INACTIVATION OF FOODBORNE PATHOGENS BY FRUIT WINES

_____

A Thesis presented to
the Faculty of the Graduate School
at the University of Missouri

_____

In Partial Fulfillment of the Requirements for the Degree

Master of Science

_____

by
TRACY J. BISH

Dr. Azlin Mustapha, Thesis Supervisor

DECEMBER 2011

The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

INACTIVATION OF FOODBORNE
PATHOGENS BY FRUIT WINES

presented by Tracy J. Bish,

a candidate for the degree of Master of Science,

and hereby certify that, in their opinion, it is worthy of acceptance.

_____

Azlin Mustapha, Ph.D., Department of Food Science

_____

Mengshi Lin, Ph.D., Department of Food Science

_____

Mark Ellersieck, Ph.D., Department of Statistics

ACKNOWLEDGEMENTS

I would like to thank my lab mates, past and present, for your all your support and understanding.

I would like to acknowledge the University of Missouri's Electron Microscopy Core Facility for assistance in preparing and imaging specimens for this research.

Thanks also to the donors of the Robert T. Marshall fellowship, the Institute of Food Technologists St. Louis chapter fellowship, the Eugene V. Nay fellowship, and the Dr. Marion Fields Graduate fellowship, all of which helped to make this possible.

I would like to express my sincere thanks to the members of my committee, and especially to Dr. Mustapha for her patience and support of my work.

Table of Contents

iv

LIST OF FIGURES

LIST OF TABLES

INACTIVATION OF FOODBORNE PATHOGENS BY FRUIT WINES

Tracy Bish

Dr. Azlin Mustapha, Thesis Supervisor

ABSTRACT

Wines produced from grapes have been found to possess bactericidal properties on a number of pathogens *in vitro*, suggesting possible health benefits that may be derived from microbiological safety. This study investigated the bactericidal effects in wines made from fruits other than grape. The foodborne pathogens *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* Typhimurium, *Shigella dysenteriae*, and *Staphylococcus aureus* were treated with fruit wine solutions at varying concentrations for 24 h using blackberry, cherry, peach, and red raspberry wine, and enumerated using a pour plate assay. At 40% fruit wine concentration, numbers of all pathogens tested were significantly reduced compared to a 0% wine solution control ($P \leq 0.05$). Increasing wine concentrations above 40% showed a greater effect. The bactericidal effect of wine extracts with ethanol removed was also tested, and a significant reduction in number for the pathogens was observed at 60% concentration compared to a control ($P \leq 0.05$). Correlation strength of the following factors to antibacterial activity of the fruit wines at 40% concentration was in the order of titratable acidity, alcohol content, pH, free sulfite concentration, anthocyanin content, total phenolic content, and tannin content. Scanning and transmission electron microscopic examinations of *E. coli* O157:H7 and *S.* Typhimurium after treatment with peach and cherry wine revealed differences in cellular morphology, specifically reduction in cell size, holes in the cell wall and membrane disruption that appeared to have caused leakage of intracellular contents.

x

CHAPTER 1

INTRODUCTION

1.1 Introduction

The food industry is always looking to find ways to add value to food, known as value added processing. Value added processing is simply the processing of an item resulting in a finished product having a value exceeding the cost of processing. The perishability of fresh fruits makes it a good candidate for processing, especially for processing methods that extend its shelf life. Though a variety of processing methods are currently used to make products like dried fruit, frozen fruit, canned fruit, fruit juice, jams and preserves, oils (for example, orange or lemon seed oil), and flavorings, the alcoholic fermentation of fruit offers a potential way to dramatically increase the value.

Historically, fermentation was a cheap and efficient way to preserve perishable raw materials. Today, fermentation is partly responsible for the greatest utilization of a single fruit product in the United States in terms of U.S. dollars. In 2008, grapes had the highest value of production for any fruit reported by the USDA (2009), with a total value of $3.3 billion. That year, apples had a value of $2.2 billion, oranges a value of $2.2 billion, and strawberries a value of $1.9 billion. In addition to grape production value, total U.S. wine sales in 2005 were estimated at $23.8 billion (MFK Research 2007). Despite the value wine can potentially bring to a fruit, wine made from fruits other than grapes is not produced in great quantity in the U.S. compared to that made from grapes. In 2010, 677.3 million gallons of still wine was produced from grapes, while only 19.3 million gallons of other special natural wines, which fruit wine is in the category of, was

produced (DOT 2011b). This constitutes less than 3% of the market, and is a valuable opportunity open to fruit processors.

While the value added to grapes through wine production is a great benefit, protection against pathogenic bacteria may potentially be another. Food safety is an important concern to public health in the United States, with microbial contamination by pathogenic bacteria being the main cause of recalls. In 2010, there were 2.3 million pounds of beef recalled in the U.S. due to 11 recalls concerning *E. coli* O157:H7 (Flynn 2010). The largest recall in 2010 was due to *Salmonella* found on eggs, with over 500 million eggs coming from two farms (Doell 2010). Despite recalls such as these, it is estimated that 1 in 6 people in the U.S. (roughly 48 million) will get sick each year from foodborne illness; approximately 128,000 people will be hospitalized from it; and 3,000 people will die (CDC 2010).

Previous research has found wines made from grapes to have a bactericidal effect on a variety of pathogens *in vitro*, including *E. coli* O157:H7 and other virulent strains (Sheth and others 1988; Weisse and others 1995; Møretrø and Daeschel 2004; Waite and Daeschel 2007; Boban and others 2010), *Listeria monocytogenes* (Møretrø and Daeschel 2004), *Salmonella* Typhimurium (Sheth and others 1988; Weisse and others 1995; Møretrø and Daeschel 2004), *Salmonella* Enteritidis (Boban and others 2010), *Staphylococcus aureus* (Møretrø and Daeschel 2004; Waite and Daeschel 2007), *Campylobacter jejuni* (Carneiro and others 2008; Gañan and others 2009), *Helicobacter pylori* (Marimon and others 1998; Daroch and others 2001), *Streptococcus pyogenes* (Chinnam and others 2010), *Shigella sonnei* (Sheth and others 1988; Weisse and others 1995), and *Vibrio parahaemolyticus* (Liu and others 2006). Although the

2

effect of grape wines has been studied on a variety of pathogenic bacteria, information about the bactericidal effect of fruit wines on pathogens is lacking. The goal of this thesis was to expand upon previous findings from red and white wines, and investigate the bactericidal properties of fruit wines.

1.2 Objectives

The objective of this study was to:

1) Determine if fruit wines possess bactericidal activity against pathogens through tube dilution assays that provide observable counts compared to a control.

2) Measure pH, titratable acidity, and ethanol concentration of the fruit wines.

3) Correlate pH, titratable acidity, and ethanol concentration to observed antibacterial activity.

4) Determine if extracts from fruit wines not containing ethanol also possess bactericidal activity against pathogens through tube dilution assays that provide observable counts compared to a control.

5) Investigate potentially related contributing factors to fruit wine bactericidal activity.

CHAPTER 2

LITERATURE REVIEW

2.1 Wine

Wine is the most popular alcoholic beverage in the United States with respect to volume produced. In the year 2010, 789 million gallons of various types of wine was produced, down from 823 million gallons in 2009 (Table 2-1).

Table 2-1 US wine production data for 2009 and 2010 as reported by the Alcohol and Tobacco Tax and Trade Bureau, given in US gallons.

Wine production (US gallons)	2010	2009
Still wine	677490922	711748757
Effervescent wines	24331584	22436954
Special natural wine (vermouth)	4900928	3594718
Other special natural wine	19374531	19451926
27 CFR 24.218 wines (wine coolers)	62627531	65859649
Total wine production	788725496	823092004

Distilled spirits were second to wine in terms of volume of production, with 373 million gallons produced and bottled for domestic use in 2010. Beer was the third largest alcoholic beverage produced by volume, with production of 195 million gallons in the US for 2010 (DOT 2011a, b).

2.1.1 Definition and varieties of wine

The legal definition of wine in the United States is defined by the Code of Federal Regulations, in Title 27, Part 4, Subpart C, §4.21, with 9 distinct classes (Table 2-2).

Table 2-2   Classes of wine defined by the CFR standards of identity.

Class 1	grape wine
Class 2	sparkling grape wine
Class 3	carbonated grape wine
Class 4	citrus wine (or citrus fruit wine)
Class 5	fruit wine
Class 6	wine from other agricultural products
Class 7	apertif wine ("vermouth")
Class 8	imitation and substandard or other than standard wine
Class 9	retsina wine

Grape wine is the most abundant class produced in terms of volume for wine, and is produced through the alcoholic fermentation of ripe grapes. This can include the post fermentation addition of condensed grape must (commonly referred to as concentrate), and grape brandy or alcohol. Addition of sugar is limited to 35% increase in total volume, 20% by weight of the sugar. The grape wine class is further categorized as grape wine, table wine, and dessert wine. The grape wine category can contain no more than 13% alcohol by volume (ABV) from fermentation. It is labeled according to the grape skin color (that is, red, white, or pink/rose).   Table wines cannot exceed an ABV of 14%, and can be labeled red table wine, white table wine, sweet table wine, or light wine. Dessert wines contain between 14% and 24% ABV.

Sparkling grape wines are distinct from Class 1 grape wines in that they are made effervescent or are carbonated from carbon dioxide produced through natural fermentation while the product is contained within a closed vessel or container.

The third class of wine, carbonated grape wines, is produced through the addition of carbonation in a different manner than class 2 sparkling wines, with addition of carbon dioxide to the wine arising from a manner other than natural fermentation in a closed vessel.

Citrus wine, or citrus fruit wine, is produced similarly to grape wine but through the alcoholic fermentation of ripe or fresh citrus fruits. Addition of citrus brandy, alcohol, and sugar are regulated the same as grape wines, and similar categories apply (for example, citrus wine, citrus table wine, sparkling citrus wine).

Fruit wines are produced through the alcoholic fermentation of fruit other than grapes and citrus fruit, including fermentation of pure fruit concentrate or "must" that may or may not have been reconstituted to volume. Fruit wines can also include the addition of fruit brandy or alcohol after fermentation, as well as sugar additions limited to 35% by volume. The labeling of fruit wines is done according to the fruit used, if a single type of fruit or berry was used (for example, cherry wine, raspberry wine and others). If more than one type of fruit was used, it would be labeled fruit wine. Fruit wines produced from berries can also be labeled berry wine (rather than fruit wine).

The sixth class of wine, wine from other agricultural products, is produced from agricultural products other than fresh or ripe grapes, citrus fruit, or fruits and berries. This class of wine can include dried grape and fruit products (for example, raisins), and the addition of water is limited to reconstituting only to correct for moisture deficiencies. The addition of sugar is limited in the same manner as the previous five classes of wine, alcohol or spirits is restricted so that the addition should not change the character of the

6

product. Sake is a product included in the sixth class; its production specifically must be from rice according to accepted or established methods.

Appertif wines is a class of wine produced from grapes, similar to grape wines, but the alcohol content must be at least 15% ABV through the addition of brandy or alcohol. Herbs or natural flavorings are also added to appertif wines. The addition of caramel is also allowed specifically for color.

The eighth class of wine, imitation and substandard or other than standard wine, includes a variety of wines produced from unconventional methods. This includes wines made from synthetic materials, wines made from mixtures of water and leftover grape/fruit pressings, wines that have altered taste, color, smell that were derived from unconventional processes, wines having excessive volatile acidity, or wines produced from fruits that were decomposed or diseased, or had color, flavor, and aroma that were not characteristic of wines produced in a normal manner.

Retsina wine, the ninth class of wine, is produced in a manner identical to grape wine, but includes the addition of resin for flavoring.

2.1.2 Wine making process

Wine making with grapes has the following steps: stemming (de-stemming), crushing, maceration, pressing, fermentation, malolactic fermentation (if necessary), maturation and natural clarification, finishing and stabilization, and finally bottling (Jackson 1994).The wine making process begins after the fruit is collected during harvest and is brought to a winery. The first processing step is stemming, in which stems, leaves, and debris is removed from the fruit. This is followed by crushing, which is simply the squeezing of grapes so that the skins are broken, and juice and contents of

the berry may run out. Maceration is the process where compounds are extracted from the skins and seeds, and is initially started through hydrolytic enzymes released through the crushing process. Maceration in white wines differs from red wines because it is very brief, and kept to a minimum. White wine grapes are gently pressed shortly after crushing, with the free run juice from crushing and that extracted from pressing combined, and then fermented. Red wine maceration occurs alongside fermentation, so the pomace, or solid remains of grapes, are present. Alcohol produced through fermentation aids anthocyanin, tannin, and phenolic extraction, and gives red wines their color and flavor qualities. The pressing for red wines occurs depending on the color desired, with rosé wines being pressed early, and red wines being pressed later nearing the end of fermentation. In both white and red wine, fermentation is mostly carried out through the inoculation of desired strains of yeast, though some fermentation may occur through wild yeasts on the grapes or the crushing equipment. In addition to the production of alcohol, yeast fermentation also contributes to the aroma and flavor of wine. Malolactic fermentation, the conversion of malic acid to lactic acid, is encouraged if wines are too acidic. Lactic acid is perceived as less harsh than malic acid, with less "bite" and a softer mouth feel. Malolactic fermentation occurs through the presence of lactic acid bacteria present in the wine, and is controlled by lower temperatures (at or below 10°C) and the addition of sulfites. Maturation and natural clarification occur with extended aging of a wine. The wine loses yeasty odors, and suspended particulate matter precipitate over time. Racking, the separation of the wine from solid sediment, preserves the quality of the wine from off-flavors. The solid sediment, or lees, consists mostly of yeast and bacterial cells, proteins, tannins, and crystallized salts of tartaric

acid. Finishing and stabilization processes follow aging and natural clarification, and include additional steps to improve the wine, like addition of fining agents and filtration for further clarification, pH adjustment or deacidification, sweetening, or blending. Sterile filtration and/or addition of sulfur dioxide are the last steps prior to bottling to reduce microbial or oxidative spoilage.

2.1.3 Differences in fruit wine production

The use of fruits other than grapes to produce fruit wines can introduce differences in processing or manufacturing methods used to produce the fruit wine. Milling, commonly used in preparation of apple juice, is also used for producing apple wine (or hard cider for ABV < 7.0%), pear wine/perry, and wines from hard fruits, in order to press juice out of the fruit. Freshly pressed juice is often treated with 50 mg/kg sulfur dioxide to prevent oxidative browning and microbial contamination, and then readjusted with the juice back to a lower residual level of 10-30 mg/kg (Jarvis 1996). The use of pectinolytic enzymes and heat treatment of 80-85 °C is also used prior to pressing to aid juice extraction. Peeling is a necessary process for fruits with undesirable skins, and is used in the production of banana wine, jack-fruit wine, and colonche, a Mexican cactus fruit wine (Battcock and Azam-Ali 1998). Chapitalization, the addition of sugar to the must to increase alcohol content, and pH adjustment, though sometimes used in winemaking with grapes, are necessary processes for fruits lacking the sugar content and acidity to make wine. In mature *Vitis vinifera* grapes, sugar concentrations of greater than 20 g/100 mL are common, and these produce wines from 10-12% ABV (Jackson 1994). The addition of sugar or glucose syrup is necessary for apple wines to reach up to 12% ABV because fresh apple juice fermenting with the fruit sugar alone would rarely

reach 6-7% (Jarvis 1996). In comparison to grapes containing 20 g/100 mL or more total sugar, apples contain 10.0-11.2 g/mL of total sugar, blackberries contain between 5.5-12.8 g/100 mL sugar, and raspberries contain between 4.7-11.6 g/mL of sugar. Total acidity also varies widely by fruit type. A desirable total acidity in wine ranges from 0.55-0.85% (Jackson 1994). Pears contain between 3.0-5.0 g/L (or 0.3-0.5%) total acidity, raspberries contain between 13.5-16.0 g/L total acidity (Jarvis 1996), both fruits falling outside the range of acceptable acidity in wine.

2.2 Health benefits of wines and fruit wines

Evidence of the health benefits of wines and fruit wines has accumulated over the last 25 years, and relates to the fruit that is used for producing it, and the phytochemicals that arise from the breakdown of chemicals by ethanol produced during fermentation. The main health benefits reported for grape and fruit wines have been reduction in oxidative stress and related benefits, primarily including the reduction in cardiovascular disease (CVD) and CVD related mortality, and antimicrobial/antibacterial properties.

2.2.1 Wine and cardiovascular disease

Friedman and Kimball (1986) were among the first to link reduced CVD mortality to wine/alcohol consumption. They had examined the data provided by a 24-year follow-up to the Framington Heart Study Cohort, initially conducted in 1948. The 24-year follow up examined the incidence and factors associated with heart disease in a population of residents in Framington, Massachusetts, between the ages of 30 and 59. There were 5,209 participants in the study, data consisting of biennial examinations of 2,106 males and 2,639 females. Friedman and Kimball had looked at the raw data, and used

multivariate analysis on the data based on Cox proportional hazards model, with age as a variable to model age-specific mortality rates. They had determined the amount of ethanol consumed based on the number of drinks of spirits, wine, or beer reported, using 2 oz, 8 oz, and 4 oz for estimating the size of drink, and 50.0, 5.0, and 16.75 percent for estimating ethanol concentration, respectively. Their findings showed a U-shaped mortality pattern in relation to ethanol consumption and cardiovascular disease mortality in both male smokers and nonsmokers. No effect was seen for nonsmoker females, a U-shaped mortality was seen in the raw data for female smokers, though this data was not analyzed using Cox multivariate analysis. Male coronary heart disease was further analyzed by modeling morbidity based on type of drink, and all types of drink showed a U-shaped curve for smokers and nonsmokers. Beer and wine consumption showed greater reduction in coronary heart disease mortality in nonsmokers roughly twice that observed with spirits.

The effect of alcohol consumption and reduction in cardiovascular disease has also been observed in women. A study by Stampfer and others (1988) distributed questionnaires to 87,526 female nurses between the ages of 34 and 59 in 1980 to assess beer, wine and spirits consumption. After 4 years, they conducted a follow up to assess incidence of cardiovascular disease, and recorded 200 cases of severe myocardial infarction (36 resulting in death), 66 ischemic strokes, and 28 subarachnoid hemorrhages. They observed a reduction in the relative risk of coronary disease in those who consumed alcohol compared to nondrinkers, 0.6 for women consuming between 5 to 14 oz of alcohol a week, 0.6 for women consuming between 15 and 24 oz of alcohol a week, and 0.4 for women who consumed over 25 oz of alcohol a week.

Similarly, alcohol consumption has been found to reduce the incidence of deep venous thrombosis and pulmonary embolism in older patients (Pahor and others 1996). Conducting in-home surveys of over 10,000 participants aged 65 and older from East Boston, Massachusetts, and in Iowa and Washington Counties in Iowa initially, they evaluated self-reported alcoholic drink consumption and calculated alcohol consumption according to the Framington study, with 1.0 oz x number of mixed drinks/cocktails, 0.67 oz x number of glasses of wine, and 0.60 oz x number of beers consumed. The participants were then classified as consuming either none, less than 1 oz a month, less than 1 oz a day, or more than 1 oz a day. Pahor and others (1996) assessed hospital discharges and deaths in 7958 participants over a 7-year span, removing cases with missing alcohol data, medical data that was not linked, or participants that had died from other causes. They found the relative risk factor for deep venous thrombosis and pulmonary embolism for those who consumed alcohol to be 0.7 for < 1 oz/mo, 0.6 for < 1 oz/day, and 0.5 for > 1 oz day compared to non-drinkers, finding that low to moderate alcohol consumption was associated with a decreased risk for the two diseases.

Alcohol consumption was also found to reduce the incidence of ischemic strokes, though a difference in reduction was found between wine and beer or spirits (Truelsen and others 1998). They examined incidence of stroke in 13,300 men and women in Copenhagen, Denmark over 16 years, and looked into differences in risk based on type of alcoholic beverage consumed, spirits, wine, or beer. Correcting for age, sex, smoking, body mass index, physical activity, systolic blood pressure, cholesterol, antihypertensive treatment, triglycerides, education, and diabetes mellitus, they found adjusted risk ratios for those who consumed wine to be significantly lower than those who did not drink, with

ratios of 1.00 for never/hardly ever consumed, 0.84 for monthly consumption, 0.66 for

weekly consumption, and 0.68 for daily consumption. The same reductions in risk were

not observed in beer, with risk ratios of 1.00, 0.95, 1.09, and 1.11, respectively; or for

spirits, 1.00, 0.99, 0.97, and 0.91, respectively. Similar findings for wine consumption

being associated with a greater reduction than beer or spirits consumption in coronary

disease related mortality in Danish populations were found by Gronbaek and others

(1995).

The inverse relationship between red wine consumption and reduced

cardiovascular disease being well founded, a study by Serafini and others (1998)

examined the relationship of polyphenol content to antioxidant activity in dealcoholized

wines to clearly identify a component in wine that was beneficial to cardiovascular

health. Antioxidants were a potential explanation for this. Oxidative stress is a general

mechanism thought to underlie a variety of diseases, and had been proposed as the

mechanism causing aging since the 1950s (Harman 1956). Harman's theory had

subsequently been revised in the 1990's, such that free radicals caused a variety of

diseases, including atherosclerosis (Harman 1992). Antioxidants interact with free

radicals to stop the formation of peroxidase by-products that were thought to cause

disease. In Serafini and others' study, they examined both the total radical-trapping

antioxidant parameter (TRAP) of plasma, as well as the total amount of phenols present

in plasma, after ingestion of alcohol free red wine, white wine, or a tap water control at 0

min, 30 min, 50 min and 120 min using 10 healthy participants over a 1 week interval.

They found that the phenolic concentration of the red wine they tested to be much higher

than the white wine fraction (3636 mg gallic acid equivalents to 31 mg GE, respectively),

and exhibited approximately 20 times greater radical trapping antioxidant capacity. The *in vivo* human study examining TRAP levels after ingestion of alcohol free red or white wine or water showed a significant increase in TRAP levels at 50 min compared to 0 min for those that consumed the red wine fraction ($P < 0.004$). No significant difference was found for those consuming either white wine fraction or water. Similarly, a significant difference in total blood plasma levels of polyphenols was found 50 minutes after consumption of dealcoholized red wine ($P < 0.05$). No differences were found after consumption of either water or dealcoholized white wine.

Tian and others' (2011) study examining red and white wine inhibition of cholesterol oxidation has supported the findings that polyphenol fractions in wine are potent antioxidants. Their study measured the oxidation product, 7-ketocholesterol and its formation in a free radical generator using 2,2'-azobis(2-methylpropionamide) dihydrochloride, and compared inhibition rates of red and white wine. The red wine inhibited oxidation products for 72 h, whereas the white wines tested inhibited 7-ketocholesterol for only 24 h at twice the concentration.

Although antioxidants have been shown to be correlated with markers that indicate a decline in stress, claims to the benefits of cardiovascular health derived directly from the antioxidant activity of polyphenols could be argued that they are not established (Hollman and others 2011). The benefits of the fruits and substances derived from them have been shown to be beneficial, but the predictive value for antioxidant activity for these benefits in humans is questioned as there is no clear evidence that antioxidant activity biomarkers cause the improvement in cardiovascular health. The antioxidant activity of the polyphenols is significantly reduced after ingestion,

and the concentration of polyphenols in the blood is comparatively low to that of other antioxidants.

2.2.2 Antioxidant content in fruit wines

The antioxidant activity of grape wines stemming from the phenolic content and polyphenols present has been consistently reported. There have been conflicting findings regarding antioxidant capacity and phenolic content in fruit wines. The differences suggest the importance of concentrations of specific phenolic compounds, rather than total phenolic content. Some fruit wines possess antioxidant activity that exceeds that of red grape wines, due to differences in the phenolic contents of the wine.

In a study of three fruit wines and one red wine, Pinhero and Paliyath (2001) found that blackberry, blueberry, and summer cherry wines had 30-40% more superoxide radical scavenging activity than the red wine tested. They also examined hydroxyl radical scavenging activity using dealcoholized phenolic components from the fruit wines, and found that summer cherry and blueberry wine extracts had greater inhibition than blackberry and red wine (with values of 5.98, 9.07, 3.57, and 2.8% inhibition/µg of gallic acid equivalent resectively). From their findings, they concluded that differences between the phenolic compound compositions explain the differences in the antioxidant activity between the fruit and grape wine fractions.

A prior study by Heinonen and others (1998) comparing the phenolic content and antioxidant activity of 33 fruit and berry wines to red and white wines had similar findings that conflicted with total phenolic content and antioxidant activity. The fruit wines they used included apple, arctic bramble, aronia, bilberry, cherry, cloudberry, cowberry, cranberry, crowberry, black, red, and green currant, honey, red raspberry, rhubarb,

rowanberry, and strawberry. The wines were dealcoholized using a rotary evaporator; phenolic content measured using the Folin-Ciocalteu procedure, and oxidation measured with a spectrophotometer after the addition of methyl linoleate. They found that fruit wines containing mixtures of black currants and crowberries or bilberries (240-275 µM GAE) were slightly superior to reference red grape wines containing a greater phenolic content (330-375 µM GAE). Their study found no correlation between total phenolic content and antioxidant activity, despite the Folin-Ciocalteu assay and oxidation assay finding a strong correlation in grape wines ($r = 0.94$) (Frankel and others 1995).

