C. *S. aureus* plates were incubated for 48 h prior to enumeration.

Treatments

The base wine for these experiments was Badger Mountain (no sulfites added) Organic Chardonnay 2002 Columbia Valley. To this wine, combinations of pH, titratable acidity, sulfur dioxide, and ethanol were added in a factorial design with 3 levels of each treatment (Table 1). The pH was adjusted using 6N hydrochloric acid to either pH 3.25 or pH 3.00 as measured by a pH meter (Digital Ionalyzer/501, Orion Research, Boston, Mass., U.S.A.). Titratable acidity was adjusted by adding 37%(w/v) tartaric acid to increase the titratable acidity by 2 or 4 g/L. Sulfur dioxide levels were adjusted by adding potassium metabisulfite to increase the total sulfur dioxide concentration by 50 ppm or 150 ppm. Ethanol levels were adjusted by adding 95% ethanol to increase the alcohol concentration by 1.5% or 3.0% (v/v).

Table 1—Factorial design of wine treatments. Letters indicated designed adjustment for wine samples: pH (A = base [3.7], B = 3.25, C = 3.00), titratable acidity (D = base [6.4 g/L], E = 8.4 g/L, F = 10.4 g/L), total sulfur dioxide (G = base [27.9 ppm], H = 77.9 ppm, I = 177.9 ppm), and ethanol (J = base [12.0%], K = 13.5%, L = 15.0%)

ADGJ	AEGJ	AFGJ	BDGJ	BEGJ	BFGJ	CDGJ	CEGJ	CFGJ
ADGK	AEGK	AFGK	BDGK	BEGK	BFGK	CDGK	CEGK	CFGK
ADGL	AEGL	AFGL	BDGL	BEGL	BFGL	CDGL	CEGL	CFGL
ADHJ	AEHJ	AFHJ	BDHJ	BEHJ	BFHJ	CDHJ	CEHJ	CFHJ
ADHK	AEHK	AFHK	BDHK	BEHK	BFHK	CDHK	CEHK	CFHK
ADHL	AEHL	AFHL	BDHL	BEHL	BFHL	CDHL	CEHL	CFHL
ADIJ	AEIJ	AFIJ	BDIJ	BEIJ	BFIJ	CDIJ	CEIJ	CFIJ
ADIK	AEIK	AFIK	BDIK	BEIK	BFIK	CDIK	CEIK	CFIK
ADIL	AEIL	AFIL	BDIL	BEIL	BFIL	CDIL	CEIL	CFIL

Wine analyses

Wine samples were stored under refrigerated conditions until analyses were completed. Final pH was measured using a pH meter (Digital Ionalyzer/501). Titratable acidity was determined following the method by Zoecklein and others (1990). Alcohol content was determined by boiling point depression with an ebulliometer. Free and total sulfite levels were determined by the pararosaniline method (Grant 1947; Morris 2003) as described (AOAC 1990), using standards of 5, 10, 15, 20, 50, 100, 150, and 200 ppm sulfur dioxide. Molecular sulfur dioxide levels were calculated using free sulfur dioxide levels and pH (Usseglio-Tomasset 1992).

Suspension tests

Stationary phase (16 to 20 h) cultures (approximately 10^9 CFU/mL) were used as the inoculum for suspension tests. A volume of 9.9 mL of wine sample was transferred to sterile 17 × 100 mm plastic culture tubes with dual position closures (VWR Intl., West Chester, Pa., U.S.A.). A volume of 0.1 mL of overnight culture was transferred to the wine. Suspensions were vortexed immediately after inoculation and again prior to plating. Samples were plated after 20 min of exposure time. Initial counts were determined from enumerative plating of overnight culture and corrected mathematically for the dilution factor used in suspension tests (approximately 10^7 CFU/mL). All enumerative plating was performed using a spiral plater (Autoplate 4000, Spiral Biotech, Norwood, Mass., U.S.A.) necessary dilutions were created using Butterfield's phosphate buffer. Suspension tests were performed in duplicate for each strain for each of the wine samples.

Statistics

Regression analysis and ANOVA (SAS 9.1., SAS Institute Inc., Cary, N.C., U.S.A.) were used for significance testing. Stepwise regression was performed to determine the impact of each parameter on inactivation of *S. aureus* and *E. coli* O157:H7.