Contrary to Heinonen and others' study (1998), a later study using fruit wines made from some of the same fruits, examined the antioxidant activity in apple, apricot, bilberry, black mulberry, blackberry, melon, quince, red raspberry, sour cherry and strawberry wine, found a significant relationship between antioxidant activity and total phenolic content ($r = 0.958$, $P = 0.001$) (Yildirim 2006). Yildirim's study measured antioxidant activity using 1,1-diphenyl-2-pikrylhydrazin instead of methyl linoleate, so differences in the methodology may account for dissimilar findings.

A study that examined differences in the type of antioxidant activity found in pomegranate juice and pomegranate wine, also found a high correlation between total phenolic content and antioxidant activity (Zhuang and others 2011). These authors produced three types of pomegranate wines using sweet pomegranate, red pomegranate, and sour pomegranate. The phenolic content of the different juices and wines was measured using the Folin-Ciocalteu procedure, while the antioxidant activity was measured using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay and a ferric reducing antioxidant power assay (FRAP). The total phenolic content

of both the pomegranate juices and pomegranate wines had significant positive

correlations to the antioxidant activity measured through DPPH and FRAP assays.

Jackfruit wine is another fruit wine produced from fruits with high antioxidant

values, and has been found to possess anti-carcinogenic properties (Jagtap and others

2011). They produced a wine by grinding cleaned and separated jackfruit pulp,

fermenting the slurry with *Saccharomyces cerevisiae* at 28 °C for 12 d, and then

clarifying using bentonite, filtering, and storing in airtight glass at 4 °C until analysis. The

antioxidant capacity of the Jackfruit wine (JFW) was assessed using 4 assays; DPPH

free radical scavenging assay, FRAP assay, N,N-dimethyl-p-phenylendiamine (DMPD)

scavenging assay, and nitric oxide (NO) scavenging assay. JFW had 55.18% and

69.44% DPPH scavenging for a 500 μL dose of wine at 12 h and 24 h, respectively and

is greater than that found in grape wine, with DPPH scavenging ranging from 31.7-

37.2% (Seeram and others 2008).The reductive capacity of JFW measured in the FRAP

assay was 0.123 and 0.316 for 100 μL and 300 μL, respectively with no significant

difference at greater concentrations. The DMPD assay showed phenolic content of JFW

to be highly correlated to DMPD radical scavenging ($r = 0.974$, $P \leq 0.002$), indicating that

the antioxidant activity of JFW relates well to concentration in an acidic environment.

NO scavenging capacity, related to reductions in oxidative stress caused by

inflammation and infection, was also strongly correlated to the phenolic content of JFW

($r = 0.993$, $P \leq 0.0003$). The authors also examined the protective effect of JFW against

H_2O_2, UV radiation, and γ–radiation damage on DNA using a pBR322 plasmid, and

found agarose gels revealed reductions in strand breaks in the plasmids with JFW

present.

17

Antioxidants possessing anti-carcinogenic properties are well established through the scavenging of radicals. Examining the antioxidant properties of blackberry extracts, Dai and others (2007) found that blackberry extract suppresses HT-29 colon tumor cell growth *in vitro*. The inhibition was dose-dependent, with 49.2 μg/mL of anthocyanins from the extract inhibiting growth of 66% of the HT-29 tumor cells over 72 h. This surpassed other studies with similar finding using strawberries (Olsson and others 2006) and blueberries (Yi and others 2005).

2.2.3 Antibacterial activity of wines/fruit wines

An early study testing the antibacterial activity of wines was conducted by Sheth and others (1988), which examined differences in the antibacterial activity of common beverages, including carbonated soft drinks, beer, wine, milk and water. These researchers inoculated *Salmonella*, *Shigella*, and enterotoxigenic *E. coli* into the beverages and counted surviving colonies over 2 days. The lowest survival rate among the pathogenic bacteria had been found in those exposed to wine, the greatest growth had occurred in the milk and water. Beer and soda allowed small numbers after 48 h, no detectable counts were found in the sour mix or diet cola after 48 h.

A study by Weisse and others (1995) compared the antibacterial properties of red and white wines to bismuth subsalicylate, both of which are traditional digestive aids, against pathogens generally responsible for traveler's diarrhea. In their study, they inoculated suspensions of *E. coli*, *Salmonella* Typhimurium, and *Shigella sonnei* into solutions of red wine, white wine, pure ethanol or tequila diluted to 10% ethanol concentration with sterilized tap water, 35 mg/L bismuth subsalicylate solution (using Pepto-Bismol), or sterilized tap water as a control. Red and white wines were found to

18

cause the greatest reductions in numbers, from 10^5-10^6 CFU to none detected (< 10 CFU). Bismuth subsalicylate was less effective than red and white wine, with a reduction in *E. coli* of 10^4 CFU after a 20 min exposure, and *S.* Enteritidis and *S. sonnei* requiring 60 and 120 min, respectively, for populations to become undetectable. Bismuth subsalicylate was more effective than the diluted tequila at reducing counts of *E.coli* and *S.* Enteritidis, but only marginally more so with *S. sonnei*. The pure ethanol diluted to 10% concentration did not exhibit any type of inhibition on the bacteria tested, and was not significantly different than inhibition by sterilized tap water.

Harding and Maidment (1996) had expanded upon the beverage study of Sheth and others (1988), and examined the antibacterial activity of red and white wines, fruit juices (grape juice, cider, and orange juice), industrial methylated spirits, and pH buffers against *E. coli*, *S.* Enteritidis and *S. sonnei*, and found the shortest survival time of the bacteria had resulted from exposure to wine. They also found that 10% methylated spirits and pH buffer had a negligible effect on the bacteria, indicating that there were additional components to wine that contributed to the wine's antibacterial activity beyond ethanol and pH. However, the addition of 10% methylated spirits to grape juice was found to have antibacterial activity greater than the two individually, suggesting a synergistic effect of ethanol and components in the wine. The antibacterial activity of the combined grape juice and methylated spirits was still less than that of a low alcohol wine they tested, so they concluded that the fermentation process added considerably to the antibacterial activity. Additionally, this study also found that the white wine had exhibited slightly greater antibacterial activity that the red wine that was tested.

Møretrø and Daeschel (2004) took a different approach to investigating wine's antibacterial activity, and examined it in conjunction with foodborne pathogens and mutants that lacked genes necessary to elicit a stress response. In the study, *E. coli* O157:H7 and an *rpoS* mutant, *Listeria monocytogenes* and a *sigB* mutant, *S.* Typhimurium and an *rpoS* mutant, and *Staphylococcus* aureus and a *sigB* mutant, were exposed to organic Chardonnay and Cabernet Sauvignon wine without added sulfites. They found the mutant strains lacking stress response genes had significantly less resistance to wine than the wild-type pathogens, except *S.* Typhimurium, which was most susceptible and was not detectable after 5 min of exposure. It was concluded that the genes encoding proteins necessary for stress response offer protection to ethanol, low pH, or organic acids. Red wine was also found to have had a greater bactericidal effect than the white wine on most strains tested. They also compared red and white wine to solutions containing a combination of organic acids, ethanol, and low pH (0.15% malic acid, 0.6% tartaric acid, 15% ethanol, and pH 3). The synthetic mixture had significantly greater bactericidal activity than the red or white wine, or the acids, ethanol, or pH components tested individually, and the authors assumed that pH, organic acids, and ethanol were largely responsible for the bacterial effect found in wine.

A similar finding was made by Daglia and others (2007), in a study that tested dealcoholized red (DRW) and white (DWW) wines, and organic acids found in wine against a variety of oral streptococci and *Streptococcus pyogenes*. DRW was found to have a lower minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) than DWW, with MIC for the DRW ranging from 10-20% volume of wine, and ranging from 20-30% in volume for the DWW. The MLC for the DRW ranged from 20-

40% volume and from 30-50% volume for the DWW. The lower concentrations needed

for MIC and MLC with the DRW for the bacteria tested suggest greater bactericidal

activity, although the differences were not significant at $P \leq 0.05$. They then separated

the fractions in the DRW using solid phase extraction into polar compounds, catechins,

oligomeric proanthocyanidins, or tannins. The polar compounds were the only fraction

that displayed bactericidal activity, and these were fractioned again with solid phase

extraction, revealing low molecular weight compounds and organic acids being

responsible for the bactericidal activity found in the DRW. Subsequently testing acetic

acid, citric acid, lactic acid, malic acid, succinic acid, tartaric acid, as well as high and

low concentration mixtures of the acids, revealed that lower MIC and MLC were needed

for the mixtures than for DRW. From their findings that wine polyphenols displayed no

activity, and low concentration mixtures of acids were more bactericidal than DRW,

Daglia and others suggested that the activity of the organic acids in wine is at least partly

inhibited.

Conflicting with this are the findings that phenolic and polyphenolic compounds

are themselves bactericidal. The antimicrobial activity of pure phenolic compounds, four

phenolic acids: gallic, vanillic, protocatechuic and caffeic, and three flavonoids: rutin,

catechin and quercetin, were tested against *Serratia marcescens*, *Proteus mirabilis*, *E.*

coli, *Klebsiella* pneumonia, *Flavobacterium* sp., and *S. aureus*. All of the bacteria

showed different sensitivities to the phenolic compounds at different concentrations, with

Flavobacterium being resistant to all compounds (Rodriguez Vaquero and others

2007a). The antimicrobial activity of wines was also tested against these bacteria, and it

was found that the wines tested were effective against all of the bacteria, even more so

21

at greater concentrations (2x, 3x, and 4x). Clarified wines were not found to be antimicrobial, and were attributed to the lack of phenolic compounds.

Nohynek and others (2006) examined the mechanism of nordic berry phenolics on the antibacterial activity against human pathogens. They exposed 7 pathogens to extracts of 12 types of berries, at 1 mg/mL of broth solution, and grown under standard conditions and plated on respective agar. They found that *Bacillus cereus* was the only pathogen tested that was susceptible to all extracts, or strongly inhibited. *Candida albicans*, a yeast, was the most resistant, but was susceptible to three types of berries, cloudberry, raspberry, and strawberry. Cloudberry and raspberry were found to be bactericidal rather than bacteriostatic, and were found to disrupt the outer membrane of *Salmonella*. Fractions of the berry extracts were chemically separated and used to determine permeability of the membrane through uptake of 1-N-phenyl-naphthylamine, and the authors observed a difference in uptake in cells treated with anthocyanins and ellagitannins from raspberry, or ellagitannins from cloudberries, and with phenolic extract and ellagic extracts from strawberries. The authors concluded from their findings that ellagic acids were primarily responsible for the antimicrobial activity of the berries they studied.

The effect of specific phenolic compounds and phenolic extracts of three red wines on the growth of *L. monocytogenes* had been investigated by Rodriguez Vaquero and others (2007b). An agar diffusion assay with the non-flavonoids gallic acid, vanillic acid, protocatechuic acid, and caffeic acid; the flavonoids rutin, quercetin, and catechin; and extracts of Cabernet Sauvignon, Malbec, and Merlot at 1x, 2x, and 4x concentration were used to compare the antimicrobial properties of the specific compounds against

22

Listeria. The wine concentrates were also clarified using activated charcoal to remove phenolic compounds for comparison to non-clarified concentrated wine. They found that all of the phenolic compounds tested inhibited growth of *L. monocytogenes*, and larger concentrations had a greater inhibitory effect. Rutin reached the greatest inhibition at the least concentration, 25 mg/L. The wine extracts also showed greater effect from higher concentration, with a 4-fold concentration showing the greatest inhibition. The Merlot tested had a greater inhibitory activity than the other wines, as measured by zones of inhibition. The clarified wines with phenolic compounds had been found to be inactive against *L. monocytogenes*, thus,the authors indicate that the polyphenolic compounds in the wine were responsible for the inhibitory effect of wine.

Rodriguez Vaquero and others (2007a) had similar findings when examining the effect of phenolics and wine extracts effect on the growth of *E. coli*, *S. marcescens*, *P. mirabilis*, *K. pneumoniae*, and *Flavobacterium*. Differences were found in the effectiveness of the different phenolic compounds, with quercetin being more inhibitory than rutin overall. *E. coli* was found to be the most sensitive, while *Flavobacterium* was the least, showing no inhibition to any of the phenolic compounds tested. The total phenolic content of the wines had been measured, and Merlot was found to be greater than the Malbec, which was greater than the Cabernet Sauvignon, and the inhibitory effects reflected this. Again, no inhibitory effect had been observed from the clarified wines on any of the bacteria studied.

The combination of polyphenols can have a synergistic or an antagonistic effect on antibacterial properties. Examining the effect of combinations of polyphenols and the effect of antibacterial activity on *Bacillus cereus* and *S*. Enteritidis, Arima and others

23

(2002) found combinations of quercitin with either quercitrin, morin, or rutin to have significantly more activity than any flavonoid by itself. Rutin alone did not demonstrate antibacterial activity, yet increased the activity in combination with other flavonoids. Similar findings have been found with phenolic phytochemical enriched alcoholic beverages on *Helicobacter pylori* (Lin and others 2005). Synergistic and antagonistic effects were found using a combination of polyphenols extracted from almond skins (Mandalari and others 2010), and bergamot peel extracts (Mandalari and others 2007).

Phenolic compounds in wine have also been found to possess antibacterial properties against *Campylobacter jejuni* (Gañan and others 2009). Red, white, and rose wines with 11.5% alcohol content were mixed with phosphate buffered saline (PBS) in concentrations of 1%, 10%, 25%, 50%, 75%, and 100%, inoculated with *C. jejuni*, incubated for 10 min in a variable atmosphere incubator, and then plated on Mueller Hinton agar with sheep's blood. A solution of 11.5% ethanol was mixed with PBS in the same concentrations, and plated to determine the effect of ethanol alone. They found that the red and rose wines were bactericidal at concentrations of 10% or greater, with no CFU present, and white wine was bactericidal at 25% and greater. Ethanol solutions were bactericidal at mixed concentrations of 25% and greater as well, and the authors concluded that part of the antibacterial activity was due to ethanol. Surprisingly, the authors also tested the influence of pH by exposing *C. jejuni* to PBS solutions at pH 3.2 and pH 7, and found no difference.

Waite and Daeschel (2007) examined the contributions of various components of wine, specifically ethanol concentration, pH, titratable acidity, and sulfur dioxide concentration, in relation to the antibacterial activity of organic white wine. Using organic

Chardonnay wine, they adjusted the pH using hydrochloric acid to either pH 3.0 or 3.25, the titratable acidity was modified with addition of either 2 or 4 g/L tartaric acid, sulfur dioxide concentration adjusted through addition of potassium metabisulfite to total sulfur dioxide levels of 50 ppm or 150 ppm, and addition of 1.5% or 3% addition of ethanol, to create a 3 level multifactorial wine treatment. The base or unmodified wine was one treatment. *E. coli* O157:H7 and *S. aureus* were inoculated into the various wine solutions and plated after a 20-min treatment. They found that *S. aureus* was significantly more resistant than *E. coli* O157:H7. Using stepwise regression, they determined that the parameters that contributed to the effectiveness of the wine solution against *S. aureus* were in the order of pH, molecular sulfur dioxide, titratable acidity, and ethanol. They did not have an order for *E. coli* O157:H7, due to samples being below detection limits, but proposed pH and ethanol being a reasonable model for prediction.

A similar study by Boban and others (2010) examined the contributing factors of wine, specifically phenolics, ethanol, and pH, to the antibacterial activity of wine. They used red wine, phenol-stripped wine, dealcoholized wine, ethanol (12.5% solution), low pH (3.1 pH solution with 0.2 g/L tartaric acid), a combination of ethanol and low pH, and a saline solution control for treatments on *Salmonella* Enteritidis and *E. coli*, with exposure time ranging from 5 min to 24 h, and subsequently plated on blood agar. Their results showed that intact wine had the greatest antibacterial activity, followed by phenol-stripped wine, dealcoholized wine, a combination of low pH and ethanol, and finally, low pH, followed by ethanol. They also examined the phenolic content and antioxidant capacity (as measured through a FRAP assay) of the solutions, and found that the two were closely related. Phenolic content and antioxidant capacity were not

found to correlate to antibacterial activity, and the phenolic stripped wine was twice as effective at reducing bacterial counts as the dealcoholized wine. The combination of ethanol and low pH was synergistic for antibacterial activity, having shown significant reduction in counts, where the ethanol and low pH treatments alone had a negligible effect.

Melanoidins, compounds formed in the Maillard reaction and are present in sweet Spanish wines, has been found to have antibacterial activity against *E. coli* and *S. aureus* (Rufián-Henares and Morales 2008). The authors had examined the antibacterial activity of a variety of melanoidins by preparing different melanoidin compounds, either synthetically or derived from food products. Synthetic melanoidins were created from aqueous Maillard reactions of glucose with the amino acids alanine, arginine, cysteine, histidine, lysine, methionine, tyrosine, tryptophan, and phenylalanine, and isolated through ultrafiltration. Melanoidins derived from food were extracted from light and medium roast coffee, a Pilsner style beer, a Belgian Abbey style beer, a dry stout beer, and a Spanish sweet wine (produced from a soleo process), by removal of fat through a dichloromethane treatment and subsequent ultrafiltration. The authors inoculated 2 mg/mL of each type of melanoidin into BHI broth prior to inoculation with *E. coli* and *S. aureus*. They determined optical density, and found that all of the melanoidins inhibited growth compared to a control. There were significant differences in inhibition between the two bacteria with melanoidins extracted from the same food ($P ≤ 0.01$). Differences were also observed from melanoidins obtained from more or less roasted food. Melanoidins from dark stout beer had greater antibacterial activity than lighter pilsner style beer; similarly melanoidins from medium roast coffee had greater activity than light

roast coffee. These differences in food type were less significant with *E. coli* than with *S. aureus*.

Sugita-Konishi and others (2001) have argued against the benefits of the antibacterial properties in wine effecting humans *in vivo*, and that the antibacterial properties of wine had been observed *in vitro*. Implementing a three-stage study that tested both *in vivo* and *in vitro* properties of the wine, they first examined the antibacterial properties of red and white wines, a 14% ethanol solution, a 350 ppm potassium pyrosulfite solution, and a phosphate buffered saline solution against *E. coli* O157:H7, *S.* Enteritidis, and *Vibrio parahaemolyticus in vitro*. The red and white wines significantly decreased colony counts within 30 min, from 10^5 to undetectable. No effect was observed with the ethanol solution, the potassium pyrosulfite solution, or the phosphate buffer. It was assumed that neither ethanol nor sulfites was directly responsible for the wine's antibacterial activity. The second stage, in which they tried to identify the antibacterial fraction of the wine, separated components through freeze drying and centrifugation. They identified a polyphenol-free section of wine, and discovered that it had the same antibacterial properties of the wine as that of the whole, un-fractionated wine, concluding that it was not the polyphenols present that gave the wine its antibacterial properties. In the third stage, they administered either a red wine, white wine, polyphenol fraction (red colored), 14% ethanol solution, or water over a one-week period into mice infected with *S.* Enteritidis. At the end, they removed the spleens from the mice to screen for infection, and found no differences between the wine, ethanol, or water solution administered mice, and concluded that wine did not prevent or provide protection against infection by *Salmonella*.

Phytochemicals from fruits acting as quorum sensing inhibitors could be another factor in wine contributing a health benefit against bacteria. Quorum sensing is a chemical signaling mechanism that transmits information about cell density and regulates the expression of genes in bacteria (Miller and Bassler 2001). The signaling compounds, called autoinducers, regulate a variety of cell behavior, including virulence, antibiotic production, sporulation, and biofilm production. Vattem and others (2007) investigated dietary phytochemicals and their role in acting as quorum sensing inhibitors, through the use of sublethal concentrations of fruit, spice and herb extracts. A series of microdilution assays was used to determine the MIC of cranberry, wild blueberry, raspberry, blackberry, strawberry, grape, oregano, rosemary, basil, thyme, kale, turmeric, and ginger extracts on *Chromobacterium violaceum* strains CVO26 and CV 31532, *E. coli* O157:H7, and *Pseudomonas aeroginosa*. Extract concentrations below the MIC were then used to measure violacein pigment production of the *C. violaceum* strains using a *Chromobacterium violaceum* bioassay system (McLean and others 2004). Swarming motility associated with the pathogenicity of *P. aeroginosa* and *E. coli* O157:H7 was measured through a point inoculation assay (Ren and others 2001) using extract concentrations below MIC and comparing inhibition in terms of diameter growth after 24 h against a control. They found that violacein production in *C. violaceum* was only inhibited by raspberry, blueberry, and grape extracts for the fruits tested (with inhibition of 60%, 42%, and 20%, respectively), and that herbs and spices were significantly more effective, with inhibition ranging from 40-78% for all but rosemary (showing no effect). However, the swarming activity of *P. aeroginosa* and *E. coli* O157:H7 was inhibited more by the fruit extracts, with inhibition ranging from 23-50%

and 25-40%, respectively. From their findings, they concluded that quorum sensing was inhibited from 2 different mechanisms, interference in the activity of the autoinducer, and the bacteria's ability to synthesize these compounds.

2.2.4 Theories on antibacterial properties

Reasons theorized for polyphenol and phenolic compounds in wines having antibacterial properties have been that the tannin compounds adhere to cell surfaces, interact with enzymes, and adhere to metal ions that are required substrates for growth (Scalbert 1991). The theory for adherence of tannin compounds to cell surfaces is largely derived from a basic definition of tannins, which are phenolic compounds with the ability to bind proteins. The perceived astringency characteristic of tannins is related to this protein's binding activity. Tannins cause the rough, tactile sensation of astringency through the precipitation of water soluble salivary proteins on the tongue (Sarni-Manchado and others 1998). Scalbert noted that complexes or interaction of tannins with microbial enzymes had already been observed in cellulases (Lyr 1965; Benoit and Starkey 1968; Mole and Waterman 1987), pectinases, xylanases (Lyr 1961), peroxidase (Lyr 1961, 1965), laccase (Lyr 1961), and glycosyltransferase (Kakiuchi and others 1986; Hada and others 1989). Phenolic compounds with great astringency would presumably have greater antibacterial activity. This has since been observed with the highly astringent phenolic epigallocatechin gallate compound.

Ikigai and others (1993) examined the antibacterial mechanism of (-)-epigallocatechin gallate (EGCg) and (-)-epicatechin (EC) on *E. coli* and *S. aureus* using an agar dilution assay to determine MIC, and fluorescence measurements using 5,6-carboxyfluorescein to determine liposomal damage from exposure to EGCg and EC. The

MIC of EGCg and EC were significantly different between the two compounds, as well between species. EGCg had a greater inhibitory effect than EC, and *E. coli* showed less inhibition to the compounds than *S. aureus*. They also found that EGCG caused leaking in phosphatidylcholine liposomes and aggregation in cells, whereas EC had no significant effect. The authors concluded that the negative charge of the lipopolysaccharide layer of gram negative bacteria (*E. coli*) was responsible for the differences observed.

Delehanty and others (2007) examined the aggregation of gram negative bacterial cells through the binding of lipopolysaccharides to proanthocyanidin fractions obtained from cranberries, tea, and grapes. A proanthocyanidin fraction from cranberries was found to have the greatest lipopolysaccharide binding activity of the three fruits. Concentration of cranberry proanthocyanidin fractions sufficient for neutralization of the LPS aggregation in *E. coli* minimized its toxicity *in vitro*. They also found that the affinity of cranberry proanthocyanidins to *E. coli* LPS were similar to that of *Salmonella*, *Shigella*, and *Pseudomonas*.

Polyphenols and beverages rich in polyphenols have also been found to produce significant amounts of hydrogen peroxide through contact with metal ions, formed through oxidation (Akagawa and others 2003). The phenolic compounds pyrocatechol, hydroquinone, pyrogallol, 1,2,4-benzenetriol, gallic acid, chlorogenic acid, and caffeic acid; were measured using a ferrous oxidation-xylenol orange assay to determine the formation of H_2O_2. They found that the hydroxyl position on the phenol limited autoxidation, and that pH had a major impact. Dissociation caused from higher pH values caused greater H_2O_2 production. The polyphenols epigallocatechin gallate,

30

epicatechin gallate, epicatechin, and catechin, were also assayed using the FOX assay, controlling for pH, and were found to produce H_2O_2 in that order, respectively. The authors concluded from this that the addition of a gallate group in the third adjacent position on the catechin was responsible for the increased H_2O_2 formation. Again, the role of pH was important, and H_2O_2 production increased at higher pH values.

The structure of the polyphenol playing an important role in antibacterial activity was supported by Taguri and others (2006). The antibacterial properties of 22 polyphenols were examined in relation to the hydroxyphenyl structure using 26 species of bacteria. Polyphenols with pyrogallol groups showed greater activity than polyphenols with catechol or resorcinol rings. The number of rings did not correlate to greater antibacterial activity, only the presence/absence of the structure.

Polyphenols have also been found to inhibit ATP synthase, necessary for cell growth and metabolism. Chinnam and others (2010) studied the effect of 17 polyphenols on the activity of purified F1 ATP synthase and F1F0 ATP synthase located on *E. coli* cell membrane, finding inhibition to be highly variable among the compounds. Morin, silymarin, baicalein, silibinin, rimantadin, amantidin, or, epicatechin completely inhibited ATP synthase activity, while hesperidin, chrysin, kaempferol, diosmin, apigenin, genistein, or rutin exerted partial inhibition in the range of 40–60%. Inhibition by galangin, daidzein, or luteolin was insignificant. The inhibition of ATP synthase by the polyphenols was completely reversible.

CHAPTER 3

MATERIALS AND METHODS

3.1 Fruit wines

The fruit wines used in the study were obtained from local grocery stores, and stored in a 4 °C refrigerator between use. The wines used for the study were peach (one sample from Montelle Winery, St. Genevieve, MO, referred to as peach #1, another from St. James Winery, St. James, MO, referred to as peach #2), red raspberry (Montelle Winery, St. Genevieve, MO), blackberry (St. James Winery, St. James, MO), and cherry (Mogen David Winery, Westfield, NY). All fruit wine samples were removed aseptically from the wine bottles using 10 mL sterilized serological pipettes for use in the study.

3.1.1 Drying procedure for dealcoholized fruit wines

Samples of fruit wines were transferred to sterilized 20 mL beakers, and 1.5 mL of the respective wine was pipetted into sterilized 2.0 mL microcentrifuge tubes and dried under nitrogen gas in the dark using an evaporating unit with 2.5" stainless-steel needles (Reacti-Vap Model 18780, Pierce, Rockford, IL) to remove ethanol. Samples were then stored in a dessicator at 4 °C until used.

3.2 Bacterial strains and growth conditions

Bacterial strains used in the study were *Escherichia coli* O157:H7 505B, *Listeria monocytogenes* Scott A, *Salmonella* Typhimurium ATCC 14028, *Shigella dysenteriae* ATCC 29028, and *Staphylococcus aureus* FRI, obtained from the culture collection of the University of Missouri Food Microbiology Laboratory. All cultures were propagated in

32

tryptic soy broth supplemented with 5% yeast extract (TSBY), except *L. monocytogenes*, which was propagated in brain heart infusion broth (BHI). All microbiological media reagents were procured from Difco Labs (Benton, Dickinson and Company, Sparks, MD). The culture isolates were all aerobically incubated at 37 °C for 24 h.

3.3 Tube dilution assay

3.3.1 Fruit wines

Two preliminary trials were performed to make a rough determination of the log reduction in the population of the foodborne pathogens that may occur due to exposure to the fruit wines. A 200 µL volume of *E. coli* O157:H7 was inoculated into 10 mL of TSBY, and the tube incubated aerobically at 37 °C for 24 h. Then, 20 µL of the broth culture was inoculated into five different concentrations (v/v), 0%, 25%, 50%, 75%, and 100%, of red raspberry wine and TSBY and the tubes incubated aerobically at 37 °C for 24h. The solutions were then serial-diluted and pour-plated with tryptic soy agar (Difco Labs.) supplemented with 5% yeast (TSAY) into sterile petri dishes, and incubated aerobically for 24 h at 37°C. No colonies grew on agar plates from the 50% or greater fruit wine concentration mixtures, so a subsequent preliminary trial was repeated for the same tube dilution assay, but with wine/broth mixtures of 0%, 40%, 50%, 60%, and 70% (v/v) in order to assess a concentration that could potentially be bactericidal. No colonies grew on agar plates from the 50% or greater wine solutions in the second preliminary trial, so the wine concentration used for primary trials were 0%, 30%, 40%, 50%, and 60%. Pathogens tested in the first primary trial were *E. coli* O157:H7, *L. monocytogenes*, *S.* Typhimurium, *S. dysenteriae*, and *S. aureus*. *L. monocytogenes* grew with countable plates at 60%, thus it was also inoculated into a 70% wine mixture

in subsequent trials. The trials were then repeated once with all of the wines and all of the bacteria.

3.3.2 Dealcoholized fruit wines

A preliminary trial using dealcoholized red raspberry wine and *E. coli* O157:H7 was conducted to estimate the log reduction in population of the pathogen that may occur due to exposure to the fruit wines that had the ethanol removed. For the preliminary trial, 200 μL of broth culture containing *E. coli* O157:H7 was inoculated into a tube containing 10 mL of TSBY, and then incubated aerobically at 37 °C for 24 h. Dealcoholized 1.5 mL red raspberry wine was reconstituted back to a volume of 1.5 mL using sterilized deionized water, and vortexed until completely dissolved. This rehydrated wine sample was then transferred to a sterilized test tube, and 1.5 mL of TSBY was added, to bring the mixture to 50% wine/broth concentration. As a control, 3 mL of TSBY was pipetted into another tube. Both tubes were inoculated with 10 μL of *E. coli* O157:H7 that had been subcultured, and the mixture and control were incubated for 24 h at 37°C. These were then serial-diluted and pour-plated in TSAY, and the plates incubated for 24 h at 37°C. There were no countable plates at 50% dealcoholized wine concentration, so the primary trials began with a 50% dealcoholized wine mixture as described in the following paragraph.

In the first primary dealcoholized fruit wine trial, 200 μL of broth culture containing *E. coli* O157:H7, *L. monocytogenes*, *S.* Typhimurium, *S. dysenteriae*, and *S. aureus* were each inoculated into 10 mL tubes with broth media for the respective culture (*L. monocytogenes* in BHI broth, the rest in TSY broth*)*, and incubated aerobically at 37 °C for 24 h. Dealcoholized red raspberry wine samples were reconstituted to 1.5 mL,

34

identical to the preliminary trial above, added to an autoclaved test tube, and 1.5 mL of the respective broth media for each culture was added to bring the wine concentration to 50%. A tube containing 3 mL of the 50% wine/broth mixture, and a 3 mL tube containing only the respective broth media were inoculated with 10 μL of the 24-h grown culture, and incubated aerobically for 24 h at 37°C. These were then serial-diluted and pour-plated in TSAY for all but those inoculated with *L. monocytogenes,* which were pour-plated with BHI agar. The plates were incubated for 24 h at 37°C, and colonies were enumerated. *E. coli* O157:H7, *L. monocytogenes*, and *S. dysenteriae* grew in up to a 50% wine mixture, so subsequent trials used the same procedure for the all of the fruit wines, but the concentration of the dealcoholized fruit wine was increased to 60%. Only 1 mL of the respective broth was added to the reconstituted wine, with a final volume of 2.5 mL, and a 2.5 mL of TSBY (or BHI for *L. monocytogenes*) was used as a control. The dealcoholized wine trials were replicated at least twice with each of the respective fruit wines and bacterium, except peach wine #2, which was not tested in the dealcoholized wine study.

3.4 Chemical analyses

3.4.1 pH measurement

The pH values of the wine samples were measured using a Corning Model 220 pH meter with a Corning Model 476086 electrode (Corning, Corning, NY), and replicated at least twice.

3.4.2 Titratable acidity

The titratable acidity (TA) of the fruit wines were determined by the titrametric procedure using NaOH described by Zoecklein and others (1995), and given in units of

g/L tartaric acid equivalents. A quantity of 100 mL of 0.1 N NaOH (Fisher Scientific, Fair

Lawn, NJ) was brought to a 0.0667 normality through the addition of 50 mL boiled and

cooled deionized water, and used as the base for all titrations. A 2 mL sample of each

fruit wine was pipetted into a beaker containing 100 mL of boiled and cooled deionized

water, and titrated to pH 8.2 using the same pH meter and probe used for pH

measurements, while being continually stirred on a magnetic stirrer. A 5.0 mL sample of

each fruit wine was then added using a volumetric pipette, and the solution titrated back

to pH 8.2 using a burette to measure the volume of NaOH added. TA of the fruit wines

was calculated with the equation:

$$TA \text{ (g/L tartaric acid equivalents)} = \frac{(mL \text{ base})(N \text{ base})(0.075)(1000)}{mL \text{ sample}}$$

The TA of the fruit wines was measured at least twice.

3.4.3 Alcohol by volume

The ethanol content of the wines was obtained from the labels on the wine

bottles.

3.4.4 Adams-Harbertson assay

3.4.4.1 Method summary

The Adams-Harbertson assay (Harbertson and others 2005) is a method used in

the U.S. to determine total phenols, tannins, anthocyanins, and polymeric pigments in

red wines through visible chemical changes that can be observed

spectrophotometrically. This method was used to assess the quantity of these

compounds present in the fruit wines studied.

36

3.4.4.2 Reagents

A washing buffer (Buffer A) was prepared by adding 20 mL of 1.0 M acetic acid to a beaker containing 60 mL of deionized water, and then adding 1.201 g NaCl. The solution was stirred, and then titrated to pH 4.9 using 1.0 M NaOH. This solution was then transferred to a 100 mL volumetric flask, and brought to 100 mL using deionized water. The stock 1.0 M acetic acid was prepared by adding 28.74 mL of glacial acetic acid to a 500 mL volumetric flask containing roughly 450 mL of deionized water, and bringing the volume to 500 mL with deionized water.

A model wine (Buffer B) was prepared by adding 1.0 g potassium hydrogen tartrate and 25.3 mL of 95% ethanol to a beaker containing 150 mL deionized water, and while stirring, titrating the solution to pH 3.30 using 1.0 M HCl. This solution was then transferred to a 200 mL volumetric flask and brought to a volume of 200 mL using deionized water.

A resuspension buffer (Buffer C) was prepared by adding 5.0 g of lauryl sulfate and 5 mL of triethanolamine to 80 mL of deionized water and titrating to pH 9.4 using 1.0 M HCl. The solution was then transferred to a 100 mL volumetric flask and brought to 100 mL using deionized water.

An acid buffer (Buffer D) was prepared by adding 4.6440 g of maleic acid and 1.9870 g of NaCl to a beaker containing 150 mL deionized water, and titrating to a pH value of 1.8 using 1.0 M NaOH.

A protein solution was prepared by adding 0.1 g of bovine serum albumin to a 100 mL volumetric flask and adding approximately 50 mL of buffer A. This was shaken to dissolve the precipitate, and then another 20 mL of buffer A was added. After this was

then shaken, solution was left to set in a 4 °C refrigerator overnight in order for foam to dissipate. The solution was then brought to 100 mL using buffer A.

A ferric chloride reagent was prepared by adding 1.0 mL of 1.0 M HCl into a volumetric flask containing 75 mL of deionized water, and then adding 0.1622 g ferric chloride. The flask was gently shaken until the contents were dissolved, and then brought to 100 mL volume using deionized water.

The bleaching reagent was prepared by adding 8.0 g to a 100 mL volumetric flask, bringing to 100 mL volume with deionized water, and shaking until dissolved.

A catechin standard was prepared by adding 0.10 g of +-catechin to a volumetric flask containing 10.525 mL 95% ethanol, and shaking vigorously. Approximately 50 mL of deionized water was added to the flask, and the contents shaken until dissolved. The flask was then brought to 100 mL with deionized water.

The bleaching reagent was made fresh every five to seven days, and the catechin standard was prepared on the day of use. The bleaching reagent and the protein solution were stored in a refrigerator while all other prepared media were kept at room temperature.

Measurements of mass were made using a Mettler AE260 Delta Range analytic scale. Measurements of pH were obtained using a Corning 220 pH meter and Corning Model 476086 electrode.

Sodium chloride, glacial acetic acid, maleic acid , hydrochloric acid and sodium hydroxide were obtained from Fisher Scientific. The potassium hydrogen tartrate was obtained from the U.S. Department of Commerce, National Bureau of Standards (Washington, D.C.). Ethanol (95%) was acquired from the University of Missouri

Chemistry Store. Triethanolamine, lauryl sulfate, ferric chloride, bovine serum albumin, and +-catechin were procurred from Sigma Chemical Co. (St. Louis, MO). Potassium metabisulfite was purchased from Mallinckrodt Chemical Workers (St. Louis, MO).

3.4.4.3 Standard curve for determination of catechin equivalents

A standard curve for catechin equivalents was constructed by measuring the visible changes observed from (+)-catechin reacting with ferric chloride. This standard curve was used to determine the equivalent amount of catechin that reacted from tannins and phenolics present in the fruit wines. Amounts of 0 µL, 50 µL, 100 µL, 150 µL, 200 µL, 250 µL, and 300 µL of the prepared catechin standard were transferred into 1.5 mL disposable cuvettes, and the volumes brought up to 875 µL with buffer C. Then, 125 µL of the ferric chloride reagent was added to each cuvette; the cuvette was then covered with paraffin and shaken, and left to sit for 10 min. The absorbance was read at 510 nm using a Varian Cary 50 UV-Vis Spectrophotometer (Agilent Technologies, Santa Clara, CA), and using buffer C as a blank.

3.4.4.4 Sample analysis

Analysis of large polymeric pigments, small polymeric pigments, tannins, and anthocyanins was performed sequentially (Fig. 3-1) at one time. Samples of each of the fruit wines were transferred to a beaker for use as an undiluted sample, and then pipetted into another beaker with an amount of the model wine (Buffer B) to dilute the sample from 1:1 to 1:9 (or from 2 times to 10 times dilution). A quantity of 500 µL of the diluted or undiluted wine sample was then pipetted into two 1.5 mL microcentrifuge tubes. The first tube had an addition of 1 mL of buffer A, and the second tube had 1.0 mL of the protein solution added. After 10 min, 1.0 mL from the first tube was pipette into

39

a cuvette, and the absorbance was measured at 520 nm using buffer A as a blank. This provided measurement A. Then, 80 µL of the bleaching solution was added to the cuvette and shaken. After 10 min, the absorbance of the solution was measured again. This provided measurement B.

After sitting for 15 min, the second tube was then centrifuged at 16,100 RCF (13,200 RPM) for 5 min using an Eppendorf, Model 5415D centrifuge. A 1.0 mL sample from the supernatant was then pipette into a 1.5 mL cuvette, 80 µL of the bleaching solution was added, and the mixture shaken. After 10 min, the absorbance was measured at 520 nm to provide measurement C.

The rest of the supernatant from the second tube was then removed carefully with a pipette, 250 µL of buffer A was added without agitating the pellet, and the suspension centrifuged at 16,100 RCF for 1 min. The supernatant was removed with a pipette, 875 µL of buffer C was added, and the tube was allowed to sit for 10 min. The tube was then vortexed until the supernatant dissolved, and sat for another 10 min. The sample was then pipetted into a cuvette, and absorbance measured at 510 nm, using Buffer C as a blank. This provided the tannin background measurement. The cuvette then had 125 µL of ferric chloride added, was shaken, and sat for 10 min. Absorbance was read again at 510 nm, which provided the tannin final measurement.

Using the same dilution of fruit wine sample as was pipetted into the two tubes, 500 µL of the fruit wine was added to a cuvette containing 1 mL of buffer D, shaken, and then let sit for 5 min. The absorbance was read at 520 nm, using buffer A as a blank. This provided measurement D.

40

For phenolics measurement (Fig. 3.2), a 25 µL to 300 µL sample of undiluted fruit wine was pipette into a 1.5 mL cuvette, brought to 875 µL with buffer C, and then set for 10 min. Absorbance was measured at 510 nm, using buffer C as a blank, which provided the total phenolics background measurement. Then, 125 µL of the ferric chloride solution was added to the cuvette, shaken, and allowed to sit for 10 min. The absorbance was measured again at 510 nm, and this provided the total phenolics final measurement.

The procedure for generating the standard curve and fruit wine sample analysis was replicated at least 3 times.
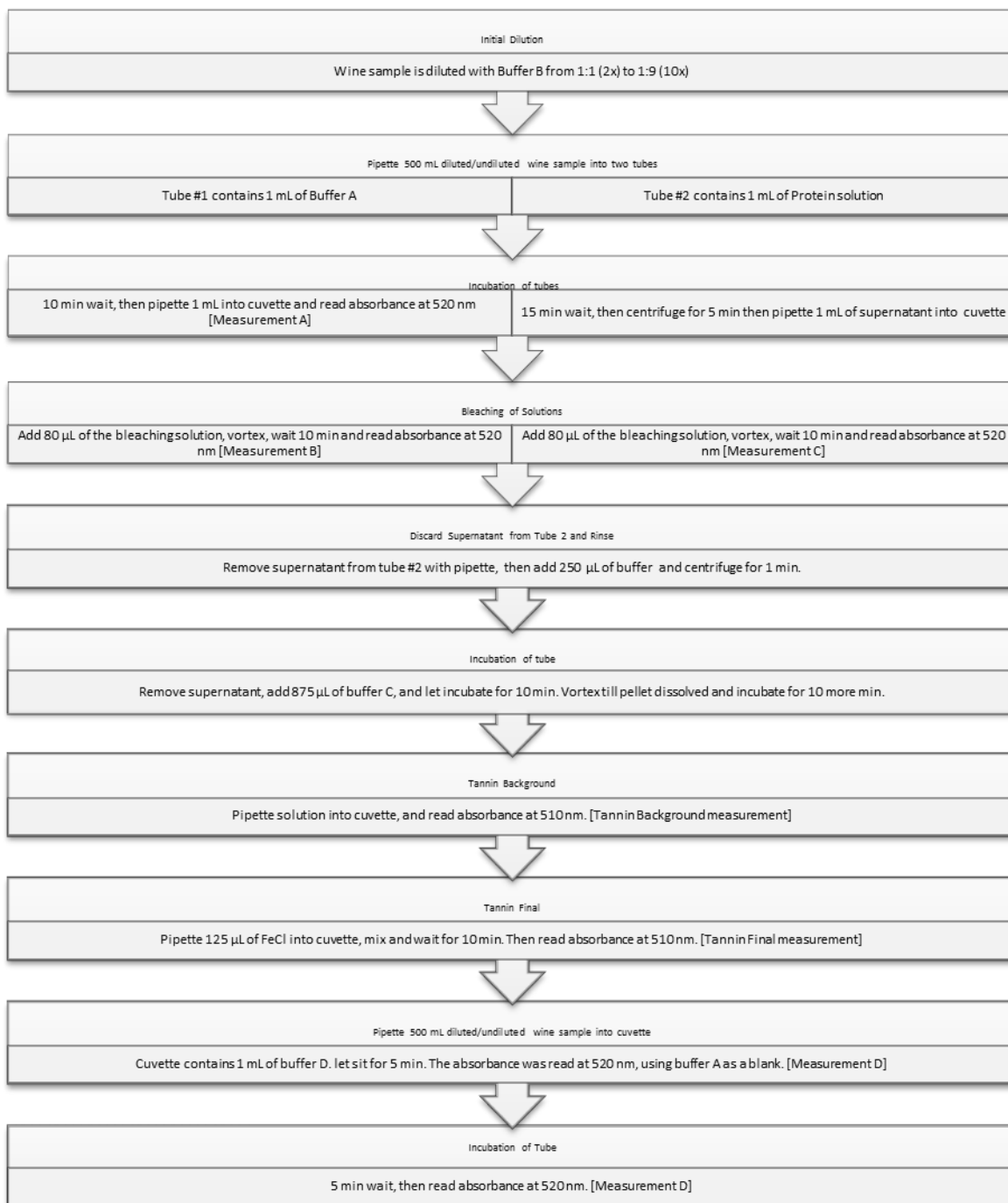
Figure 3-1   Adams-Harbertson assay for measuring large polymeric pigments, small polymeric pigments, tannins, and anthocyanins.
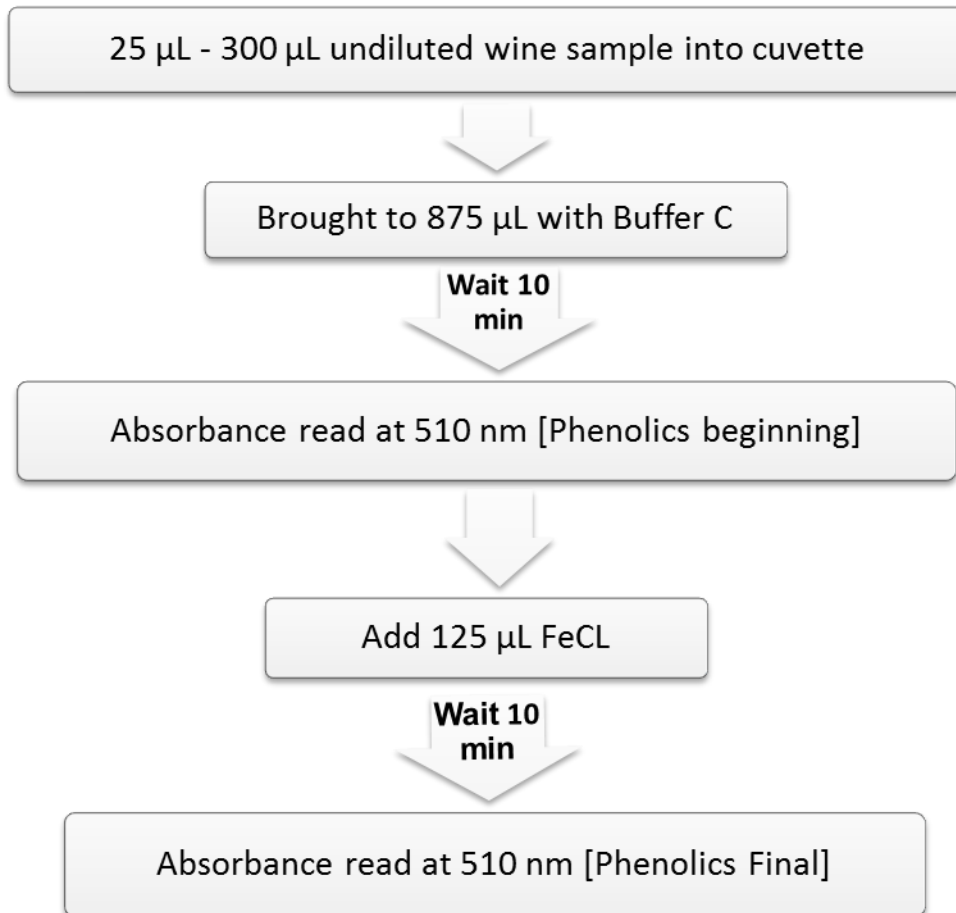
Figure 3-2   Adams-Harbertson assay for measuring phenolics.

3.4.4.5 Determination of large and small polymeric pigments, anthocyanin, tannin, and total phenolic content

The following equations were used to determine the content of compound present based on absorbance measurements of samples:

Large polymeric pigment (LPP)[1,2]:

$$(1.08) *(\text{dilution factor})*(4)*(B-C)$$

Small polymeric pigment (SPP)[3]:

$$(1.08)*(\text{dilution factor})*(30/7)*(C)$$

Anthocyanin:

$$\frac{[(\text{wine dilution}) \times (D) - (\text{wine dilution}) \times (A)]}{0.0102 \; ^{L}/_{mg\,AU}}$$

The absorbance due to tannin is:

[(Tannin Final A510) – (A510 from zero tannin)] – (Tannin background A510*0.875)

General equation for catechins equivalent of tannins:

$$2 \times \left(\frac{Abs - Intercept}{Slope}\right) \times (Dilution)$$

The absorbance due to total phenolics is:

[(Total Phenolics Final A510) – (A510 from zero tannin)] – (Total phenolics background 510 nm *0.875)

---

[1] The value of 1.08 used for LPP and SPP is a correction factor for samples that were diluted with sulfur dioxide.

[2] The value of 4 used for LPP is a correction factor for sample dilution and a bleaching correction coefficient.

[3] The value of 30/7 used for SPP is a correction factor for sample dilution and a bleaching correction coefficient.

General equation for catechins equivalent of phenolics:

$$\left(\frac{Abs - Intercept}{Slope}\right) \times \left(\frac{1000\ \mu L}{sample\ size\ in\ \mu L}\right)$$

3.4.5 Free sulfur dioxide

The free sulfur dioxide concentration of the fruit wines were measured using a

Chemwell 2902, automated chemical analyzer (Unitech Scientific, Hawaiian Gardens,

CA), with the free sulfites kit (Unitech Scientific Flex-Reagent Free Sulfites Kit, and

Standards Free-SO_2). All reagents from the kit were prepared fresh each day prior to

use. The Chemwell machine was first turned on, all self-tests were performed, then Start

of Day routines were run to prime pumps. The machine was then calibrated using the

Free SO_2 5 standard routine, using the following sulfite standards from Unitech

Scientific, 10 ppm, 20 ppm, 50 ppm, 80 ppm, and 100 ppm. After the calibration

procedure and activating values for standard curve, wine samples were then removed

aseptically from wine bottles using sterile serological pipettes, and pipetted into 1.5 mL

centrifuge tubes. The samples were then centrifuged for 3 min at 16,100 RCF (13,200

RPM). After centrifugation, 1 mL of supernatant was pipetted into a cuvette and sample

analysis begun. The samples were prepared within 30 min of analysis to prevent

elevated free sulfite levels from occurring due to warmer incubations. The free sulfite

analysis was repeated at least 3 times.

3.5 Scanning electron microscopy and transmission electron microscopy

3.5.1 Twenty four h wine exposure sample preparation

A 24 h exposure of 2 pathogens to two of the fruit wines used in the fruit wine

tube dilution assay was performed to provide samples that were to be used for scanning

electron microscopy (SEM) and transmission electron microscopy (TEM) observation. A

200 µL volume of *E. coli* O157:H7 and *S.* Typhimurium were inoculated into 10 mL of

TSBY, and the tubes incubated aerobically at 37 °C for 24 h. A one mL sample of each

culture was then inoculated into a 10 mL tube of TSBY, a 10 mL tube of 60% (v/v) peach

wine #1 and TSBY, and a 10 mL tube of 60% (v/v) cherry wine and TSBY. TSBY was

used as a control, and the peach wine #1 and cherry wine were used for observation

because they had been the most and least bactericidal wines tested, respectively. *E. coli*

O157:H7 and *S.* Typhimurium were chosen for the bacteria to be observed because the

first was found to be very resilient to the fruit wines and the latter very sensitive, and a

difference in the wine treatments using SEM/TEM was predicted to be observed. The

tubes were then incubated aerobically at 37 °C for 24 h. These were then serial-diluted,

pour-plated in TSAY, and the plates incubated for 24 h at 37 °C and colonies counted.