Results and Discussion

Wine analyses

The base wine was adjusted for each of the 80 modified wine samples. Values of pH, titratable acidity, total sulfur dioxide, free total sulfur dioxide, molecular sulfur dioxide, and ethanol were determined for each wine sample and are given in Table 2. As expected, decreases in pH led to increases in titratable acidity and vice versa. Decreasing the pH and/or increasing the titratable acidity also caused an increase in the concentration of molecular sulfur dioxide concentration. Increasing concentrations of sulfur dioxide or ethanol did not affect the values for other parameters measured in this study.

Comparison of inactivation of *S. aureus* and *E. coli* O157:H7 by base wine

Inactivations of *S. aureus* and *E. coli* O157:H7 as observed by suspension test in the base wine (ADGJ) are shown in Figure 1. *S. aureus* was significantly more resistant to the base wine treatment than *E. coli* O157:H7. Similar results were found by Moretro and Daeschel (2004) with *S. aureus* and *L. monocytogenes* being more resistant to inactivation by wine treatments than *E. coli* O157:H7 and *Salmonella typhimurium*. *E. coli* O157:H7 was more sensitive to all of the wine treatments than *S. aureus*. A majority of the wine samples elicited inactivation to the detection limit of the assay.

Table 2—Average values of pH, titratable acidity, sulfur dioxide levels (total, free, and molecular), and ethanol of adjusted wine samples

Sample	pH	Titratable acidity (g/L)	Total sulfur dioxide (ppm) (G, H, I)	Free sulfur dioxide (ppm) (G, H, I)	Molecular sulfur dioxide (ppm) (G, H, I)	Ethanol (% v/v) (J, K, L)
AD	3.72	6.42			0.05, 0.08, 0.13	
AE	3.51	8.73			0.15, 0.22, 0.29	
AF	3.27	10.55			0.26, 0.34, 0.43	
BD	3.21	7.65			0.30, 0.48, 0.82	
BE	3.03	9.69	27.9, 78.6, 191.1	3.9, 24.5, 115.0	0.94, 1.40, 1.84	12.1, 13.3, 14.7
BF	2.91	11.09			1.64, 2.15, 2.70	
CD	2.96	8.37			1.39, 2.23, 3.84	
CE	2.84	10.04			4.39, 6.59, 8.64	
CF	2.74	12.10			7.71, 10.11, 12.67	

Therefore, the analysis of the data from these experiments will focus primarily on *S. aureus* with conclusions being valid for both species tested unless otherwise indicated.

Analysis of combination treatments

A stepwise regression statistical analysis of *S. aureus* inactivation data was performed and the following parameters contributed significantly to the efficacy of the wine treatment in the following order: pH, molecular sulfur dioxide, titratable acidity, and ethanol (model $R^2 = 0.76$). An identical statistical analysis was performed with inactivation of *E. coli* O157:H7 by wine treatments; however, results were skewed due to the large number of samples resulting in inactivation beyond detection limit due to the relative sensitivity of *E. coli* O157:H7 to the treatment, resulting in a poor R^2 value (model $R^2 = 0.40$). These data points were removed and the remaining data were used to perform the stepwise regression. Inactivation of *E. coli* O157:H7 could be reasonably predicted by pH and ethanol concentration (model $R^2 = 0.74$).

pH

Inactivation of *S. aureus* grouped by pH values is shown in Figure 2. pH was the most contributory factor that explained the inactivation of *S. aureus* and *E. coli* O157:H7 by various wine samples. Significant differences were seen between samples with different pH values. Samples with pH values below 2.84 were the most efficacious at inactivating *S. aureus*, with an average log reduction (CFU/mL) of 6.13. Samples with pH values above 3.27 were the least effective against *S. aureus* with an average log reduction (CFU/mL) of 1.29.

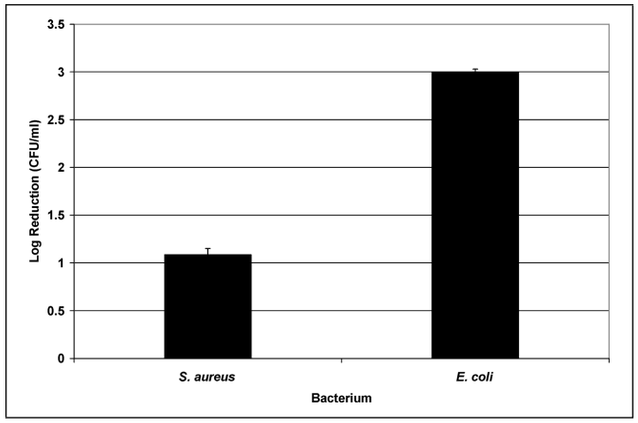

Figure 1—Inactivation of *S. aureus* and *E. coli* by suspension in base wine (ADGJ) for 20 min. Error bars indicate standard error, $n = 2$.