The bacterial culture remaining in the test tubes after pour plating were immediately

transferred to primary fixative for electron microscopic examinations.

3.5.2 Primary fixation

Samples *of E.coli* O157:H7 and *S.* Typhimurium that had been exposed to either

TSBY, a 60% (v/v) peach wine and TSBY, or 60% (v/v) cherry wine and TSBY treatment

for 24 h were centrifuged in sterilized 10 mL centrifuge tubes using a Beckman Model

J2-21 Centrifuge with a Beckman Model JA-20.1 rotor at 12,857 RCF (10,000 RPM) for

10 min. The supernatant was then discarded, and 1.0 mL of primary fixative was

pipetted into the tube and the mixture vortexed until the pellet was completely dissolved.

Primary fixative used for preparing SEM/TEM samples had the composition of

2% glutaraldehyde, 2% paraformaldehyde, 0.1 M sodium cacodylate

(Na(CH3)$_2$AsO$_2$•3H$_2$O), pH 7.35, and was purchased from the University of Missouri Electron Microscopy Core Facility. The cell suspension in primary fixative was then transferred to sterilized 1.5 mL microcentrifuge tube and refrigerated until used.

3.5.3 Critical point drying and sample mounting for SEM

Critical point drying was performed to reduce all moisture from samples in order to minimize artifacts during SEM observation. The procedure began with preparation of a slip that would be used for mounting the fixed cells to. A 13 mm Thermanox plastic coverslip (Nalge Nunc International, Rochester, NY) was folded with tweezers at an edge so the slip could be easily picked up with tweezers. Approximately two drops of poly-L-lysine solution was pipetted onto the middle of the coverslip, beginning at the crease bent into the slip. The slips sat for 40 min to allow poly-L-lysine to adhere to the Thermanox coverslip, then rinsed with ultrapure water from a Millipore Milli-Q Ultrapure water system (Millipore, Billerica, MA), and dried at room temperature.

Samples in primary fixative were then centrifuged using a Phenix Quickspin centrifuge (Phenix Research Products, Candler, NC) for 2 min and the supernatant was discarded using a pipette. The sample was then rinsed by pipetting 1 mL of 0.1 M sodium cacodylate [Na(CH3)$_2$AsO$_2$•3H$_2$O] buffer (pH 7.35) on the pellet, vortexing the sample, and centrifuging again for 2 min. The supernatant was again removed with a pipette, diluted with 0.1 M sodium cacodylate, and 1.5 mL added to the pellets of *E. coli* O157:H7 and *S.* Typhimurium exposed to TSBY, while 0.4 mL was added to the samples exposed to the peach and cherry wine mixtures. The difference in dilutions was because the pellets for the wine exposed samples were significantly smaller in size. The slips were then placed in small petri dishes, three drops of each of the diluted sample

47

solutions was pipetted onto the middle of the prepared slips, and left to sit for 30 min so

cells would stick to the poly-L-lysine area of slip. After setting, excess solution was

removed with a pipette, and the attached cells rinsed twice with 5-6 drops of 0.1 M

sodium cacodylate buffer, leaving the buffer on the slip for 5 min before removing each

time. Then, 300 mL of 0.1 M sodium cacodylate and 300 mL of 4% osmium tetroxide

(OsO_4) solution were pipetted onto the slips, to bring the OsO_4 solution to 2%, and the

petri dishes containing the slips were placed into a Pelco Biowave Microwave Sample

Processing System (Ted Pella, Inc., Redding, CA). The unit was started with the

following conditions: vacuum on, 100 watts, 60 s off, 80 s on, 3 min off, 40 s on. After

the unit finished venting, the petri dishes with slips were removed, and the slips were

rinsed two more times for 5 min each with 5-6 drops of 0.1 M sodium cacodylate buffer.

This was followed by 3 rinses with 5-6 drops of ultrapure water, 15 s each, then one

rinse for 5 min. After the fourth pure water rinse, slips were rinsed with a series of diluted

200 proof ethanol (Decon Laboratories, King of Prussia, PA) and ultrapure water,

followed by three pure ethanol rinses, each rinse being 3 min (Table 3-1). These rinses

were performed to remove water before using a critical point dryer. The ethanol was not

removed from the slips for the last rinse until they were ready to be loaded into sample

holder for the critical point dryer that also contained 200 proof ethanol.

Table 3-1   Ethanol rinse for dehydration in critical point drying procedure

Rinse #	Ethanol	Time
1	20%	3 min
2	50%	3 min
3	70%	3 min
4	90%	3 min
5	100%	3 min
6	100%	3 min
7	100%	3+ min

Following the last rinse with pure ethanol, slips were loaded into a steel 13 mm sample holder for a Tousimis Auto-Samdri 815 automatic critical point dryer (Tousimis Research Corporation, Rockville, MD), and the machine purge time was set to 3.5. After critical point dryer completed purging and venting, the sample holder was removed, and slips were removed. Slips were then mounted to standard 12.7 mm x 8 mm SEM pin stub SEM mounts using Silver Paste Plus (SPi Supplies, West Chester, PA) as an adhesive. A metallic coat using Pelco Colloidal Silver Liquid (Ted Pella Inc., Redding, CA) was applied around the edge of the slips to prevent charging during SEM observation. Mounted samples were then placed in a small box and kept in a 40 °C drying oven until used for observation.

The 0.1 M sodium cacodylate buffer, 4% OsO_4 solution were prepared and/or purchased from the University of Missouri Electron Microscopy Core Facility.

3.5.4 Sputter coating samples for SEM

Prior to SEM observation, samples on SEM stubs were sputter-coated with platinum using an Emitech K575x Turbo Sputter Coater (Emitech LTD, Kent, England), with 20 mA current, 1.0 min sputter time for settings, and using argon gas.

3.5.5 SEM observation

The samples mounted on SEM stubs were observed using a Hitachi S-4700 Field-Emission Scanning Electron Microscope (Hitachi, LTD., Tokyo, Japan). The beam setting was 3.0 kv, 20.0 mA, and the working distance was 6.0 mm. Images were captured using High Res setting (2560 × 1920).

3.5.6 Microwave processing for TEM

HistoGel specimen processing gel (Richard-Allen Scientific, Kalamazoo, MI) was melted by transferring approximately 3 mL to a 5 mL cryovial tube, putting the tube in boiling water for approximately 15-20 s, and then removing after the gel liquefied. Samples in primary fixative were then centrifuged one at a time using a Labnet Force 7 microcentrifuge (Labnet International Inc., Edison, NJ) for 2 min at 7,200 RCF (10,000 RPM) and the primary fixative/supernatant was discarded using a pipette. Two drops of warm HistoGel was pipette into the tube, and the pellet was lifted from the bottom using a sterile applicator stick to encase it with the HistoGel. The sample was then centrifuged again for 30 s, and then put into refrigerator for 10 min. Using an applicator stick, solidified HistoGel with concentrated cell pellet was removed from the tube onto a petri dish, and then sliced into thin slivers using a razor, and using a Leica S8AP0 microscope (Leica Microsystems GmbH, Wetzlar, Germany) with a Schott Modulamp (Schott North America, Inc., Elmsford, NY) as an aid. A drop of HistoGel was added to encompass slivers, and the sliced samples were then refrigerated again. The encased slivers were then cut on the outside edges with a razor to remove excess HistoGel. The pieces of HistoGel-encased cells were then put into labeled 1.5 mL centrifuge tubes with 1 mL of 0.1 M sodium cacodylate buffer (pH 7.35) for 30 s, the buffer pipetted out and replaced

50

with another 1 mL of 0.1 M sodium cacodylate buffer, and the samples refrigerated for 16 h. The samples were then rinsed 3 times for 20 min each with a 2-ME buffer containing 0.1 M sodium cacodylate, 0.13 M sucrose, and 0.01 M 2-mercaptoethanol, incubated at room temperature and placed on a Boeckel Rocker II (Boeckel Scientific, Feasterville, PA). Samples were then postfixed with 1% osmium tetroxide by adding 300 µL of 2-ME buffer, then adding 100 µL of 4% OsO_4 solution and microwaving under vacuum using a Pelco Biowave Microwave Sample Processing System at 100 watts for 1 min off, 80 s on, 3 min off, and 40 s on. After the microwave treatment, samples were put a in 4°C refrigerator for 30 min. Samples were then rinsed again with 2-ME buffer, 3 times for 5 min each, with room temperature incubations on a rocker. This was followed by 3 quick rinses with Milli-Q pure water, 50 s each, followed by 3 pure water rinses with 5 min incubation on the rocker. The samples were then dehydrated with a series of ethanol and acetone rinses (Table 3-2). The ethanol and acetone dehydration rinses used the microwave under vacuum at 100 Watts for 40 s after pipetting the solution into a tube, then incubating at room temperature on the rocker for the specified time below.

Table 3-2 Ethanol and acetone rinses for dehydration step for TEM microwave processing

Rinse #	Ethanol	Time
1	20%	3 min
2	50%	3 min
3	70%	3 min
4	90%	3 min
5	100%	3 min
6	100%	3 min
Acetone		
7	100%	3 min
8	100%	3 min
9	100%	3 min

Following dehydration, the samples were then infiltrated with resin by pipetting increasing ratios of resin to solvent, using Epon/Spurr's resin and acetone, and microwaving under vacuum at 250 Watt for 3 min. The ratio of acetone to Epon/Spurr's resin and incubation time at room temperature on a rocker is given below, in Table 3-3.

Table 3-3 Resin infiltration rinses for TEM microwave processing

Solvent:Resin	Incubation time
2 solvent:1 resin	5 min
1 solvent:1 resin	1 h
1 solvent:2 resin	1 h
pure resin	1 h
pure resin	17.5 h
pure resin	2 h
pure resin	4.5 h

After the fourth pure resin rinse with microwave treatment, a Pelco Model 105 flat embedding mold (Ted Pella, Inc., Redding, CA) was prepared by putting paper labels detailing the sample facing up, and then transferring the resin infiltrated samples in

HistoGel to the end of the wells in the mold using sterile wooden applicator sticks. Pure resin was then pipetted into the well of the mold until full. Adjustment with an applicator stick was made again after resin addition to position samples at the end of the well, and the amold was placed in a 60 °C oven for 24 h to polymerize the resin.

The 0.1 M sodium cacodylate buffer, 2-ME buffer, 4% OsO_4 solution, Epon/Spurr's resin were prepared at and/or purchased from the University of Missouri Electron Microscopy Core Facility.

3.5.7 Sectioning and staining

Embedded cell samples were sectioned using a Leica Ultracut UCT ultramicrotome. Using a glass blade made that was prepared using an LKB 7801A knifemaker (LKB Vertriebs GmbH, Vienna, Austria), the ultramicrotome sectioned to the sample area in the resin using 2.5 μm cuts. At the sample area, either two or three sections of 2.5 μm cut resin sample was fixed onto a clean glass slide by putting the section flat on the slide and heat-fixing on a 60 °C hotplate until the water evaporated. Alkaline toluidine blue dye was then applied to samples on the slide and put on a 60 °C hotplate until green rings appeared. The slide was promptly removed from the hotplate and rinsed with Milli-Q pure water. After rinsing, the slide was returned to the 60 °C hotplate to dry. An Olympus BX40CY microscope (Olympus Corporation, Tokyo, Japan) was used to observe the thick section stain, to determine areas of embedded sample that was to be cut out for thin sectioning. The area identified for thin sectioning was a 0.5 mm x 1.00 mm trapezoid shape so that the sections could be applied to a TEM grid.

In preparation for thin sectioning, the embedded sample was fastened into a chuck in the Leica ultramicrotome, and the resin around the trapezoid area desired was

cut away using a razor blade, leaving the sample area raised over the rest of the embedded sample. The thin sectioning was prepared by cutting the resin sample at 85 nm thickness, at a speed of 1.4 mm/s, and using a Ultra 45°C Diamond Section Diatome Diamond Knife (Diatome US, Ft. Washington, PA) in the Leica ultramicrotome instead of the glass blade previously used. Initial rough cuts were removed from the diamond knife well. Subsequent cuts were then collected on 3.05 mm diameter, model #: T200-cu, Gilder copper grids (Electron Microscopy Sciences, Hatfield, PA), and dried in a 40 °C incubator for 24 h.

The grids were stained by putting parafilm on the bottom of a petri dish, and putting drops of Sato's triple lead stain on the parafilm in the middle of the dish, with sodium hydroxide pellets on the outside edge to absorb carbon dioxide, and then putting the grids in the triple lead stain for 5 min. The grids were rinsed with Millipore pure water for 15 s, and a new layer of parafilm with drops of 5% uranyl acetate were put into the petri dish. Grids were put in the uranyl acetate for 15 min, and were again rinsed with pure water. The grids were then stained again with the lead stain for 8 min and rinsed with pure water. After the last pure rinse, the grids were put into a grid box and put into a 40 °C incubator to dry.

The alkaline toluidine blue stain, the Sato's triple lead stain, and 5% uranyl acetate stain were prepared at and purchased from the University of Missouri Electron Microscopy Core Facility.

3.5.8 TEM observation

The samples on sectioned TEM grids were observed using a JEOL 1400 Transmission Electron Microscope (JEOL Ltd, Tokyo, Japan), operating at 80 kV.

54

3.6 Statistical analysis

The data from the microbiological and chemical tests were analyzed with Student's t-test for significant differences within a bacterial population, and Tukey's Honest Significant Difference to determine differences among multiple groups, using Microsoft Excel (Redmond, WA) and R (R Development Core Team, Vienna, Austria), respectively. Plotting of data was performed using Microsoft Excel.

CHAPTER 4

RESULTS

4.1 Tube dilution assay preliminary studies

4.1.1 Fruit wine, 24 h exposure

The two preliminary trials that exposed *Escherichia coli* O157:H7 to red raspberry

wine for 24 h showed a significant decrease in bacterial counts at wine concentrations of

40% or more, as shown in Table 4-1.

Table 4-1 Numbers of *E. coli* O157:H7 exposed to red raspberry wine for 24 h
(preliminary study).

Wine concentration Trial 1	Bacterial count (CFU/mL)	Wine concentration Trial 2	Bacterial count (CFU/mL)
0	5.2×10^9	0	3.9×10^9
25	2.5×10^8	40	1.2×10^5
50	$< 2.5 \times 10^4$	50	0
75	< 250	60	0
100	< 25	70	0

4.1.2 Dealcoholized fruit wines, 24 h exposure

The preliminary trial examining the effect of dealcoholized Red Raspberry wine

(ethanol removed) showed a complete inhibition of *E. coli* O157:H7 as compared to a

control, as shown in Table 4-2.

56

Table 4-2   Numbers of *E. coli* O157:H7 exposed to dealcoholized red raspberry wine for 24 h (preliminary study).

Dealcoholized Wine Concentration	Bacterial count (CFU/ml)
0	3.8×10^9
50	0

4.2. Tube dilution assay primary study

4.2.1 Exposure to fruit wines for 24 h

The primary study examining the effect of fruit wines on the pathogens demonstrated a significant log reduction in colony forming units for all of the bacteria studied, except *Listeria monocytogenes,* at concentrations of 30% or greater ($P \leq 0.05$). A significant log reduction of *L. monocytogenes* was found at fruit wine concentrations of 40% and greater ($P \leq 0.05$).

Of the wines tested, the Montelle red raspberry, shown in Figure 4-1, demonstrated the second greatest reduction in CFU on the exposed bacteria, with an average log reduction of 7.23 at 40% wine concentration. *E. coli* O157:H7 was, on average, reduced by 6.4 log CFU at that concentration, and was completely inhibited at 60% concentration. *L. monocytogenes* was the most resilient to the Red Raspberry wine, and was reduced, on average, by 2.1 log CFU at 40% concentration. It was completely inhibited at 70% wine concentration. *Salmonella* Typhimurium and *Shigella dysenteriae* were both completely inhibited from a 40% concentration of the red raspberry wine, with a log reduction of 8.9 and 9.1, respectively. *Staphylococcus aureus* was completely inhibited after exposure to 40% and greater concentrations, with an average log reduction of 8.2 CFU.
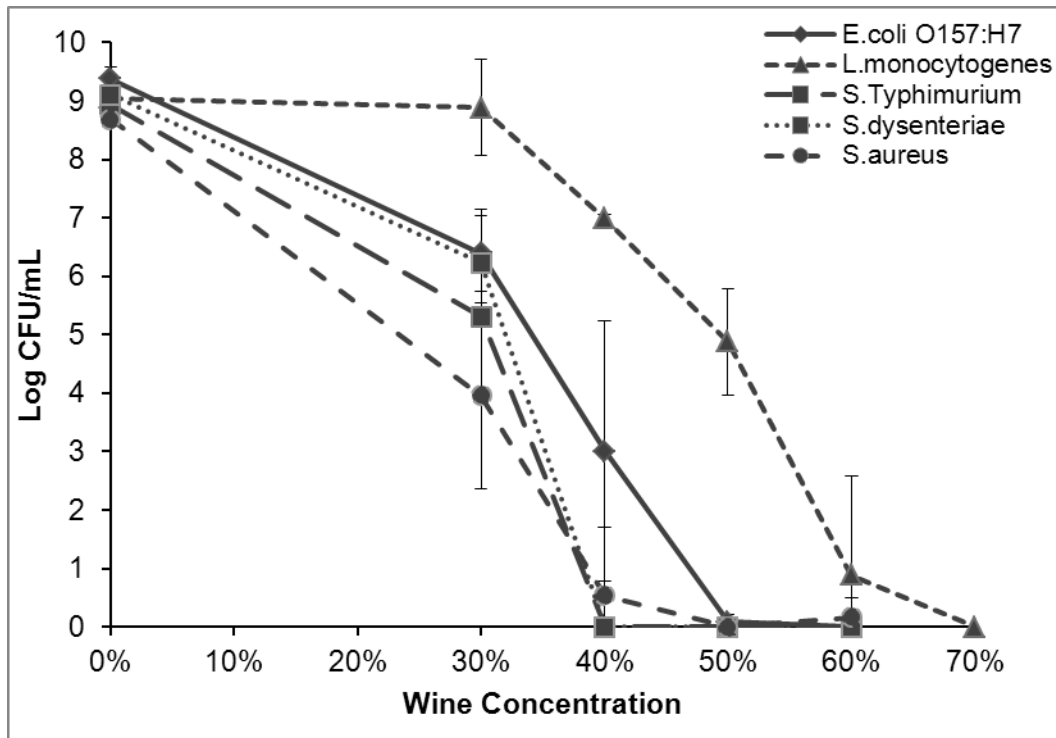
Figure 4-1   Number of pathogens exposed to red raspberry wine for 24 h.

The blackberry wine, shown in Figure 4-2, demonstrated the third greatest

reduction in CFU on the exposed bacteria, with an average log reduction of 5.12 at 40%

wine concentration. *E. coli* O157:H7 was, on average, reduced by 3.8 log CFU at a 40%

concentration, and was not completely inhibited by the wine at the concentrations tested.

L. monocytogenes was not completely inhibited at any concentration, and demonstrated

a log reduction of 0.4 CFU at 40% concentration. This pathogen was the most resistant

to the wine. *S.* Typhimurium, *S. dysenteriae*, and *S. aureus* were also not completely

inhibited at 40% concentration, with a log reduction in CFU's of 5.6, 5.5, and 7.2,

respectively. They were completely inhibited at 50% concentration and greater. *S.*

aureus demonstrated the greatest log reduction from the blackberry wine.
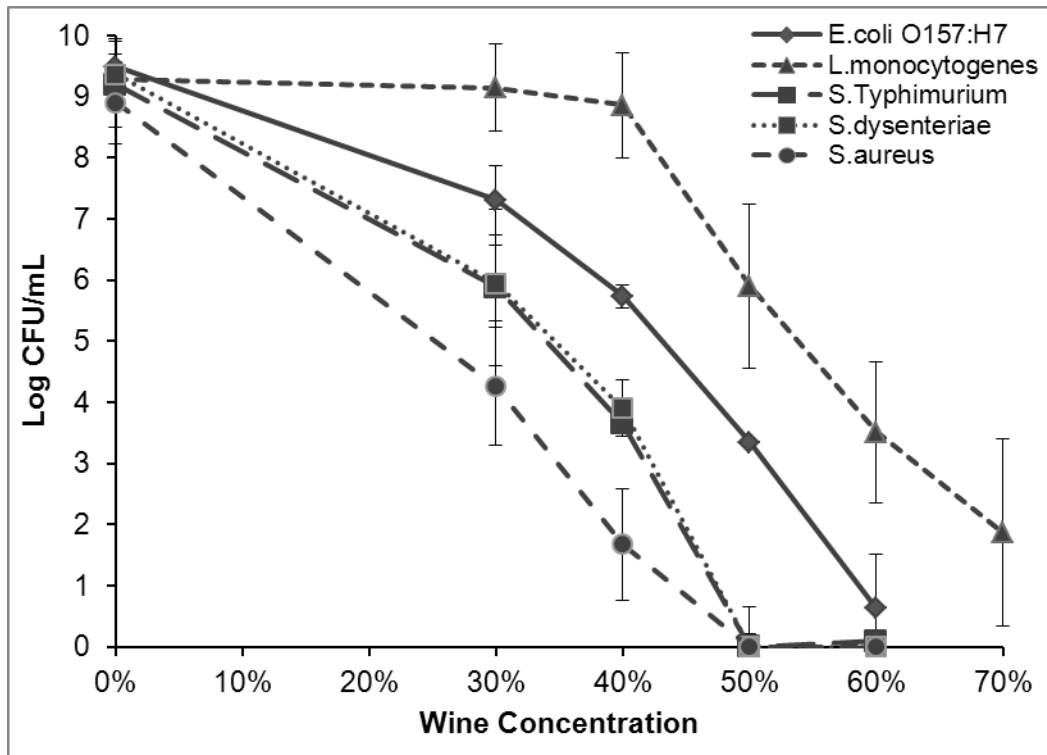
Figure 4-2   Number of pathogens exposed to blackberry wine for 24 h.

Peach wine #1, shown in Figure 4-3, demonstrated the greatest reduction in CFU on the exposed bacteria for all the wines tested, with an average log reduction of 8.43 at 40% wine concentration. At 30% concentration, peach wine #1 had an average log reduction of 5.29 CFU, demonstrating a greater reduction than all the other wines besides the red raspberry at a 40% concentration. Except for *L. monocytogenes*, all of the bacteria were completely inhibited by the peach wine at concentrations of 40% or greater. *L. monocytogenes* was the most resistant, with a log reduction of 4.6 CFU at 40% concentration, and was completely inhibited at 70% concentration. *S. aureus* and *S.* Typhimurium were the most susceptible to the effects of peach wine #1, with log reductions of 8.2 and 7.8 CFU, respectively at 30% wine concentration.

59
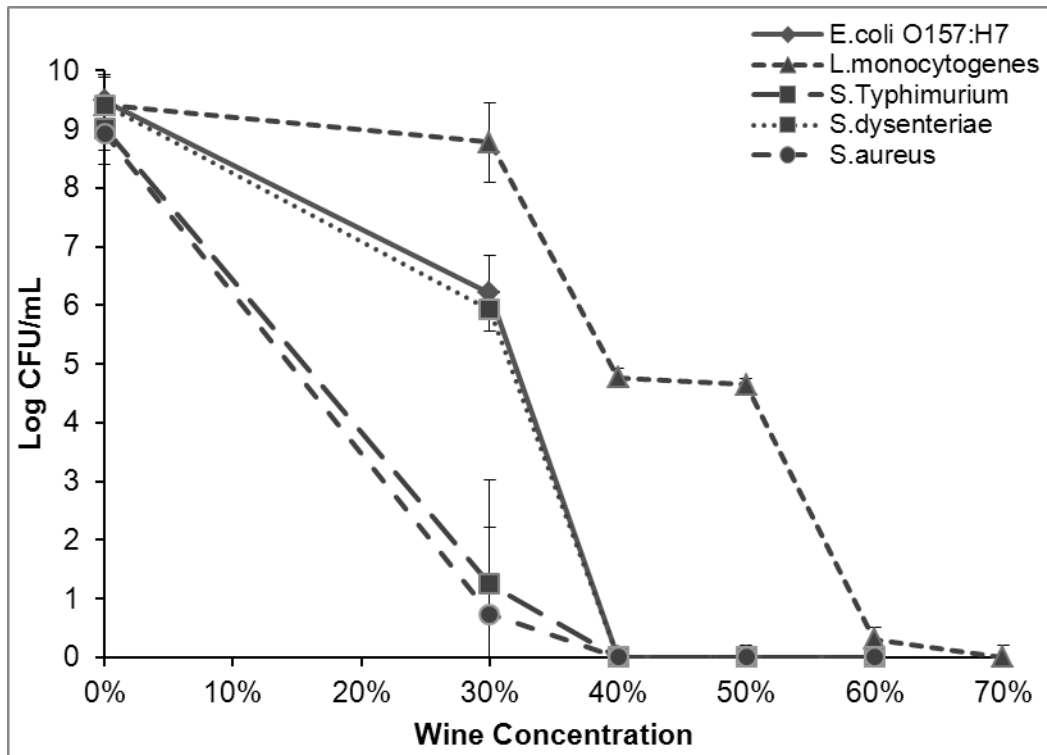
Figure 4-3   Number of pathogens exposed to peach wine #1 for 24 h.