Sulfur dioxide

Following pH, molecular sulfur dioxide concentration was the next most significant factor in determining efficacy of wine samples against *S. aureus*. Figure 3 displays the inactivation of *S. aureus* by various pooled wine sample treatments. Molecular sulfur dioxide levels are calculated based on free sulfur dioxide levels and pH of the sample solution. Samples were grouped by pH so that direct comparisons could be made between samples that only differed in sulfur dioxide levels. Samples are presented in order of decreasing pH. Within the pH groups, only two of these groups showed significant differences between sulfur dioxide levels. CDG (molecular sulfur dioxide = 0.26 ppm) was significantly less effective against *S. aureus* than CDH (1.64 ppm), which was significantly less effective than CDI (7.71 ppm) with average log reductions (CFU/mL) of 1.82, 4.84, and 6.85, respectively. CFG (0.43 ppm) was significantly less effective than both CFH (2.70 ppm) and CFI (12.67 ppm) with average log reductions (CFU/mL) of 4.58, 6.36, and 6.87, respectively. Other pH groups displayed this same trend with inactivation increasing with increasing molecular sulfur dioxide concentrations; however, the differences were not statistically significant. While molecular sulfur dioxide levels are dependent on total sulfur dioxide and free sulfur dioxide levels, these values did not contribute significantly to inactivation by wine samples.

Titratable acidity

Titratable acidity (TA) was significant in predicting inactivation of *S. aureus* by wine samples; however, like molecular sulfur dioxide, there is a relationship with pH. This relationship between titratable acidity and pH was apparent in the analysis of the wine samples. Figure 4 displays the impact of pH and titratable acidity on inactivation of *S. aureus*; bubble size indicates the extent of inactivation with the average log reduction (CFU/mL) shown numerically within the bubble. As titratable acidity increases and pH decreases, effectiveness of the treatment is enhanced; however, pH is the predominant factor statistically. The most effective treatments were those with titratable acidities of 10.04 and 12.10 g/L, which were the samples with the lowest pH values of 2.84 and 2.74, respectively. With small changes in pH, increases in titratable acidity caused increases in inactivation. This is especially apparent with the B pH family. BD (pH = 3.21, TA = 7.65 g/L) was significantly less effective than BE (3.03, 9.69 g/L), which was significantly less effective than BF (2.96, 11.09 g/L), causing average log reductions (CFU/mL) of 2.34, 3.73, and 4.50, respectively. Large changes in titratable acidity may not have much impact on inactivation of bacteria if the pH of the wine is high ($\geq$ 3.27). This is demonstrated by the A pH family, where increasing the titratable acidity from 6.42 g/L to 10.55 g/L did not significantly enhance