The cherry wine, shown in Figure 4-4, demonstrated the fourth greatest reduction in CFU of the exposed bacteria, with an average log reduction of 3.30 at 40% wine concentration. The pathogens were not completely inhibited at any of the concentrations tested. *E. coli* O157:H7 had, on average, been reduced by 3.3 log CFU at 40% concentration. *L. monocytogenes* demonstrated the greatest resistance to the effects of the wine, with a reduction of 0.1 log CFU at 40% concentration, and 1.1 log CFU at 50% concentration. *S.* Typhimurium, *S. dysenteriae*, and *S. aureus* were reduced by 4.5, 3.3, and 5.1 log CFU at 40% wine concentration, respectively.
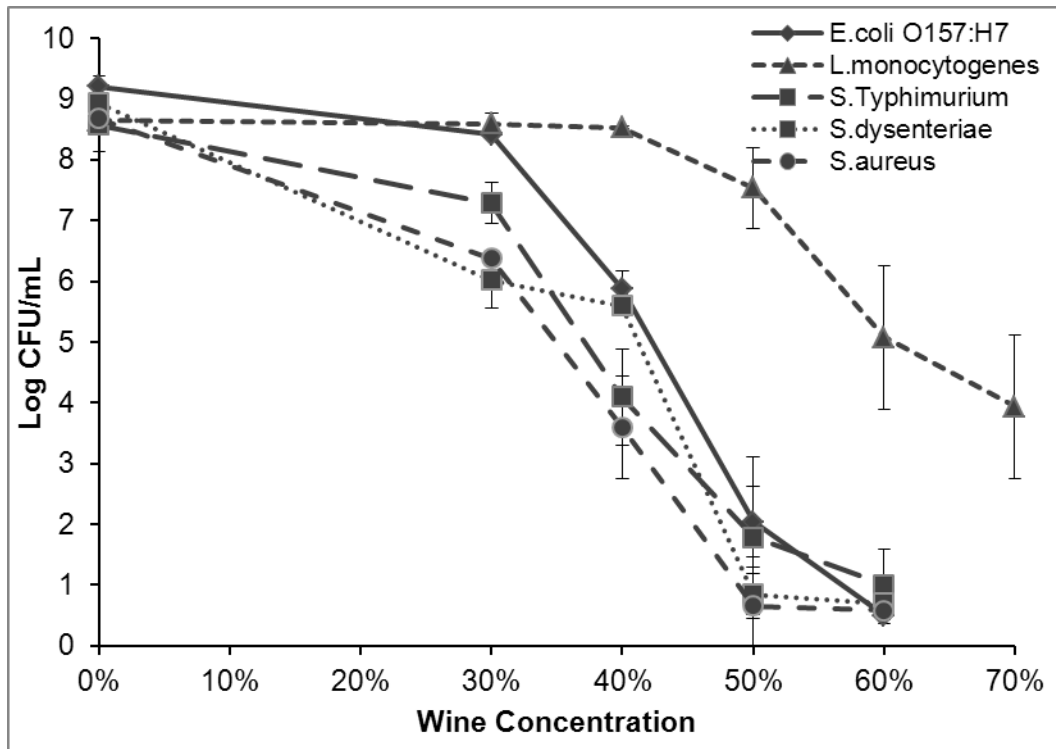
Figure 4-4   Number of pathogens exposed to cherry wine for 24 h.

Peach wine #2, shown in Figure 4-5, demonstrated the least reduction in CFU of the exposed bacteria for all the wines tested, with an average log reduction of 2.46 at 40% wine concentration. *E. coli* O157:H7 and *L. monocytogenes* exhibited, on average, log reductions of 4.2 and 0.7 CFU at that concentration, respectively, with *E. coli* O157:H7 exhibiting greater reduction in CFU after exposure than *L. monocytogenes*.

The trials using peach wine #2 used only the two most resistant bacteria, *E. coli* O157:H7 and *L. monocytogenes*, as compared to the other studies using all five species. The trials with peach wine #2 were conducted after performing trials of all the other wines, and finding that peach wine #1 had significantly greater bactericidal activity than the other wines. To see if the results were an anomaly for just that particular bottling of

peach wine and not the fruit type, another brand of peach wine was used. Though the average log reduction in CFU after exposure to peach wine #2 was the least of the wines tested, it would not be the most accurate indicator for the bactericidal activity because of the pathogens tested compared to using all of them. Based on the results of the log reduction of the wine at 40% concentration compared to other wines for specifically these two bacteria, peach wine #2 would have been the third most bactericidal wine, after peach wine #1 and red raspberry wine. The cherry wine exhibited the least bactericidal activity of the wines in terms of log reduction of CFU based on these two pathogens.
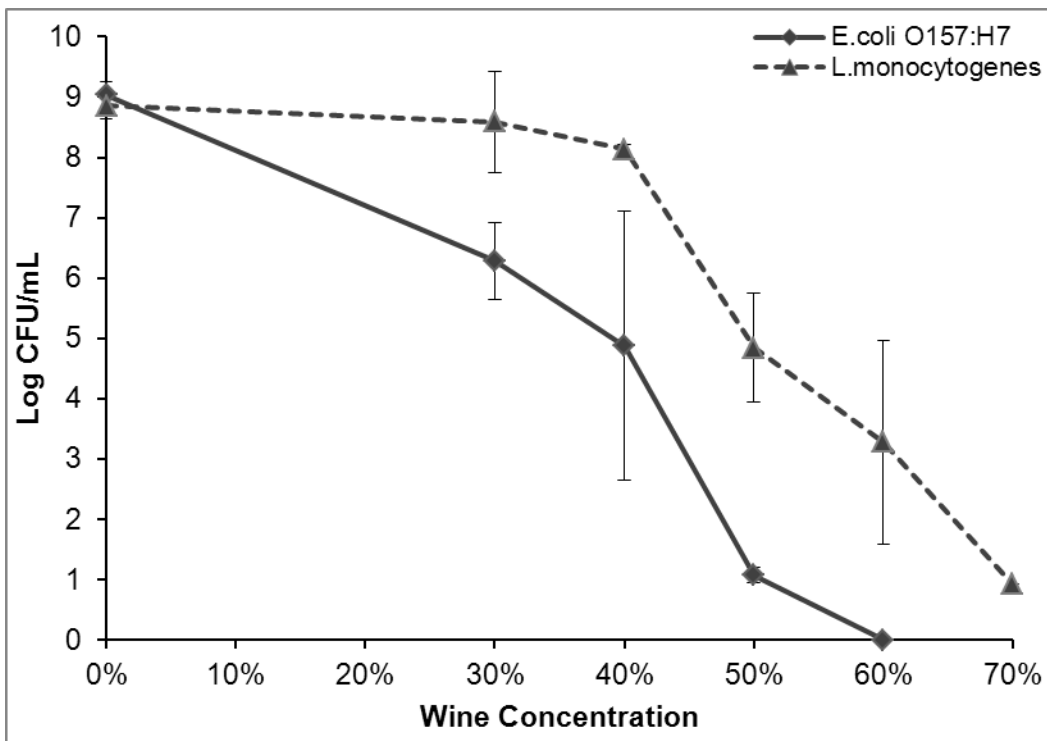
Figure 4-5   Numbers of *E. coli* O157:H7 and *L. monocytogenes* exposed to peach wine #2 for 24 h.

Analysis of variance (ANOVA) was used to compare the effects of bacterial species and wine concentration on numbers of bacteria as shown in Table 4-3, using all species and wines, except peach wine #2, and for concentrations between 0-60%. Peach wine #2 was not included in the ANOVA, because not all species of bacteria were tested using it. The 70% concentration was not included, because only *L. monocytogenes* was treated with fruit wines at that concentration. Wine concentration was the most significant factor in log reduction of the bacteria, followed by bacterial species and type of wine used ($P \le 0.01$). Interactions between species and wine concentration, type of wine and concentration, species and type of wine, and the combination of species, wine, and concentration were also found to be significant ($P \le 0.01$).

Table 4-3   Analysis of variance table examining the factors and interactions between the factors; using all species, 4 types of fruit wine, concentrations between 0-60%, and using CFU as response after 24 h exposure.

Source	Df	Sum Sq	Mean Sq	F value	Pr(> F)
Species	4	527.91	131.98	390.0541	< 2.2×10^{-16}
Wine	3	115.44	38.48	113.7245	< 2.2×10^{-16}
Concentration	4	2499.19	624.80	1846.5663	< 2.2×10^{-16}
Species*Wine	12	12.29	1.02	3.0274	0.0007701
Species*Conc.	16	211.57	13.22	39.0813	< 2.2×10^{-16}
Wine*Conc.	12	106.44	8.87	26.2161	< 2.2×10^{-16}
Species*Wine*Conc.	48	95.77	2.00	5.8969	< 2.2×10^{-16}
Residuals	153	51.77	0.34		

Analysis using Tukey's HSD ($P \le 0.05$), shown in Table 4-4, indicated that the trend for a 24-h wine exposure at the various concentrations for *L. monocytogenes* was

significantly different than all other pathogens tested when comparing differences in

species based on log reduction of CFU. Overall, there was less reduction in log CFU

after exposure to the various wine types and concentrations for *L. monocytogenes* than

the other pathogens. *E. coli* O157:H7 exhibited the second least reduction in log CFU

overall, and was also significantly different from all other bacteria. *S. dysenteriae and S.*

Typhimurium were significantly different from *L. monocytogenes, E. coli* O157:H7, and

S. aureus when examining differences in log CFU among species, but were not different

from each other at α = 0.05. *S. dysenteriae* had the third least reduction in log CFU after

exposure to the various wines at various concentrations, and was ranked in the middle

of the species for log CFU reduction. *S.* Typhimurium was ranked fourth for log reduction

in CFU. *S. aureus* demonstrated the greatest log reductions in CFU of the pathogens

tested, and was significantly different than all other species.

Table 4-4   Comparison of significant differences among all species with 4 types of
fruit wine, and concentrations between 0-60%, using Tukey's HSD ($P \leq$
0.05), with identical letters showing no significant differences.

Species		Wines		Concentrations	
Listeria monocytogenes	a	peach #1	a	0	a
Escherichia coli O157:H7	b	red raspberry	b	30	b
Shigella dysenteriae	c	blackberry	c	40	c
Salmonella Typhimurium	c	cherry	c	50	d
Staphylococcus aureus	d			60	e

Comparing the wines through the changes observed in bacterial numbers after

the pathogens were exposed to them in various concentrations, peach wine #1 showed

a significant difference in inhibition from all of the other wines tested (Table 4-4) in the

study. Peach wine #1 had reduced the log CFU the most for all pathogens and

concentrations tested. The red raspberry wine was also significantly different from all other wines at reducing log CFU of the pathogens for all wines. The red raspberry wine had reduced log CFU on average second-most of the wines tested, following behind peach wine #1. The blackberry wine was the third most effective wine at reducing log CFU, and was significantly different from peach wine #1 and red raspberry wine, but was not significantly different the cherry wine. The cherry wine was also significantly different than the peach and raspberry wine for reducing log CFU, and trailed behind the blackberry wine, though the difference was not significant.

The various concentrations of wine were all found to be significantly different than each other for the log reduction of pathogenic bacteria ($P \leq 0.05$).

An ANOVA for all of the wines, including peach wine #2, compared data for *E. coli* O157:H7 and *L. monocytogenes* at concentrations between 0-60%, as shown in Table 4-5. Wine concentration was again the most significant factor in log reduction of the bacteria, followed by bacterial species and type of wine used ($P \leq 0.01$). All interactions that included concentration as a factor were significant ($P \leq 0.01$); and included interactions between species and wine concentration, type of wine and concentration, and the combination of species, wine, and concentration. The interaction between the species and type of wine was not found to be significant.

Table 4-5   Analysis of variance table examining the factors and interactions between the factors; using *E. coli* O157:H7 and *L. monocytogenes* for species, all types of fruit wine, concentrations between 0-60%, and using CFU as response after 24 h exposure.

Source	Df	Sum Sq	Mean Sq	F value	Pr(> F)
Species	1	152.45	152.446	307.6924	$< 2.2 \times 10^{-16}$
Wine	4	62.05	15.512	31.3091	6.011×10^{-15}
Concentration	4	937.98	234.494	473.2952	$< 2.2 \times 10^{-16}$
Species*Wine	4	0.95	0.237	0.4793	0.7508038
Species*Conc.	4	76.93	19.232	38.8164	$< 2.2 \times 10^{-16}$
Wine*Conc.	16	58.42	3.651	7.3699	1.137×10^{-9}
Species*Wine*Conc.	16	24.18	1.511	3.0498	0.0006598
Residuals	70	34.68	0.495		

A revised Tukey's HSD analysis ($P \leq 0.05$), using the model that included *E. coli* O157:H7 and *L. monocytogenes* for species, and all of the fruit wines, shown in Table 4-6, indicated that the trend for a 24-h wine exposure at the various concentrations for *L. monocytogenes* was significantly different than for *E. coli* O157:H7 based on log reduction of CFU. Peach wine #1 was significantly different than the other wines when comparing *E. coli* O157:H7 and *L. monocytogenes*. Red raspberry wine and peach wine #2 were not found to be different ($P = 0.89$), though they were different from peach wine #1, blackberry wine, and cherry wine. Blackberry wine and cherry wine were not found to be different from each other, as was also seen with the previous model using all species of bacteria, but the difference found was negligible ($P > 0.99$). The concentrations of fruit wine were all again significantly different from each other for log CFU reduction.

66

Table 4-6 Comparison of significant differences among *E. coli* O157:H7 and *L. monocytogenes* for all fruit wines, and concentrations between 0-60%, using Tukey's HSD ($P ≤ 0.05$), with identical letters showing no significant differences.

Species		Wines		Concentrations	
Listeria monocytogenes	a	peach #1	a	0	a
Escherichia coli O157:H7	b	red raspberry	b	30	b
		peach #2	b	40	c
		cherry	c	50	d
		blackberry	c	60	e

4.2.2 Exposure to dealcoholized fruit wines for 24 h

The primary study examining the dealcoholized fruit wines found that the extract from the wines with ethanol removed demonstrated a significant log reduction in CFU/ml for all of the bacteria studied at a 60% concentration ($P ≤ 0.05$).

Of the dealcoholized fruit wines tested, the dealcoholized red raspberry wine, shown in Figure 4-6, demonstrated the second greatest reduction in log CFU for the pathogenic bacteria exposed, with an average log reduction of 7.04 CFU/ml at the 60% concentration. At this concentration, *E. coli* O157:H7 and *L. monocytogenes* demonstrated the greatest resistance to the dealcoholized wine, with log reductions of 4.2 and 4.9, respectively. *S.* Typhimurium, *S. dysenteriae*, and *S. aureus* exhibited a greater log reduction in CFU after exposure to the dealcoholized red raspberry wine, with log reductions of 9.3, 8.8, and 8.5 at 60% concentration. Both *S.* Typhimurium and *S. aureus* were completely inhibited at this concentration.
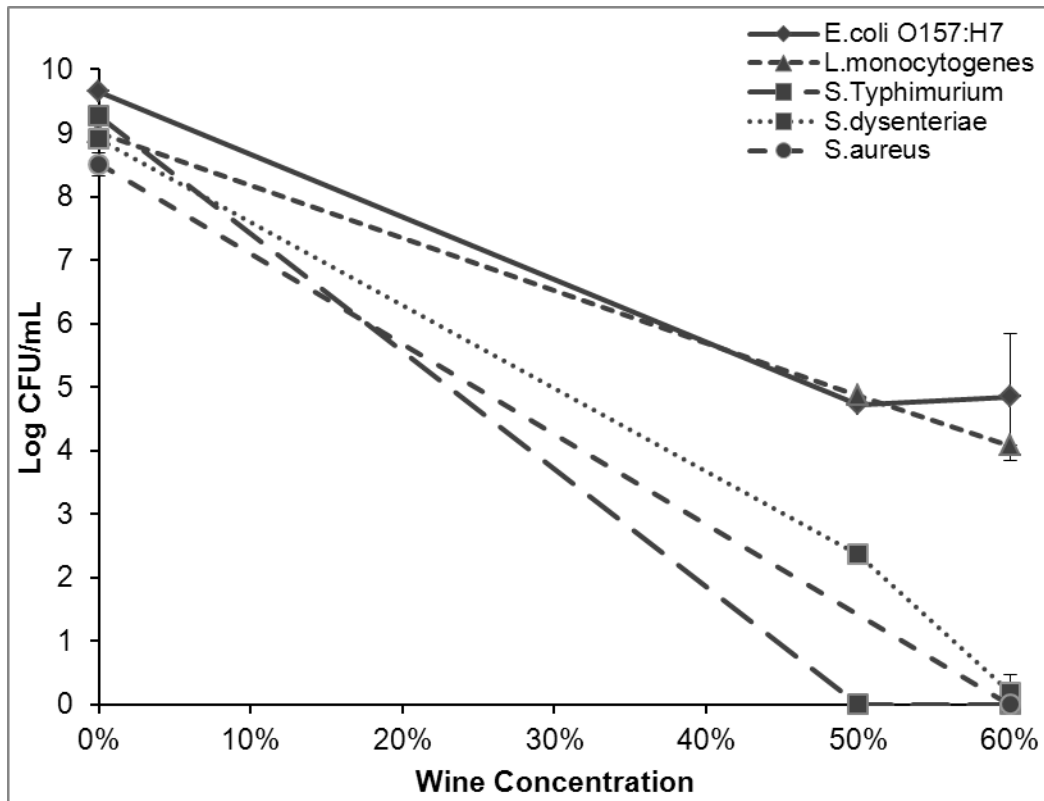
Figure 4-6   Number of foodborne pathogens exposed to dealcoholized red raspberry
wine for 24 h.

The dealcoholized blackberry wine, shown in Figure 4-7, exhibited the least

reduction in log CFU for the pathogens of all the wines tested. At 60% concentration,

the average log reduction was 4.31 CFU. *E. coli* O157:H7 showed the greatest

resistance to the dealcoholized blackberry wine, with an average log reduction of 3.0

CFU. *S.dysenteriae*, *L. monocytogenes*, and *S.* Typhimurium exhibited slightly reduction

after exposure to the dealcoholized blackberry wine, with an average log reduction at

60% concentration of 3.1, 3.3, and 3.9 CFU/ml, respectively. *S. aureus* exhibited the

greatest sensitivity to the dealcoholized blackberry wine at the 60% concentration, with

an average log reduction of 8.2 CFU. None of the pathogens tested were completely

inhibited by the dealcoholized blackberry wine at the 60% concentration.
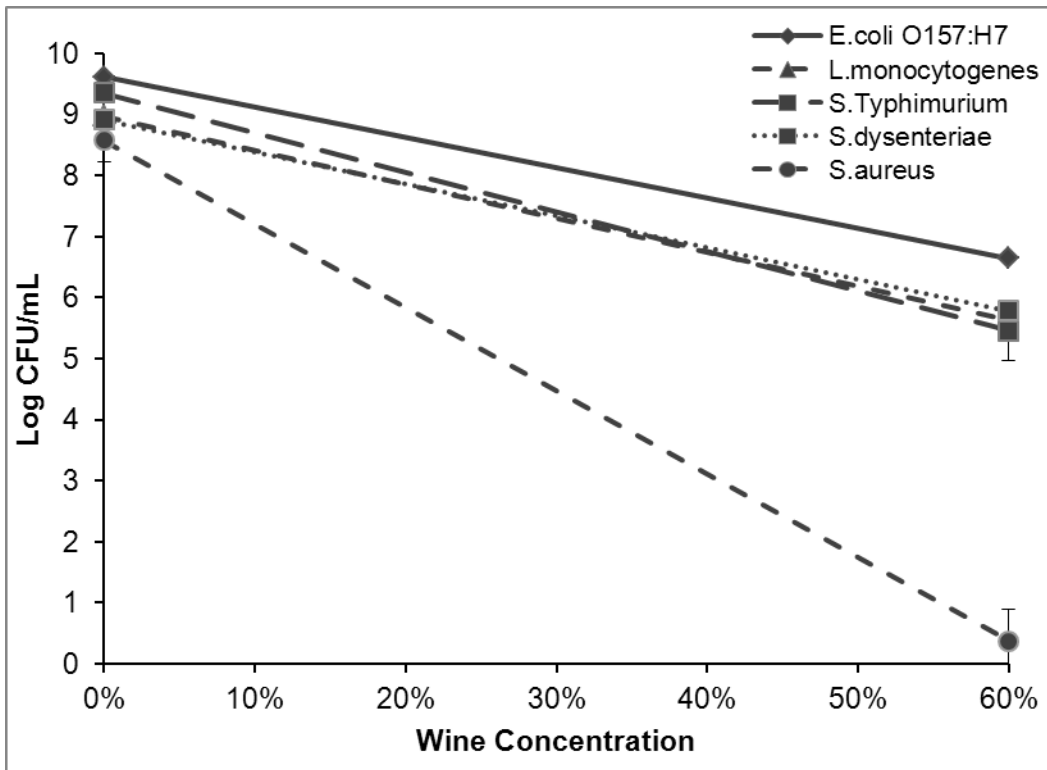
Figure 4-7   Number of foodborne pathogens exposed to dealcoholized blackberry
wine for 24 h.

The dealcoholized peach wine #1, shown in Figure 4-8, demonstrated the

greatest reduction in log CFU for the pathogens exposed, with an average log reduction

of 7.84 at 60% concentration. *L. monocytogenes* showed the greatest resistance to

dealcoholized peach wine #1, with an average log reduction of 4.1 CFU. *E. coli*

O157:H7 exhibited an average log reduction of 8.0 at this concentration, while *S.*

Typhimurium, *S. dysenteriae*, and *S. aureus* were completely inhibited by the

dealcoholized peach wine #1, with average log reductions of 9.3, 8.9, and 8.8 CFU, respectively.
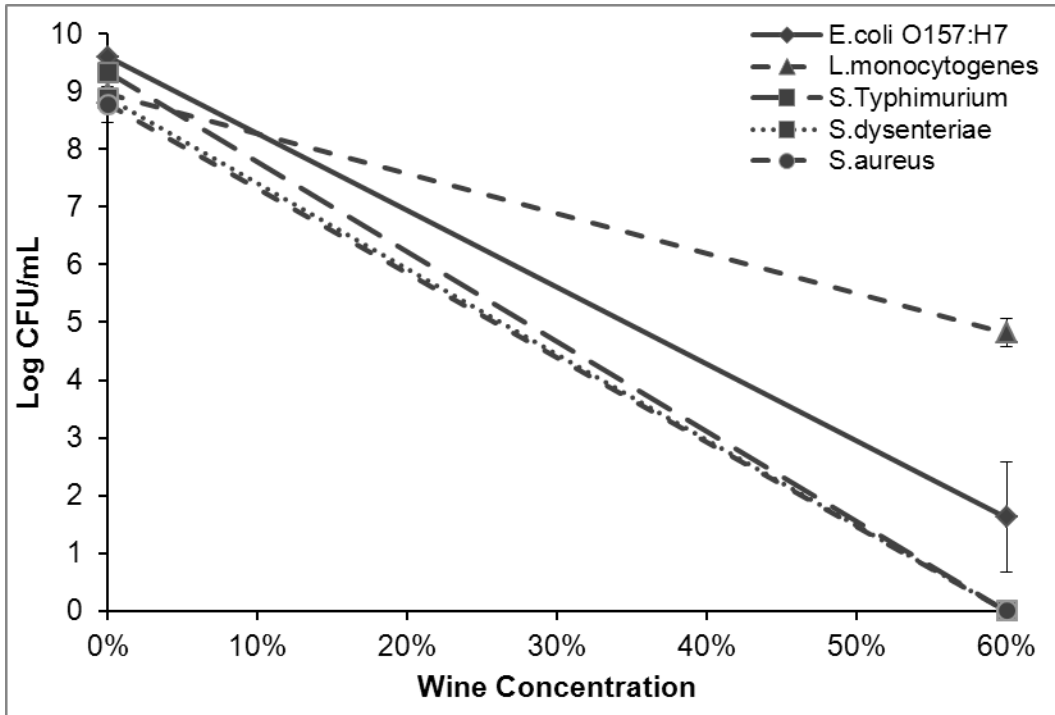
Figure 4-8   Number of foodborne pathogens exposed to dealcoholized peach wine #1 for 24 h.