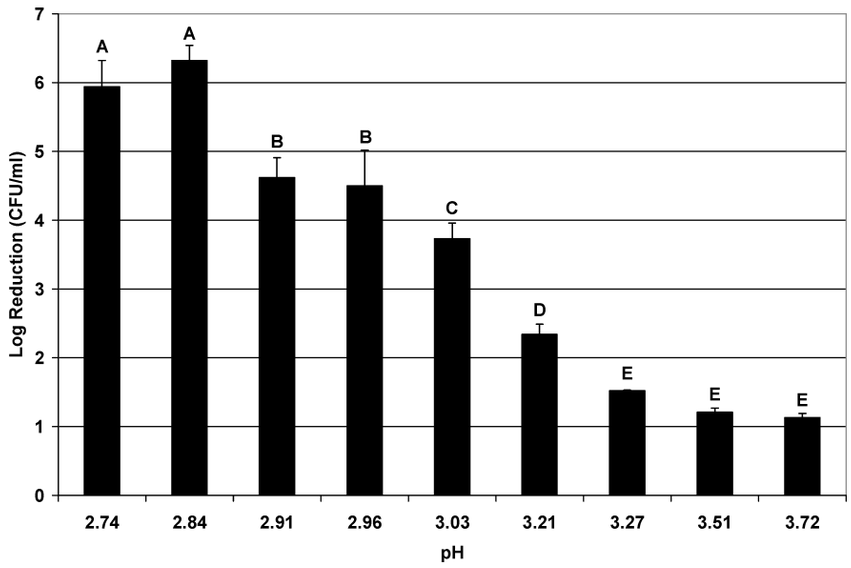

Figure 2—Inactivation of *S. aureus* in wine samples grouped by pH. Error bars indicate standard error, *n* = 18. Bars with the same letter are not significantly different.

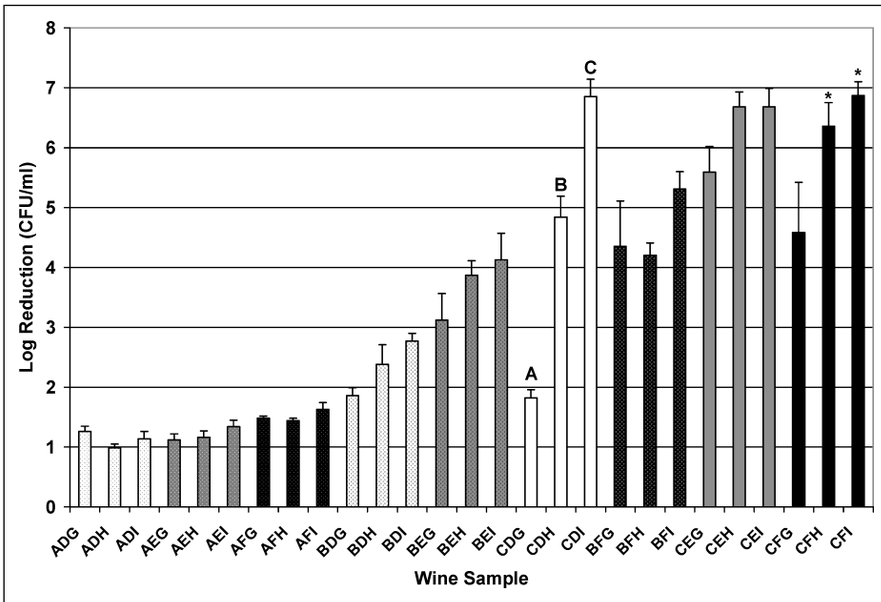

Figure 3—Impact of molecular sulfur dioxide on inactivation of *S. aureus* by various wine samples. Wine samples were pooled for each molecular sulfur dioxide concentration and are presented in order of decreasing pH; error bars indicate standard error, *n* = 12. Significant differences are expressed within the pH/titratable acidity groups (that is, AE). Log reduction bars with different letters indicate significant differences within the group (that is, CD). *indicates significant differences between sample CFG and CFH/CFI.

inactivation even with the corresponding decrease in pH (from 3.72 to 3.27).

Ethanol

Ethanol concentration contributed to inactivation of *S. aureus* and *E. coli* O157:H7 by wine samples. Figure 5 displays the effect of ethanol on pooled samples against *S. aureus*. The trend displays correlation between increasing ethanol concentration and increased inactivation. An ethanol concentration of 14.66% was significantly more effective than either 13.28% or 12.08%; the same was true for inactivation of *E. coli* O157:H7.

Discussion

Wine samples tested in this study were effective at inactivating *S. aureus* and *E. coli* O157:H7. *S. aureus* was significantly more resistant to inactivation by wine than *E. coli* O157:H7. Previous studies have shown Gram-positive organisms (*S. aureus* and *L. monocytogenes*) to be more resistant than Gram-negative organisms (*E. coli* O157:H7 and *Salmonella typhimurium*) when treated with wine (Moretro and Daeschel 2004).