The dealcoholized cherry wine, shown in Figure 4-9, demonstrated the third greatest reduction in log CFU in the pathogens exposed at the 60% concentration, with an average log reduction of 4.69 CFU. *E. coli* O157:H7 exhibited the greatest resistance to the dealcoholized cherry wine, with an average log reduction of 3.2 CFU. *L. monocytogenes*, *S. dysenteriae*, and *S.* Typhimurium had demonstrated average log reductions of 3.3, 3.4, and 5.9 CFU, respectively. *S. aureus* showed the greatest log reduction in CFU to the dealcoholized cherry wine at 60% concentration, with an

average log reduction of 7.1 CFU. None of the pathogens tested were completely

inhibited by the dealcoholized cherry wine at the 60% concentration.
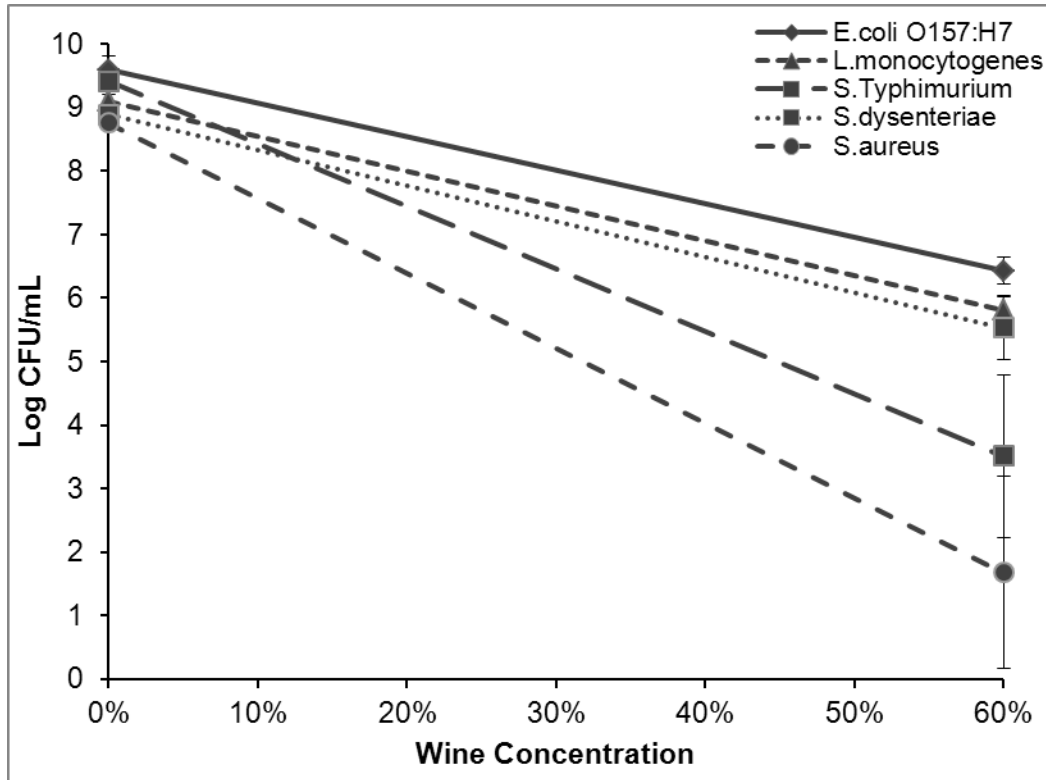
Figure 4-9   Number of foodborne pathogens exposed to dealcoholized cherry wine
for 24 h.

ANOVA of the dealcoholized fruit wines, shown in Table 4-7, compared the

effects of species, dealcoholized fruit wine, concentration, and interactions of these

factors on numbers of the pathogens exposed for 24 h. The concentration of the wine

was the most significant factor in log reduction of the bacteria, with 0% and 60%

concentration being used for the ANOVA. Data for 50% concentration was not used

because that concentration was only used in the first dealcoholized red raspberry wine

trial, and not with any of the other dealcoholized fruit wines. Species of the bacteria

71

exposed, followed by the type of wine used, were both significant factors in the reduction

of CFU of the pathogens ($P \leq 0.01$). Interactions among the factors of species and wine,

species and concentration, wine and concentration, and the combination of species,

wine, and concentration were also significant at $P \leq 0.01$.

Table 4-7   Analysis of variance table examining the factors of bacterial species, type
of dealcoholized fruit wine, concentration of dealcoholized fruit wine, and
interactions between the factors using CFU as response after 24 h
exposure**.**

Source	Df	Sum Sq	Mean Sq	F value	Pr(> F)
Species	4	71.28	17.82	76.2378	$< 2.2 \times 10^{-16}$
Wine	3	52.65	17.55	75.0813	$< 2.2 \times 10^{-16}$
Concentration	1	767.75	767.75	3284.5797	$< 2.2 \times 10^{-16}$
Species*Wine	12	24.40	2.03	8.6985	2.561×10^{-8}
Species*Conc.	4	43.68	10.92	46.7204	1.226×10^{-15}
Wine*Conc.	3	51.26	17.09	73.0992	$< 2.2 \times 10^{-16}$
Species*Wine*Conc.	12	23.65	1.97	8.4327	4.009×10^{-8}
Residuals	46	10.75	0.23		

Analysis using Tukey's HSD ($P < 0.05$) with the same linear regression model

used for ANOVA, shown in Table 4-8, showed a significant difference in the trend for *E.*

coli O157:H7 and *L. monocytogenes* and the trend for *S.* Typhimurium, *S. dysenteriae*,

and *S. aureus. S.* Typhimurium and *S. dysenteriae* were also found to have significantly

different log reductions than *S. aureus*, which was different than all other pathogens

tested.

Table 4-8   Comparison of significant differences among species, type of dealcoholized wine, and concentrations using Tukey's HSD ($P \leq 0.05$) with identical letters indicating no significant differences.

Species		Dealcoholized Wine Type		Concentrations	
Escherichia coli O157:H7	a	peach #1	a	0	a
Listeria monocytogenes	a	red raspberry	a	60	b
Salmonella Typhimurium	b	blackberry	b		
Shigella dysenteriae	b	cherry	b		
Staphylococcus aureus	c				

Among the dealcoholized fruit wines tested, both the dealcoholized red raspberry wine and peach wine #1 were significantly different than the dealcoholized blackberry and cherry wine, based on the resulting log CFU after exposure. The pathogens exposed to dealcoholized peach wine #1 had the greatest reduction in population, with exposure to the dealcoholized red raspberry wine being the second greatest reduction in population, though the difference was not significant. A significant difference was not found between the dealcoholized blackberry and cherry wine, both having a lesser effect on reducing log CFU after exposure than dealcoholized peach wine #1 and red raspberry wine.

The 0% concentration of wine was found to be significantly different than the 60% concentration of dealcoholized wine, as determined by the change in log CFU after exposure.

73

4.3 Chemical analysis

4.3.1 pH measurement, titratable acidity, and alcohol content by volume, and free sulfur dioxide content of fruit wines

As shown in Table 4-9, the pH values of the fruit wines tested ranged from 3.0 to 3.4, and would be considered acidic, like grape wines with similar pH values. Grape wines tend to have pH values ranging from 2.8 to 3.8, with the lower pH values more common in white wines, with red wines having slightly higher pH values (Rajković and Sredović 2009). The amount of total acids of the fruit wines, as measured by titratable acidity (TA) in relation to tartaric acid equivalents, ranged from 5.4 to 10.9 g/L tartaric acid. Tartaric acid is one of the predominant acids in grape wines, and is the unit used for equivalence when estimating total acidity. The cherry wine, at 5.4 g/L tartaric acid, had a considerably lower TA than the other wines. The TA of grape wines generally ranges from 4-8 g/L tartaric acid, so the blackberry wine, peach wine #1, and red raspberry wine had TA values greater than what would typically be found in a grape wine. The alcohol content of the fruit wines was relatively similar, between 10.5-11%, except the Mogen David cherry wine, which had an ABV of 9.0%. The free sulfur dioxide content of the fruit wines tested ranged from 6.7 – 27.7 ppm. Peach wine #1 had the greatest free sulfite concentration of the wines tested. At 27.7 ppm, the free sulfur content of peach wine #1 was 1.7 times greater than that of the second highest wine tested, peach wine #2, and more than 4 times greater than that of the lowest concentration, 6.7 ppm, found in the cherry wine.

Table 4-9   pH values, titratable acidity, alcohol content by volume, and free sulfur dioxide content of fruit wines.

Wine	pH	Titratable Acidity (g/L Tartaric acid)	ABV	Free Sulfur Dioxide (ppm)
red raspberry	2.99±0.07	10.87±0.24	11.0%	11.00±1.41
blackberry	3.38±0.08	10.02±0.30	10.5%	7.30±1.25
peach #1	3.20±0.04	10.11±0.37	11.0%	27.70±1.70
cherry	3.21±0.08	5.40±0.35	9.0%	6.70±2.00
peach #2	3.40±0.04	7.19±0.02	10.5%	16.30±1.42

4.3.4 Polyphenol (Adams-Harbertson) assay

4.3.4.1 Standard curve using +-catechins

The standard curve for +-catechins (Figure 4-10) that was generated to determine tannin and phenolic values of the fruit wines had an R-square value of 0.9997. The high correlation value indicated consistency for the absorbance readings across all concentrations of the +-catechin standard tested.
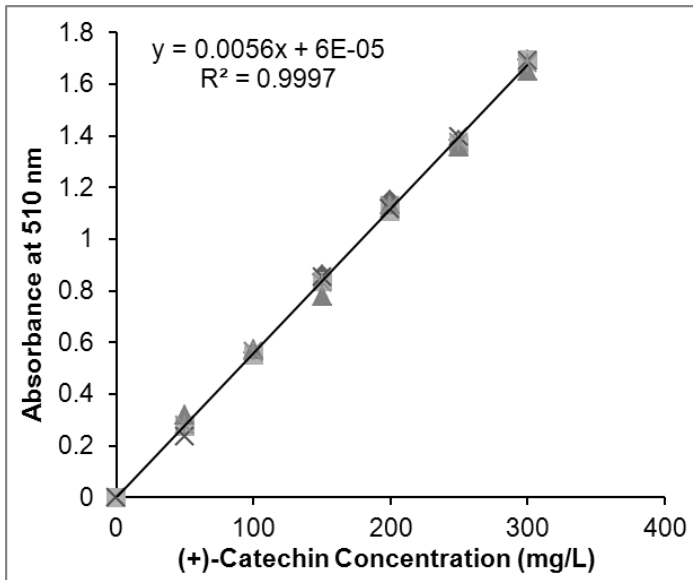
Figure 4-10   Standard curve for determination of catechin equivalents in $FeCl_3$ tannin assay.

75

4.3.4.2 Large polymeric pigment (LPP), small polymeric pigment (SPP), anthocyanin,

tannin, and phenolic concentrations of fruit wines

The results of the Adams-Harbertson assay for LPP, SPP, anthocyanin, tannin, and phenolic concentrations of the fruit wines are shown in Table 4-10. Large polymeric pigments, anthocyanin pigments bound to tannins that precipitate, had values ranging from 0.00 – 0.89 in the fruit wines tested, the highest value found in the blackberry wine with a value of 0.89. The other fruits wines tested had marginal to no LPP values (0 - 0.11).

Small polymeric pigments, anthocyanins bound to tannins that do not precipitate, had values ranging from 0.03 to 1.59. The cherry and blackberry wine had the greatest SPP content, 1.59 and 1.56, respectively. The red raspberry wine had a small amount of SPP, with a value of 0.64, whereas both peach wines tested had no or almost no detectable amount.

The anthocyanin concentrations of the fruit wines ranged from 0.00 – 163.34 mg/L malvidin-3-glucose equivalents (M3GE). Again, the blackberry and cherry wine had the greatest content measured, with values of 163.3 and 149.6 mg/L M3GE respectively. The red raspberry wine had low anthocyanin content at 27.5 mg/L M3GE, and both peach wines contained negligible amounts (or none detected).

Table 4-10 Classes of phenolic compounds in the fruit wines measured by Adams-Harbertson assay.

Wine	LPP	SPP	Anthocyanin (mg/L M3GE)	Tannin (mg/L CE)	Phenolic (mg/L CE)
red raspberry	0.11±0.20	0.64±0.07	27.52±3.35	4.54±1.72	271.11±23.66
blackberry	0.89±0.33	1.56±0.51	163.34±19.46	458.22±20.71	1614.71±97.09
peach #1	0.01±0.01	0.08±0.07	1.32±3.26	2.50±1.81	145.24±56.05
cherry	0.11±0.14	1.59±0.24	149.59±2.94	16.32±6.47	526.02±57.18
peach #2	0.00±0.00	0.03±0.00	0.00±0.00	6.14±6.40	10.09±0.29

The tannin concentrations, measured in mg/L of catechin equivalents (CE), ranged from 2.50 – 458.22 mg/L CE. The blackberry wine had the greatest amount of tannin at 458.22 mg/L CE, containing almost 30 times the concentration of tannin as the cherry wine, which had the second greatest amount. All fruit wines other than the blackberry wine had very small amounts of tannin, measuring less than the 50 mg/L +- catechin standard that was used for the lowest point on the standard curve.

The phenolic content of the fruit wines ranged from 10.1 – 1614.7 mg/L CE. The blackberry wine had the highest phenolic content measured of the wines, at 1614.7 mg/L CE. This was three times greater than the second highest phenolic content found in the cherry wine, at 526.0 mg/L CE. The red raspberry wine had the third greatest phenolic content at 271.1 mg/L CE, followed by peach wine #1 with 145.2 mg/L CE. Peach wine #2 had a negligible phenolic content at 10.1 mg/L CE.

4.4 Antibacterial activity of 40% concentration fruit wine and 60% dealcoholized fruit wine related to chemical analysis

4.4.1 Correlation of pH to antibacterial activity

The pH values of the fruit wines were moderately correlated with their antibacterial activity, both in the fruit wine at 40% concentration (R^2 = 0.374), and in the 60% concentration of the dealcoholized wine (R^2 = 0.4161), as seen in Figures 4-11 and 4-12, respectively. The lower pH values were associated with a greater log CFU reduction, or greater antibacterial activity.
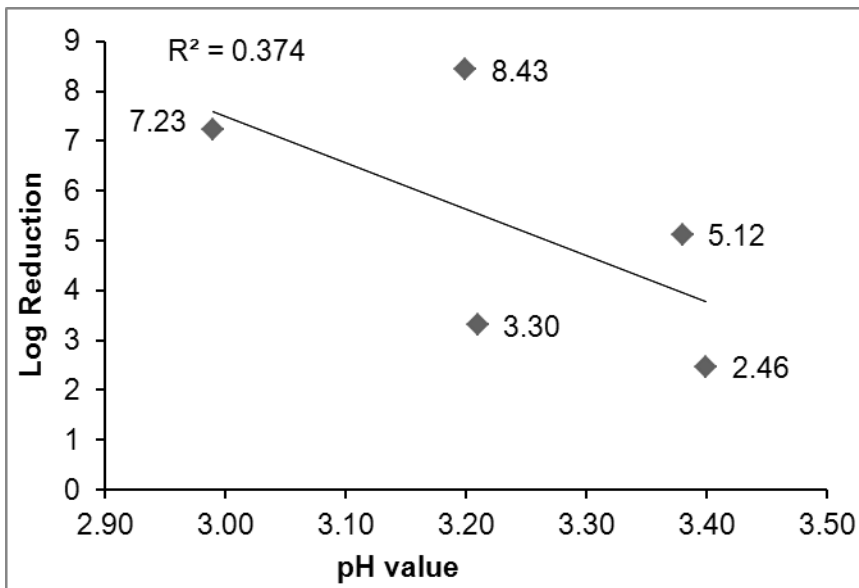
Figure 4-11   pH and log reduction of pathogens in 40% fruit wine concentration.
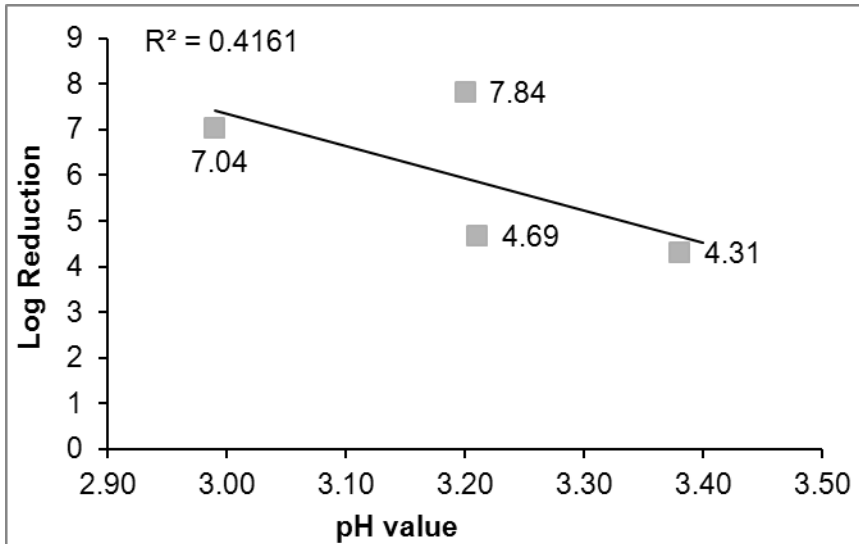
Figure 4-12   pH and log reduction of pathogens in 60% dealcoholized fruit wine concentration.

4.4.2 Correlation of titratable acidity to antibacterial activity

There was a strong correlation between TA and the antibacterial properties of the fruit wine at a 40% concentration (Figure 4-13), R^2 = 0.6695. A much weaker relationship (R^2 = 0.2894) is seen between TA and the cell log reduction in the 60% concentration dealcoholized fruit wine (Figure 4-14). With the ethanol removed, the total acid content of the fruit wine was less closely related to the log reduction of pathogens. The TA measurements for total acid content, while given in tartaric acid equivalents, do not reflect any specific type of acid contained by the fruit. Using 4 different fruit types, the presence of greater concentrations of specific acid(s) could potentially have played a role in the antibacterial properties of fruit wine, without being reflected in the TA measurement.
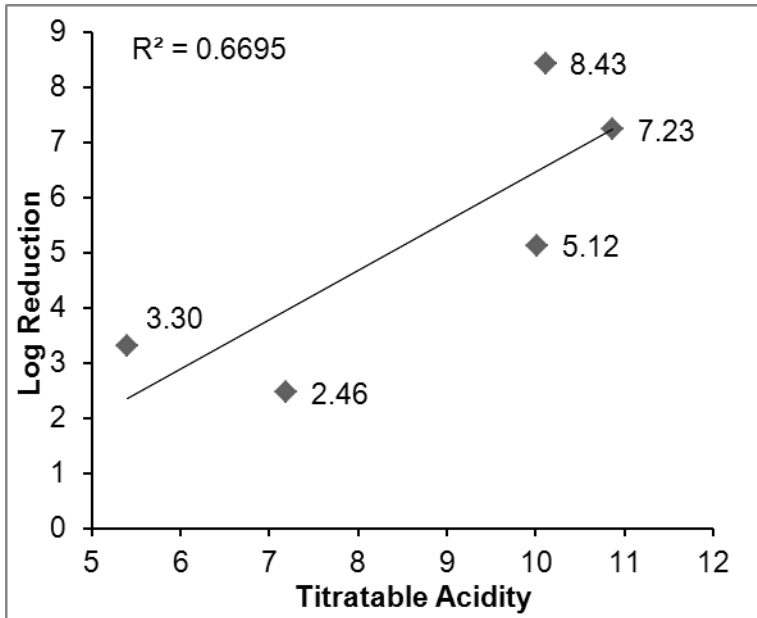
Figure 4-13   Titratable acidity and log reduction of pathogens in 40% fruit wine concentration.
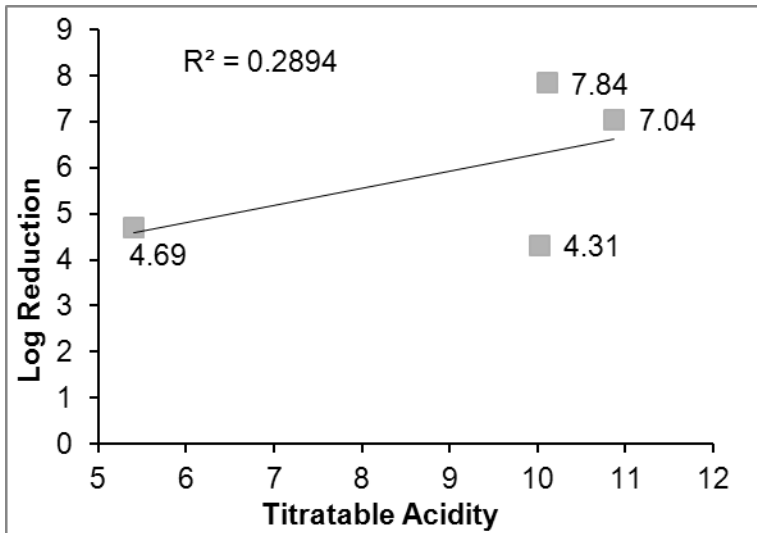
Figure 4-14   Titratable acidity and log reduction of pathogens in 60% dealcoholized fruit wine concentration.

80

4.4.3 Correlation of alcohol content to antibacterial activity at 40% wine concentration

The alcohol content of the fruit wines had a moderate correlation to its antibacterial activity, seen in Figure 4-15, with higher concentrations being related to greater antibacterial activity (R^2 = 0.4427). Because four of the five fruit wines tested were within 0.5% ABV range, there was not a wide range of concentrations to provide a clear linear relationship.
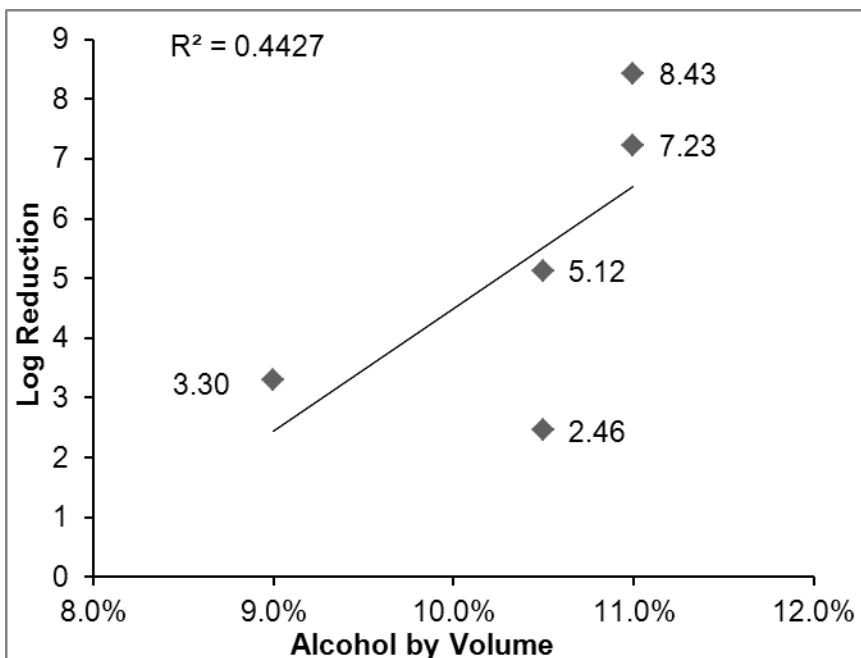
Figure 4-15   Alcohol by volume and log reduction of pathogens at 40% wine
concentration.

4.4.4 Correlation of free sulfite concentration to antibacterial activity

The addition of sulfites to wine, used to control spoilage from oxidation and microorganisms, inhibits bacterial growth when sulfur dioxide is in the free, unbound form. There was a moderate correlation (R^2 = 0.2798) between free sulfur content and

log reduction of pathogens at 40% fruit wine concentration, shown in Figure 4-16. The

correlation of free sulfur content to log reduction was stronger with 60% dealcoholized

fruit wine, R^2 = 0.6964 (Figure 4-17). Part of the difference might be attributed to peach

wine #2 contributing a dissimilar R-square value in the 40% fruit wine study, and not

contributing to the 60% dealcoholized fruit wine value in the dealcoholized wine study,

as it was not used.
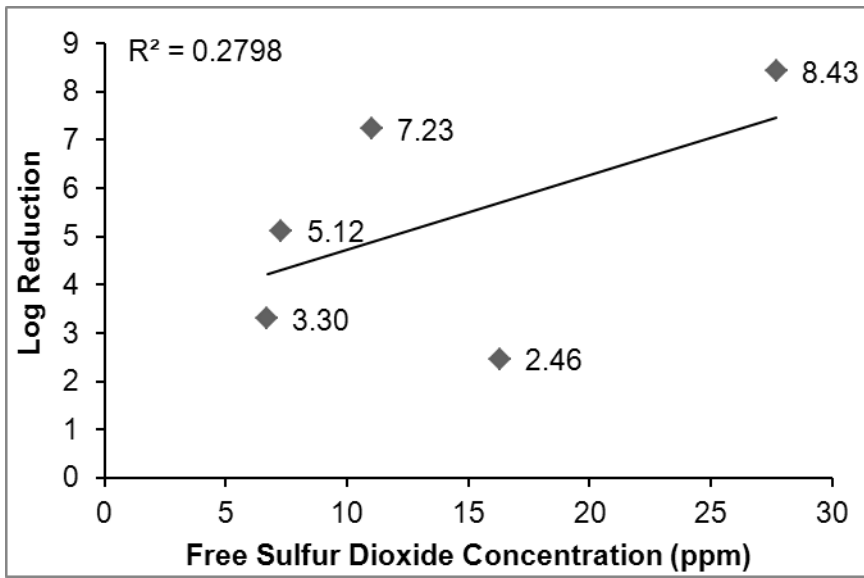
Figure 4-16   Free sulfur dioxide concentration and log reduction of pathogens at 40%
fruit wine concentration.
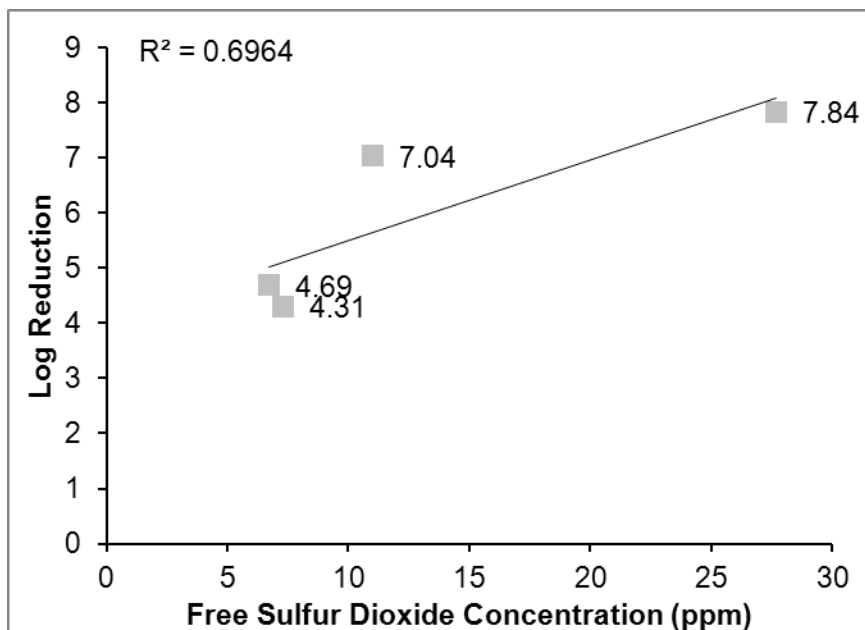
Figure 4-17 Free sulfur dioxide concentration and log reduction of pathogens at 60% dealcoholized fruit wine concentration.

4.4.5 Correlation of anthocyanin, tannin, and phenolic concentration to antibacterial activity

In addition to pH, TA, alcohol content, and free sulfur content, the study correlated phenolic compounds, tannins and anthocyanin had to the antibacterial content of the wines, shown in Figures 4-18 to 4-23. There was very little, or moderately weak negative correlation found between log reduction and anthocyanin content in the 40% concentration fruit wine (R^2 = 0.1174). In contrast to this, there was a very strong negative correlation (R^2 = 0.992) between these two factors in the 60% concentration dealcoholized fruit wine, indicating a relationship between greater anthocyanin content and lower log CFU reduction. The dissimilar correlation values between the fruit wine and dealcoholized fruit were also found in comparisons for tannin and phenolic concentrations and log reduction. Tannin concentration and log reduction in 40%

concentration fruit wine had an R-square value of 0.0034, the 60% concentration

dealcoholized wine had an R-square value of 0.4331. For phenolic concentrations and

log reduction, the 40% concentration fruit wine had an R-square value of 0.004; the 60%

dealcoholized wine had a 0.642 correlation value.

Similar to the free sulfite analysis, the difference between the strength in the

relationship seen in the fruit wine and the dealcoholized fruit wine might in part have

occurred due to the inclusion of peach wine #2 in the fruit wine data, but not in the

dealcoholized wine study. Peach wine #2 did not have measurable anthocyanin, tannin,

or phenolic content, as seen in Table 4-8, and its inclusion with a relatively low log

reduction value in the fruit wine may negate the negative relationships found between

the presence of these compounds and log reduction.
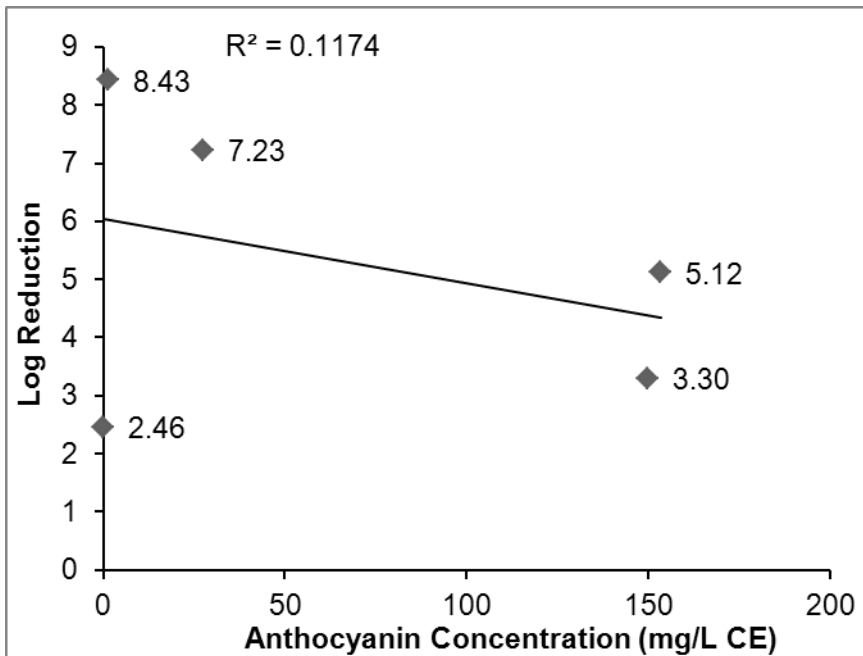
Figure 4-18   Anthocyanin concentration and log reduction of pathogens at 40% fruit
wine concentration.
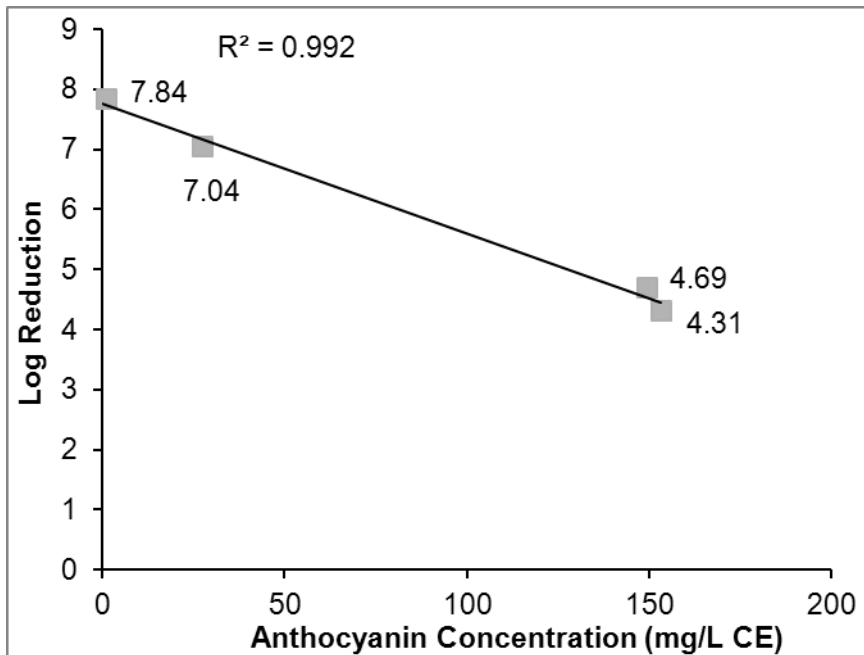
Figure 4-19   Anthocyanin concentration and log reduction of pathogens at 60% dealcoholized fruit wine concentration.
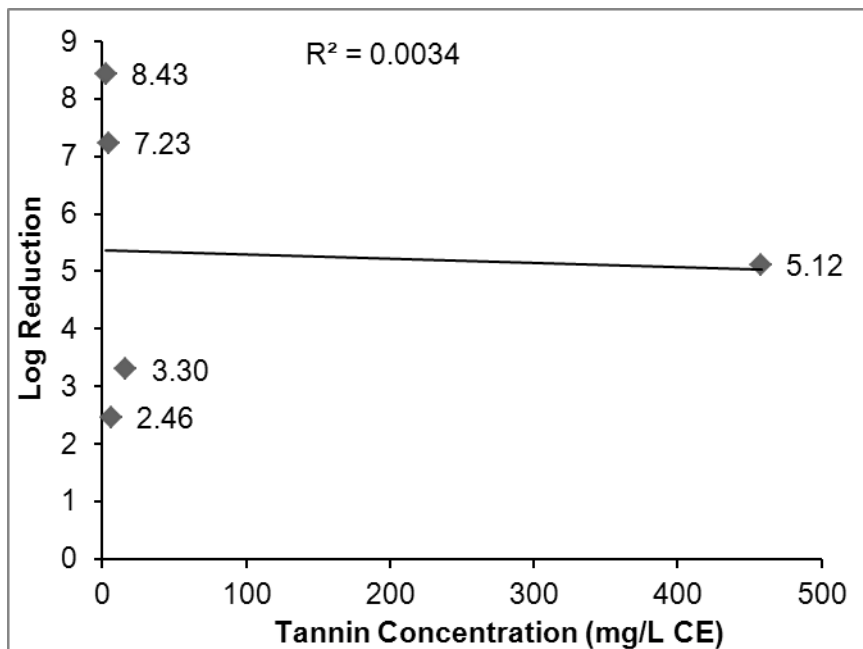
Figure 4-20   Tannin concentration and log reduction of pathogens at 40% fruit wine concentration.

85
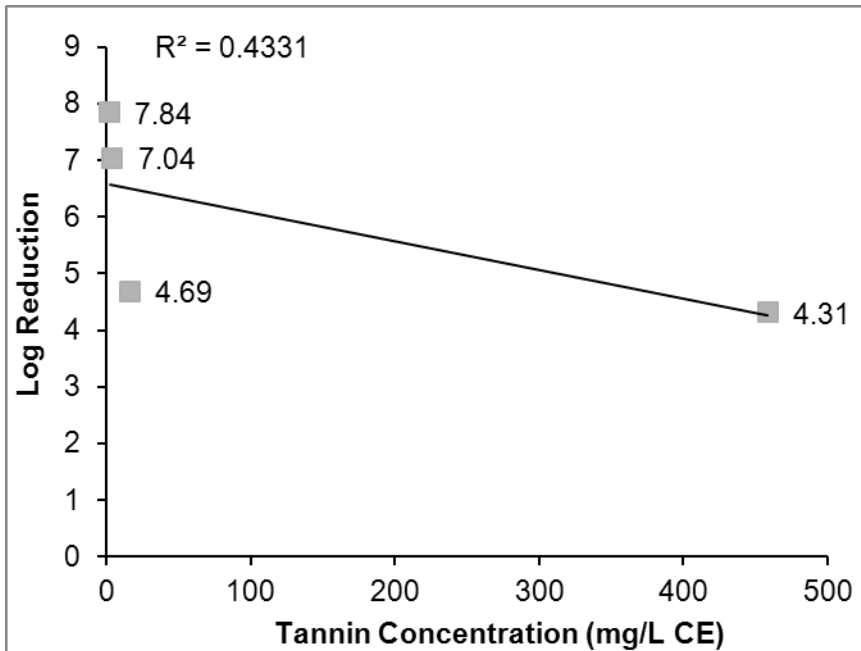
Figure 4-21   Tannin concentration and log reduction of pathogens at 60% dealcoholized fruit wine concentration.
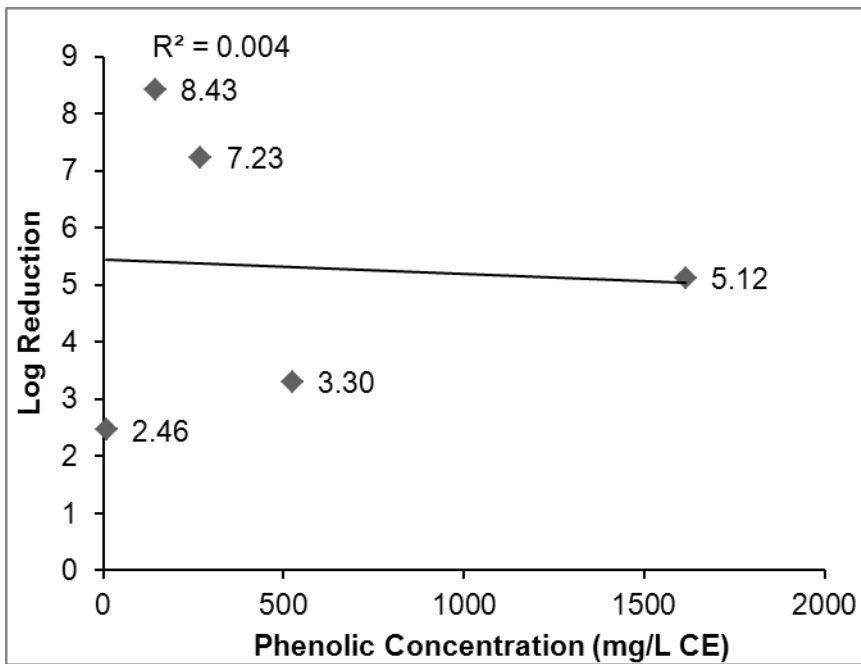
Figure 4-22   Phenolics concentration and log reduction of pathogens at 40% fruit wine concentration.
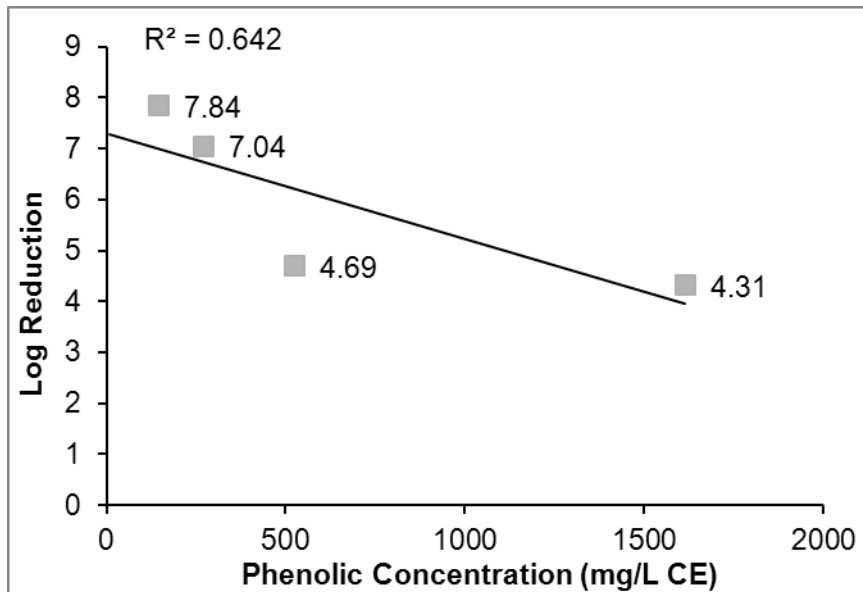
Figure 4-23   Phenolics concentration and log reduction of pathogens at 60% dealcoholized fruit wine concentration

4.5 Scanning electron microscopy and transmission electron microscopy

4.5.1 Scanning electron microscopy (SEM)

SEM pictures of *E. coli* O157:H7 treated with TSBY for 24 h (Figure 4-24) showed the cells to be naturally rod-shaped, with variations in size consistent with regular growth of the cell. Both medium and long length rods were present, and flagella remained intact. *E. coli* O157:H7 treated with 60% peach wine #1 (Figure 4-25) were much shorter, with creases and indentations present on the outer membrane. Flagella were still present on some cells, but were not as common. The length of flagella was in general much shorter. Cell division appeared to have occurred in cells that were not fully grown. *E. coli* O157:H7 treated with 60% cherry wine for 24 h (Figure 4-26) also appear shorter than the control group grown in TSBY. Cell aggregation occurred, and the outer membrane of cells appeared to have adhered to material in the cherry wine that precipitated out during centrifugation. Indentations and rough deformations appear

87

in the outer membrane, with some collapse of cell ends observed. The flagella were also shorter than is seen in the control.

S. Typhimurium grown in TSBY for 24 h (Figure 4-27) were medium to very long slender shaped rods, with smooth cell surfaces. Flagella and fimbriae were present, the latter appearing with loose aggregation of cells. SEM observation of S. Typhimurium treated with 60% peach wine #1 for 24 h (Figure 4-28) showed much less variety in length of cells, which were all medium length with no long cells present. The flagella and fimbriae organelles were also absent, and aggregation of cells was very close. The outer membranes of the cells were very rough and deformed, with some holes visible, and intracellular contents appearing to have leaked out. S. Typhimurium treated with 60% cherry wine for 24 h (Figure 4-29) were also much shorter than the control grown in TSBY. Flagella were present on most cells, but fimbriae were not visible, and aggregation of cells was not visible. Material from the cherry wine adhered to the cell's outer membrane, and it appeared that cells were torn open, or deformation at the ends of the cells occurred during cell division. The outer membrane was rough, and loss of intracellular content was apparent in some cells.

Figure 4-25 SEM picture of *E. coli* O157:H7 treated with 60% peach wine #1 for 24 h, 3 kV accelerating voltage and magnification taken at 5,000x (a), 15,000x (b), and 35,000x (c).
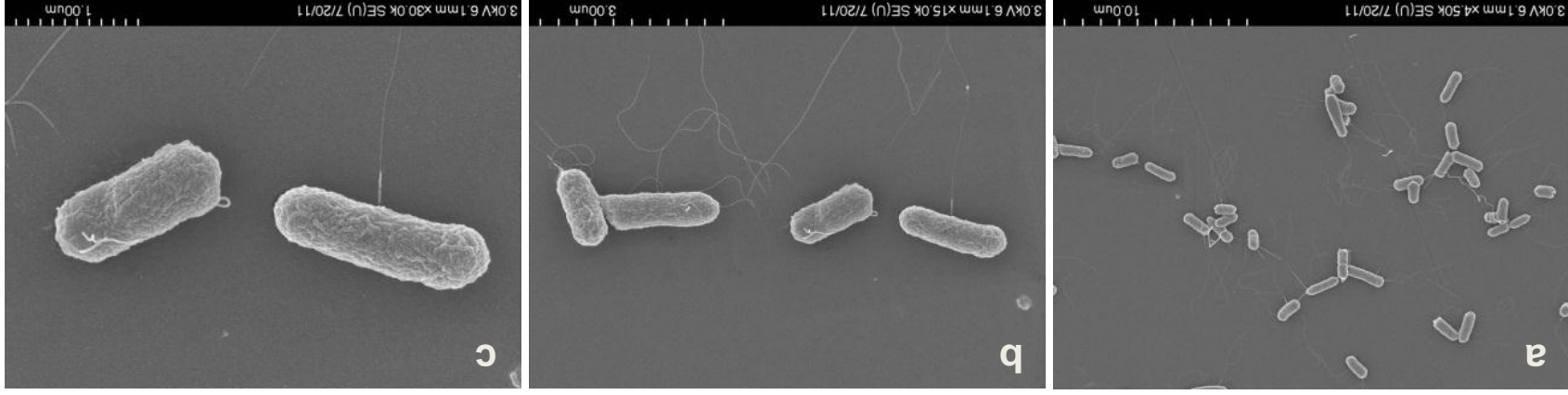
Figure 4-24 SEM picture of *E. coli* O157:H7 treated with tryptic soy broth for 24 h, 3 kV accelerating voltage and magnification taken at 4,500x (a), 15,000x (b), and 30,000x (c).

Figure 4-27 SEM picture of *S*. Typhimurium treated with tryptic soy broth for 24 hours, 3 kV accelerating voltage and magnification taken at 5,000x (a), 10,000x (b), and 40,000x (c).
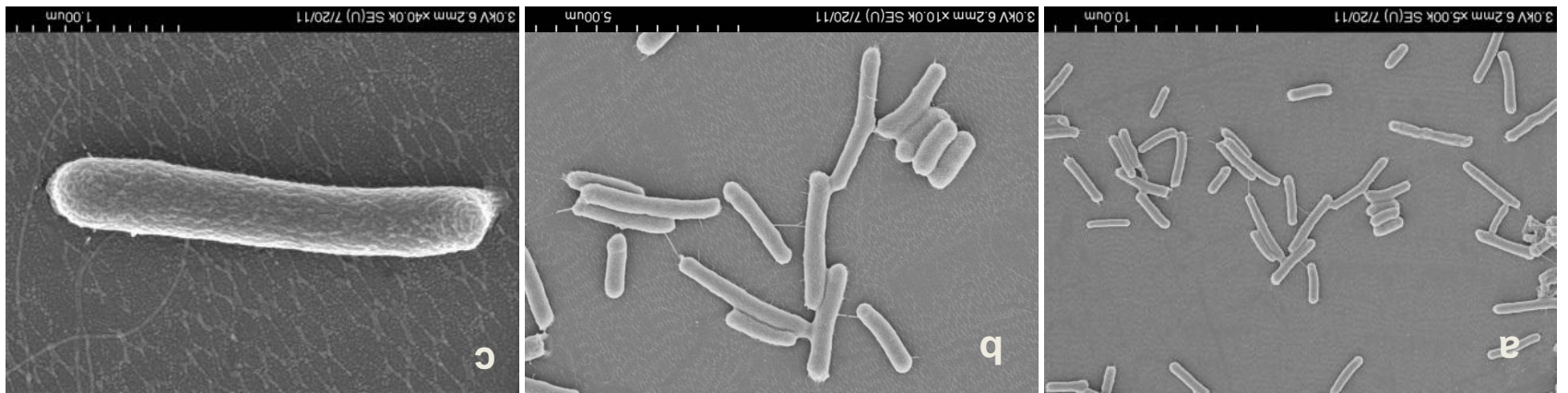
Figure 4-26 SEM picture of *E. coli* O157:H7 treated with 60% cherry wine for 24 h, 3 kV accelerating voltage and magnification taken at 1,800x (a), 9,000x (b), and 30,000x (c).

90
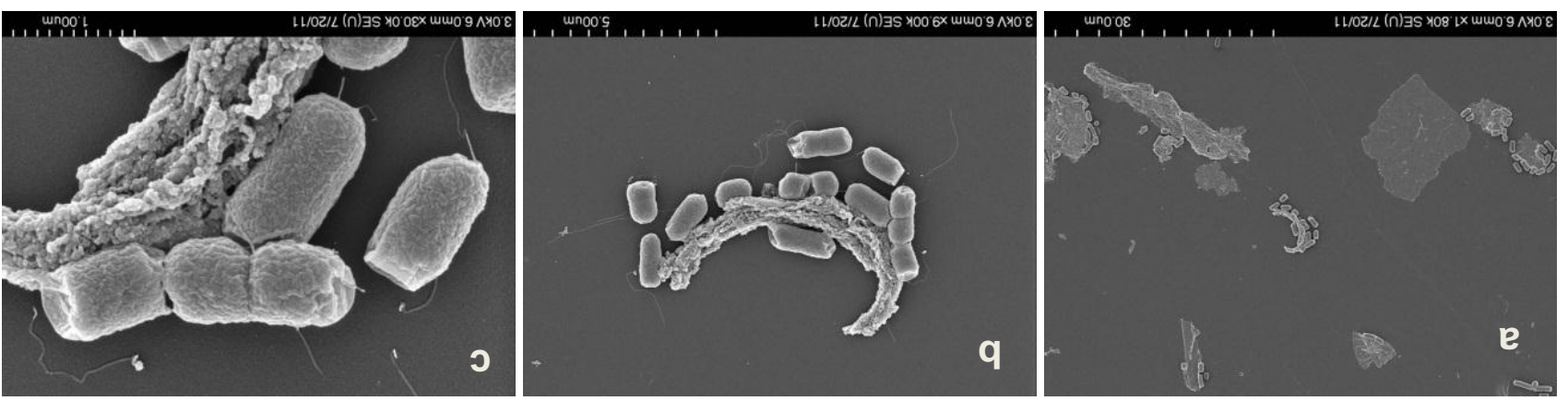
Figure 4-29 SEM picture of *S.* Typhimurium treated with 60% cherry wine for 24 h, 3 kV accelerating voltage and magnification taken at 3,000x (a), 9,000x (b), and 35,000x (c).
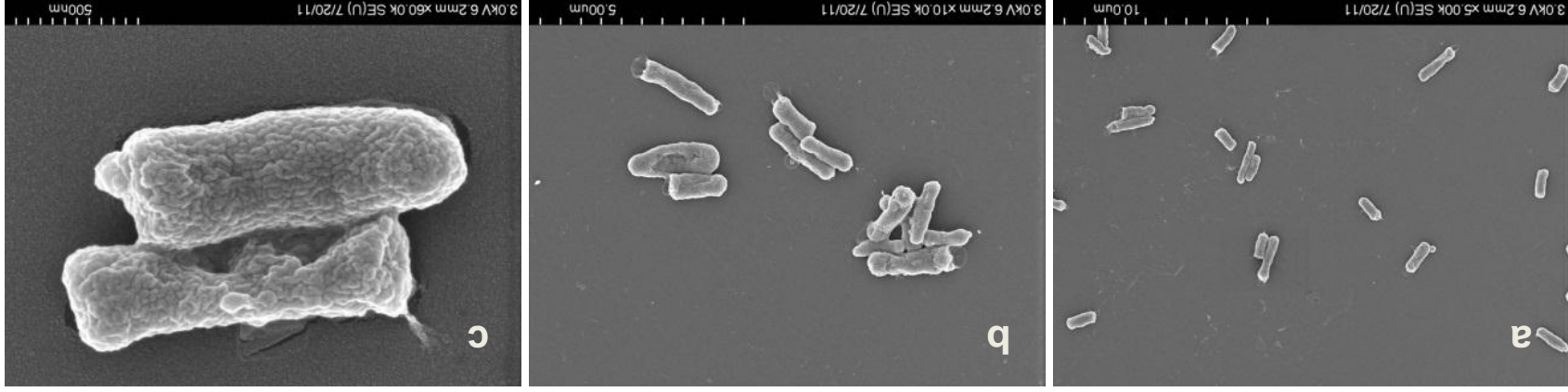
Figure 4-28 SEM picture of *S.* Typhimurium treated with 60% peach wine #1 for 24 h, 3 kV accelerating voltage and magnification taken at 5,000x (a), 10,000x (b), and 60,000x (c).

4.5.2 Transmission electron microscopy (TEM)

TEM images of *E. coli* O157:H7 treated with TSBY, 60% concentration peach wine #1, and 60% concentration cherry wine for 24 h, shown in Figure 4-30, revealed differences in shape and intracellular content leakage of cells treated with the wine. The outer membrane of *E. coli* O157:H7 cells grown in TSBY were intact, and cytoplasmic material was dispersed throughout the cell. The outer membrane of cells treated with 60% peach wine appeared to have been disrupted, with resulting loss of intracellular material. Treatment with 60% cherry wine did not appear to disrupt the cell membrane, but loss of intracellular material occurred, resulting in deformation and collapse of the cell. Remaining cytoplasmic material had separated from the cell wall.