This study determined in a stepwise manner the impact of selected wine components on inactivation of 2 food-borne pathogens. In order of importance, pH, molecular sulfur dioxide, titratable acidity, and ethanol concentration predicted inactivation of *S. aureus* in suspension tests with a treatment time of 20 min. For studies with *E. coli* O157:H7, inactivation was predicted by pH and ethanol concentration. Weisse and others (1995) found the combination of ethanol and low pH to be important when determining inactivation of *E. coli, Salmonella* sp., and *Shigella sonnei*. Marimon and others (1998a) found similar results with ethanol and pH combinations against *H. pylori*. Moretro and Daeschel (2004) found the combination of organic acid concentrations (malic and tartaric), ethanol (15%), and low pH (≤3.0) had significantly stronger antimicrobial activity than the effect of these components individually against various food-borne pathogens, indicating potential synergistic interactions between these components leading to an

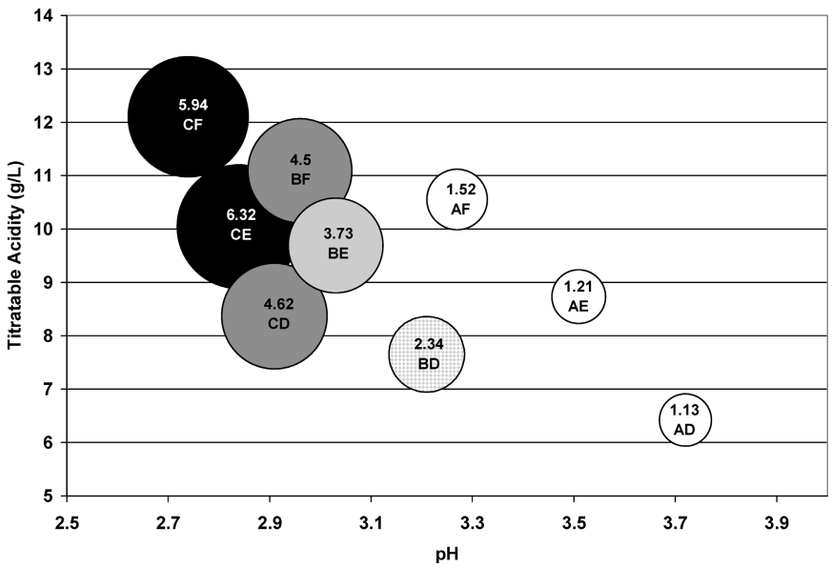

Figure 4—Impact of pH and titratable acidity on inactivation of *S. aureus* in wine samples. Area of bubble and number within circles indicate average log reduction (CFU/mL) for pooled samples with the same pH and titratable acidity, n = 18. Letters on circles indicate pooled samples. Bubbles with the same color/pattern are not significantly different.

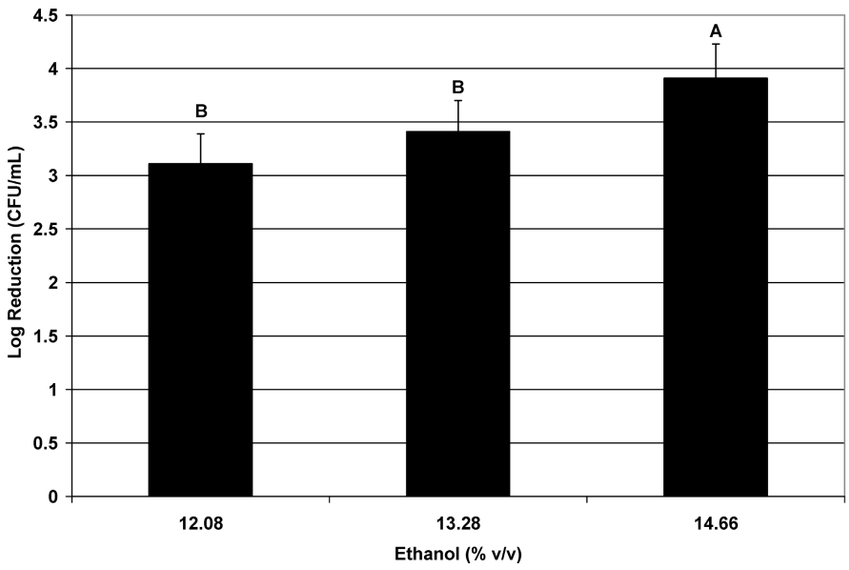

Figure 5—Impact of ethanol concentration on inactivation of *S. aureus* by wine treatment. Bars indicate mean of pooled samples within the same ethanol concentration. Error bars indicate standard error, *n* = 27. Bars with the same letter are not significantly different.

enhancement of antimicrobial activity. Decreases in pH will lead to an increase in the undissociated form of organic acids, which are considered to be the antimicrobially active species (Doores 1983). Ethanol is known to damage the cytoplasmic membrane, causing an increase in permeability of the membrane. These changes in membrane permeability may lead to enhanced efficacy of organic acids and may partly explain the difference in antimicrobial activity between grape juice and wine (Harding and Maidment 1996; Barker and Park 2001; Just and Daeschel 2003).

Additional components of the wine may impact inactivation, including phenolic compounds; however, this was not evaluated in this study. Soleas and others (1997) quantified the levels of various phenolic compounds (*cis*- and *trans*-resveratrol, gallic acid, caffeic acid, *p*-coumaric acid, vanillic acid, ferulic acid, and gentisic acid) in a number or red and white wines made in Ontario. Red wines, in general, contain much higher levels of both *cis*- and *trans*-resveratrol, gallic acid, vanillic acid, ferulic acid, and genistic acid. These differences have been used to explain the additional effectiveness often seen with red wines compared to white wines (Moretro and Daeschel 2004). Papadopoulou and others (2005) investigated the effectiveness of phenolic extracts of red and white wines at inactivating *S. aureus*, *E. coli*, and *Candida albicans*. Vaquero and others (2007) performed a similar study using red wines and investigating effectiveness against 7 different bacterial species. Phenolic fractions from wines showed marked antimicrobial activity, indicating some contribution of the phenolic compounds in inactivation of microorganisms by wine treatment. Several studies have investigated the impact of specific phenolic compounds on inhibiting microbial growth. Mahady and Pendland (2000) and Mahady and others (2003) determined a MIC_{50} value of 12.5 μg/mL of resveratrol against *H. pylori* strains using an agar disk diffusion assay. Chan (2002) used a broth dilution assay to determine the MIC of resveratrol against *S. aureus*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa* to be 171 to 342 μg/mL. Aziz and others (1998) investigated the inhibitory effect of several phenolic compounds against *E. coli*, *Klebsiella pneumoniae*, and *Bacillus cereus* using a suspension test. Caffeic acid and protocatechuic acid were effective at inhibiting the growth of *E. coli* and

K. pneumoniae at levels of 0.3 mg/mL. Vanillic acid and *p*-coumaric acid were capable of inhibiting growth of *E. coli, K. pneumoniae*, and *B. cereus* at levels of 0.4 mg/mL. While efficacy of phenolic compounds has been observed against various bacterial species, these studies have been performed using concentrations 10 to 1000 times greater than found in commercially available Charnonnay wines. Efficacy of the phenolic compounds in the wine may be enhanced by the inherent environment present in the wine (that is, pH, ethanol concentration, and so on); however, this impact is beyond the scope of this study as the concentration of phenolic compounds in all treatments was presumed to be identical.

Volatile acidity may also impact the efficacy of a specific wine treatment against various microorganisms. Sugita-Konishi and others (2001) found that the majority of antibacterial effect of wine against *Salmonella* Enteritidis, *E. coli* O157:H7, and *Vibrio parahaemolyticus* was due to the volatile components of wine. Preliminary results from additional experiments in our lab indicate the importance of volatile acidity when determining effectiveness of commercial Chardonnay wine samples against *S. aureus* (data not shown).

Alternatively, the efficacy of a wine-based disinfectant could be enhanced by the addition of antimicrobial compounds not traditionally associated with wine. Friedman and others (2006) investigated the impact of adding various essential oils derived from plants to Chardonnay, Pinot Noir, and Sherry. They found the effects of the wine and essential oils on inactivation of bacterial species to be additive.

Conclusions

The antimicrobial properties of wine have been confirmed against food-borne pathogens *S. aureus* and *E. coli* O157:H7. Of the factors tested in this study, pH was found to be the most critical factor in predicting inactivation of both *S. aureus* and *E. coli* O157:H7. Molecular sulfur dioxide, titratable acidity, and ethanol concentration also contributed to the inactivation of *S. aureus*. Ethanol concentration was also found to contribute the efficacy of wine treatments on *E. coli* O157:H7. Total sulfur dioxide and free sulfur dioxide were not predictive of wine efficacy against either pathogen tested. These findings indicate the importance of each parameter in wine to be used for potential disinfection purposes. Processors of a wine-based disinfectant may make adjustments to the wine to enhance the efficacy of the solution.

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