S. Typhimurium treated with TSBY, 60% concentration peach wine #1, and 60% concentration cherry wine, shown in Figure 4-31, showed similar effects of the wine as seen with *E. coli* O157:H7. *S.* Typhimurium treated with TSBY for 24 h were regular rod shaped with evenly distributed intracellular material. The 60% concentration peach wine #1 treatment appeared to have disrupted the cell membrane and cell wall, resulting in loss of intracellular material. S. Typhimurium treated with 60% concentration cherry wine were irregular, not definitively rod shaped, and loss of intracellular material was visible. Cell walls remained mostly intact, though holes in spots of cell wall did occur. It was not discernible if material from the cherry wine that adhered to the surface caused the holes, or if some other component in the wine might have caused this to occur. Separation of the intracellular material from the cell wall was also visible in *S.* Typhimurium treated with the cherry wine.

Figure 4-30 TEM picture of *E. coli* O157:H7 treated for 24 h with TSBY (a), 60% peach wine #1 (b), and 60% cherry wine (c). Scale bar equal to 200 nm.
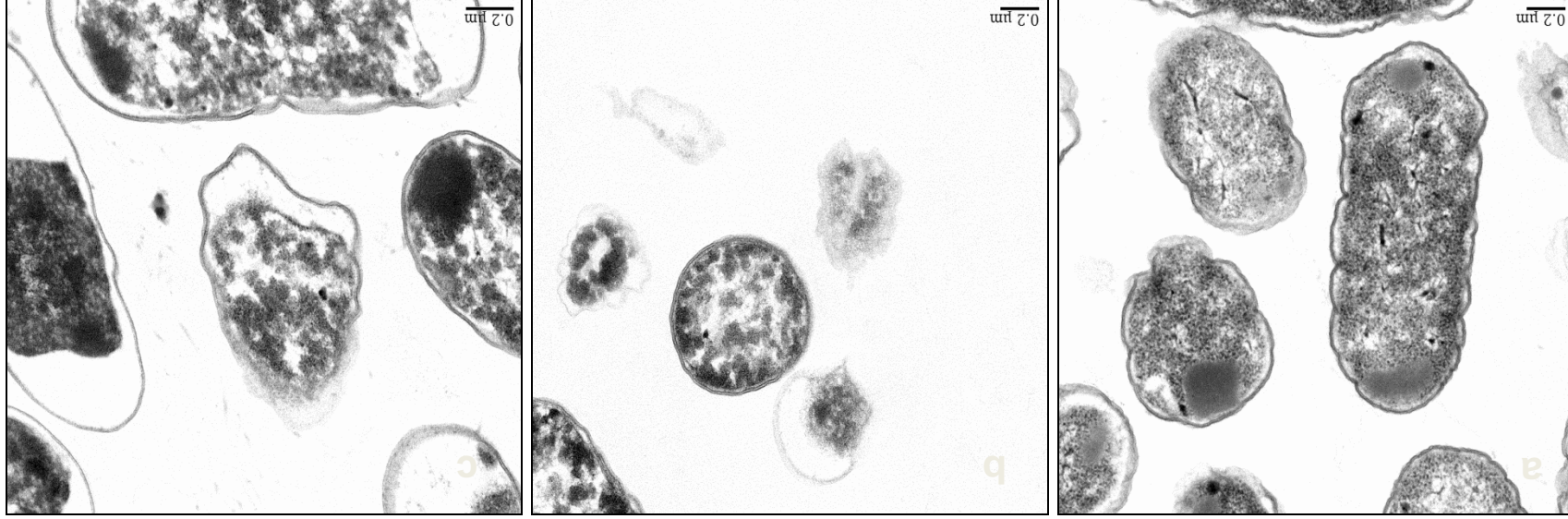
Figure 4-31 TEM picture of *S.* Typhimurium treated for 24 h with TSBY (a), 60% peach wine #1 (b), and 60% cherry wine (c). Scale bar equal to 200 nm.
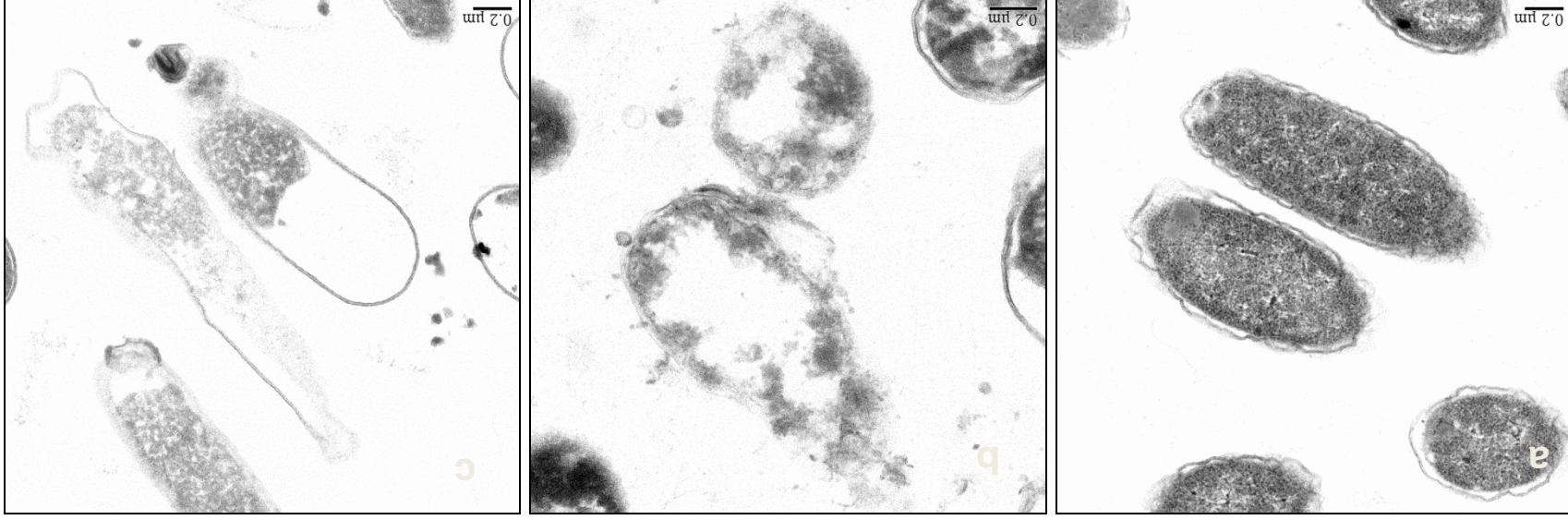
CHAPTER 5

DISCUSSION

5.1 Fruit wine and dealcoholized fruit wine tube dilution assay

The results from the preliminary and primary tube dilution assays showed significant log reduction of pathogens after 24 h exposure to the fruit wines. Research with grape wines has already provided ample evidence to the antibacterial properties of grape wine (Sheth and others 1988; Weisse and others 1995; Harding and Maidment 1996). The findings of this study showed that wines produced from red raspberry, peach, blackberry, and cherry fruit also possess antibacterial properties that can significantly reduce the number of pathogens.

The tube dilution assay using fruit wine with ethanol removed also showed significant log reduction of pathogens after treatment. This is consistent with other findings showing an antimicrobial effect on pathogens by wine phenolic extracts (Papadopoulou and others 2004; Vaquero and others 2007; Gañan and others 2009), and berry extracts (Puupponen-Pimiä and others 2005; Nohynek and others 2006), but extends to extracts found in red raspberry, peach, blackberry, and cherry wine.

The most significant factor in log reduction of CFU found in the tube dilution studies; shown in ANOVA Table 4-3, Table 4-5, and Table 4-7; was the concentration of fruit wine or dealcoholized fruit wine that the bacteria were exposed to. This is reasonable, as fruit wine was found to be antibacterial. The less diluted the antibacterial agent is, the more effective it is. The significance of concentration as a factor in log CFU reduction may also be influenced in part due to the design of the study; concentrations

95

above 60% were not used, except 70% concentrations used with *L. monocytogenes* and fruit wine, and were not used for ANOVA. Fruit wine concentrations greater than 60% would not have made a difference on the resulting CFU after exposure, with presumably all counts being very close to 0, and their inclusion in the study would reduce the overall significance of concentration as a factor. The concentration of dealcoholized fruit wines could still have been significant at concentrations greater than 60%, as many of the resulting CFU counts were not 0.

While not being more significant than concentration, finding species to be a more significant factor than wine type for log CFU reduction in the fruit wine assay was surprising. The design of the present study, to examine the reaction of various pathogens to different types of fruit wine at increasing concentrations, intended to look at the impact fruit type had on a wine's antibacterial properties. Weisse and others' (1995) study had found that wine was more effective than the equivalent amount of ethanol or pH, and raised the issue of other contributing factors, namely presence of polyphenols being a possible factor. Subsequent studies by others have investigated the importance of contributing factors to wine's bactericidal activity, but the variation among species and its importance to experimental design is not stressed. Species as a factor for log reduction in the dealcoholized wine assay was still more significant than wine type, but not drastically so.

The type of fruit used for producing the wine was found to be a significant factor to the log reduction of pathogens in both the fruit wine and dealcoholized fruit wine assays. Despite the significance of wine type as a factor, actual differences between wine types could be misleading. In Table 4-6, comparisons of significant difference

96

among wine types show peach wine #1 being significantly different from all other fruit wines, including peach wine #2. Red raspberry wine and peach wine #2 were not found to be different, nor were blackberry and cherry different from each other. The practical importance of this is that differences in the wine making process and quality of fruit used could possibly affect the antibacterial properties of the wine more than the type of fruit used.

5.2 Chemical analysis

The pH, titratable acidity, alcohol by volume content, and free sulfur dioxide content measurements of the red raspberry wine, peach wines, blackberry wine, and cherry wine all appeared to be within acceptable measurements for wine, and are standard practice.

The Adams-Harbertson assay for measuring large polymeric pigment, small polymeric pigment, anthocyanin, tannin, and phenolic content was developed for red wines (Harbertson and others 2005). Its choice for measuring the content of these compounds in fruit wines may have been flawed, as the phenolic and polyphenolic content of the two peach wines and the red raspberry wine studied were negligible, and may not have been accurately measurable with the assay. The assay's validity and reliability for tannin measurements has also been questioned. In one study, tannin measurements between wineries and among analysts had been found unacceptable, with mean measurements between wineries ranging from 71 to 178% from that of a commercial laboratory (Brooks and others 2008). The method also has not yet been approved by the AOAC.

5.3 Antibacterial activity of fruit wine and dealcoholized fruit wine related to chemical analysis

The pH values, alcohol content, and free sulfur content were found to be moderately correlated with the antibacterial activity of 40% concentration fruit wines; titratable acidity was strongly correlated with antibacterial activity. With 60% concentration dealcoholized fruit wine, antibacterial activity was moderately correlated with pH and titratable acidity, and strongly correlated with free sulfur dioxide concentration. Looking at the red raspberry and cherry wines as examples, the red raspberry had a lower pH (2.99), T.A. (10.87 g/L), ABV (11.0%), and free sulfur dioxide content (11.0 ppm) compared to the cherry wine (3.21 pH, 5.40 g/L TA, 9.0% ABV, and 6.7 ppm Free SO2), and had approximately 8,500 times greater antibacterial activity than the cherry wine at 40% concentration (log CFU reductions of 7.23, 3.30, respectively). With the removal of ethanol, the red raspberry wine was only 220 times more bactericidal than the cherry wine (7.04, 4.69 log CFU reduction, respectively). The titratable acidity for the fruit wines having the highest correlation value might be seen in the trend with the fruit wines having more than 10 g/L T.A. (peach #1, red raspberry, and blackberry) having greater log CFU reductions (ranging from 5.1-8.4) than the cherry wine and peach #2 wine (with log CFU reductions ranging 2.5-3.3, and having T.A values of 5.4-7.2).

The strength of correlation (R^2 = 0.6964) between free SO_2 concentration and log reduction in the dealcoholized wines may have resulted from the values of the dealcoholized blackberry wine and the dealcoholized cherry wine being so similar. Removal of either the red raspberry wine or the peach wine data in Figure 4-17 would have resulted in much stronger correlation (R^2 = 0.9849 or 0.9349, respectively).

Additional dealcoholized wine data would likely have diminished the correlation strength to moderate, rather than strong. The difference in the strength of the relationship between these factors with and without ethanol may result from the complexity of the components and their interactions on antibacterial activity.

Lower pH values have been found to increase the antibacterial properties of sulfite and bisulfite, by preventing the dissociation of free SO_2 and the formation of its bound form (Babich and Stotzky 1978). The combination of low pH and the presence of ethanol have also been found to enhance the antibacterial activity of both properties (Jordan another others 1999). Similarly, increased ethanol concentrations or additions of sulfur dioxide have been found to have a greater antibacterial effect for the other component (Britz and Tracey 1990). A combination of different organic acids, ethanol, and low pH also had a synergistic antibacterial effect (Møretrø and Daeschel 2004). Modification of wine treatment through either lowering pH, increasing titratable acidity, increasing ethanol concentration, or increasing sulfur dioxide concentration in organic wine were all found to increase antibacterial activity, but stepwise regression analysis found that alteration of pH and ethanol provided the greatest predictive value (Waite and Daeschel 2007). The combination of these factors could also be species specific. The resistance of *E. coli* O157:H7 surpassed *L. monocytogenes* in three of the four dealcoholized fruit wine assays, specifically the dealcoholized red raspberry, dealcoholized blackberry, and dealcoholized cherry wines, whereas *L. monocytogenes* had shown to be more resistant to all of the fruit wines containing alcohol.

The presence or absence of ethanol in fruit wine also changed the correlations of anthocyanin, total phenolic, and tannin content to the antibacterial activity at 40% wine

concentration and 60% dealcoholized wine concentration. Anthocyanin, phenolic, and tannin content were not found to be correlated to antibacterial activity in wines with ethanol, yet had strong to very strong negative correlations to antibacterial activity in wines without it. The inclusion of peach wine #2 log reduction data and Adams-Harbertson assay data for fruit wine, and its omission for dealcoholized wine probably explains part of the differences. Peach wine #2 had no measurable amount of anthocyanin, phenolic, or tannin content, and it had a lower log reduction on average than the other wines. It was not tested in the dealcoholized wine study, so its inclusion to the dealcoholized wine study could have negated the relationship found. The log reduction values of peach wine #1 for all assays, and lack of measurable content in the Adams-Harbertson assay, may have contributed largely to the negative correlation found as well. Peach wine #1 had been found to be the most antibacterial wine of those tested. Testing a second peach wine to repeat the fruit type did not produce similar findings. Peach wine #1 had demonstrated bactericidal activity that was more than 900,000 times that of peach wine #2 at 40% concentration (with log CFU reductions of 8.43 and 2.46, respectively). The accuracy of the log reduction values associated with peach wine #2 using only data from *E. coli* O157:H7 and *L. monocytogenes*, as alluded to in section 4.2.1, would still not account for such a difference. If the two peach wines had been compared using only *E. coli* O157:H7 and *L. monocytogenes,* peach wine #1 would still have shown over 60,000 times the bactericidal activity to that of peach wine #2 (log CFU reductions of 7.24 and 2.46, respectively). Ignoring the results from the Adams-Harbertson assay, as the values were negligible for both peach wines, chemical analysis of the wines had revealed substantial differences in the pH, titratable acidity, and free

sulfur dioxide concentrations of the two peach wines. Peach wine #1 had a pH of 3.2, a

T.A. of 10.11 g/L, 11.0% ABV, and a free sulfur dioxide content of 27.7 ppm, while peach

wine #2 had a pH of 3.4, T.A. of 7.19, 10.5% ABV and 16.3 ppm free sulfur dioxide

content. Essentially, peach wine #1 was more acidic (1.5x), had a higher total acid

content (1.4x), slightly higher alcohol content, and a considerably higher free sulfur

dioxide content (1.7x) than peach wine #2.

Other compounds existing in fruit could be a contributing factor to a fruit wine's

antibacterial activity. Chlorogenic and neochlorogenic acids were both found to be the

primary phenolic compound in three cultivars of peach, and their content has been found

in extremely high concentrations in immature peaches (Villarino and others 2011).

Chlorogenic acid has been found to possess significant antibacterial activity through a

mechanism that increased plasma membrane permeability, resulting in cell leakage (Lou

and others 2011).

The lack of correlation found between phenolic content in the wines with

ethanol and antibacterial activity is surprising, but not unique. Daglia and others (2007)

found no difference in wine components from those of synthetic acids against oral

streptococci. A study that examined wine, phenols-stripped wine, dealcoholized wine,

ethanol, and pH, was unable to relate antimicrobial activity to phenolics, ethanol, or pH

(Boban and others 2010).

5.4 Scanning electron microscopy and transmission electron microscopy

SEM and TEM observation had shown a change in cell structure in *E. coli*

O157:H7 and *S.* Typhimurium treated with 60% concentration peach wine and 60%

concentration cherry wine compared to a TSY control. The changes visible after

101

treatment appeared to have been greater with *S.* Typhimurium than with *E. coli* O157:H7, although no significant difference in resistance was seen at the 60% concentration with these wines in the tube dilution assay. The difference seen through SEM and TEM observation did reflect differences in resistance observed between the two species at different concentrations though, with *E. coli* O157:H7 being more resistant to the wines overall.

The patterns observed with cherry and peach #1 wine treatment using SEM and TEM showed an interesting difference between the two wines on both species. SEM observation of peach wine #1 revealed holes and shriveling of the cells, indicating cellular leakage. The surface of *E. coli* O157:H7 and *S.* Typhimurium cells treated with peach wine were also rougher than that seen with TSBY and cherry wine. TEM observation showed a similar pattern from the peach wine, with complete disruption of the cell wall and a resulting loss of intracellular contents, especially visible in *S.* Typhimurium. The cherry wine treatment observed through SEM showed considerable material from the wine remaining and adhering to the outer membrane of cells. *S.* Typhimurium was seen to have smaller fragments of cherry wine material adhering, while *E. coli* O157:H7 appeared to have aggregated in groups, with greater accumulation of the material among cells. TEM observation of cells treated with the cherry wine did not show the same cell wall disruption as seen with peach wine, and there was much less intracellular leakage. These differences may have occurred due to differences in the severity of the mechanisms underlying the fruit wine's antibacterial activity. The lower pH, higher ethanol concentration, and higher free SO_2 concentrations of the peach wine appear to have caused a greater disruption to the cell membrane than

102

the cherry wine. Ethanol in high concentrations destroys membranes through solubilizing lipids and denaturing proteins, and, in lower concentrations, can cause leaking of small molecules through the membrane (Ingram and Buttke 1985). Ethanol also has been found to weaken hydrophobic interactions of the outer membrane (Ingram 1981), and potentially increases the effects of other antimicrobial agents. The effect of lower concentrations of ethanol on membrane permeability leaking small molecules has been found to be molecular weight specific, as differences in uptake in ethidium bromide and propidium bromide uptake in the presence of ethanol have been observed (Barker and Park 2004). At acidic pH, membrane disruption has been found to be enhanced in cationic antibacterial substances through their action on the anionic lipid components of the membrane (Mason and others 2006). Though changes in membrane permeability might be small, the uptake of protons, organic acids, and molecular sulfur dioxide due to their low molecular weight and small size could be substantial. The increased uptake and enhanced effect of organic acids on the membrane, as well as increased uptake of molecular sulfur dioxide due to the presence of ethanol and the acidity of wine, might best explain the differences in cellular leakage observed.

The role of tannins and phenolic compounds in the wines is unclear. Particulate matter from the cherry wine did adhere to the cell membrane of *E. coli* O157:H7 and *S.* Typhimurium. However, damage to the cell membrane was less pronounced than with cells treated with the peach wine, as seen in TEM observation. Changes in gross cell morphology, as visible between the two wine treatments and the TSBY control, were not as clear between wine treatments. Substantial elongation and inhibition of cell division, like that seen in SEM and TEM observation of *E. coli* O157:H7 treated with nut gall

103

extracts possessing high tannin and gallic acid content (Suwalak and Voravuthikunchai 2009), was not observed in cells treated with the cherry wine. It is possible that different components in the wine acted upon the bacteria differently, but this was not apparent through SEM and TEM observation.

CHAPTER 6

CONCLUSIONS

To conclude, the study found that fruit wines, both with and without ethanol, possess bactericidal properties for the pathogenic bacteria tested *in vitro*. The type of fruit wine, while very significant, was not as important of a factor in reduced cell counts as the species of pathogenic bacteria that had been exposed. This has important implications for generalizing the antibacterial benefits of fruit wine to other bacteria. Differences in the properties of wine are often stressed in the literature, but not enough attention is given to the types of bacteria studied. Sensitivity to the fruit wines among gram negative and gram positive classification of bacteria was not clearly distinguished in the study. The pH, titratable acidity, alcohol content, and free sulfur dioxide content of the wines were moderately to strongly correlated to cell count reduction. Unlike other wine studies that have found phenolic content being positively related to the bactericidal properties of wine, this study did not.

The study and its findings are significant in that it adds to a growing body of literature on wine as a potential antibacterial/bactericidal agent. It supports existing research that has found pH, ethanol, and free sulfur dioxide content of wine to be predictive of *in vitro* cell count reduction of pathogenic bacteria. The study also makes novel contributions to the research by using wines produced from different types of fruit other than grape, and offering evidence of the bactericidal activity of wine through SEM and TEM observation.

The study's findings suggest the potential application of fruit wine and fruit wine extracts as a possible antibacterial agent or additive that could be used in food, especially as a marinade. Wine has been found to be an effective solvent for essential oils, and enhances the bactericidal activity of oils found in oregano, thyme, cinnamon, and lemon grass (Friedman and others 2006). Marinades made with wine alone (Isohanni and other 2010), or with wine and either oregano leaves or oil, or with garlic are also bactericidal (Friedman and others 2007). Fruit wines could potentially do the same, and further research could investigate or assess this.

The study examined wines made from four types of fruit, further research in this area using different types of fruit or fermentable materials could confirm the findings of this study, and offer a potential way to use other types of surplus food material.

Another area of research that has not been explored is the use of wine, made from grape or other fruit, for inactivation of *Staphylococcus aureus* enterotoxin. *S. aureus* was found to be inactivated by the fruit wines in this study, and in other studies using wine made from grapes. The enterotoxin it produces is heat stable, and is a major cause of food poisoning in the U.S. Inactivation of the *S. aureus* enterotoxin by wine could provide another possible protective effect in food.

APPENDIX A

R data analysis and output

A-1. R data for Fruit Wine exposure analysis for all species of bacteria

Commands input are in courier font, output and comments are in ariel.

```
fwine = read.table("H:/fwine.txt",header=T) #data doesn't include
SJP, or 70% conc
attach (fwine)
fwine   #outputs all data that was read in R as a table
```

Data not included

```
species = factor(Species)
wine = factor(Wine)
conc = factor(Conc)   #This could probably be changed to ordered.
results = Result + 1
cfu=log10(results)
pairs (fwine)
```
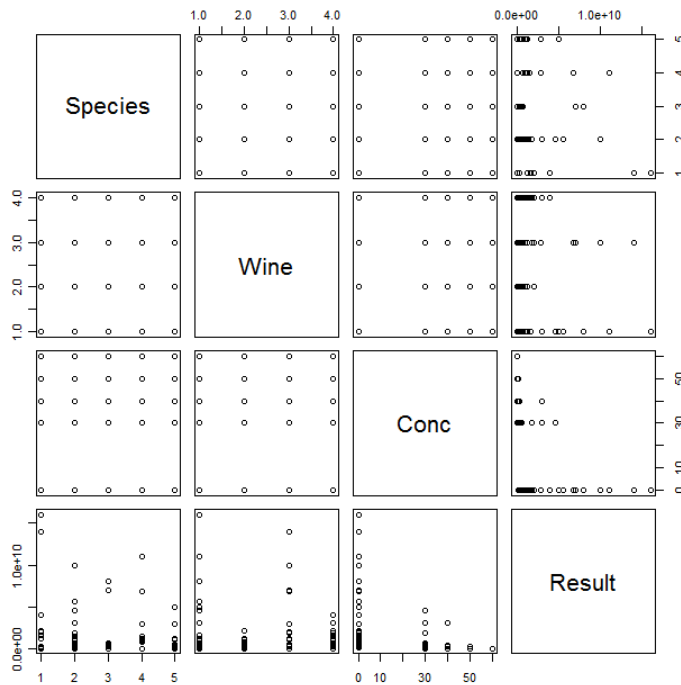
```
fwinemodel=lm(cfu ~species + wine + conc + species:wine +
species:conc + wine:conc + species:wine:conc)

summary (fwinemodel)
```

Call:
lm(formula = cfu ~ species + wine + conc + species:wine + species:conc +
   wine:conc + species:wine:conc)

Residuals:
Min   1Q Median   3Q   Max
-1.5663 -0.1760 0.0000 0.1308 2.2160

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)	
(Intercept)	9.49581	0.33584	28.275	< 2e-16	***
speciesLm	-0.13515	0.53100	-0.255	0.799438	
speciesSa	-0.58479	0.44427	-1.316	0.190045	
speciesSd	-0.12819	0.47494	-0.270	0.787593	
speciesST	-0.29254	0.53100	-0.551	0.582496	
wineC	-0.29511	0.53100	-0.556	0.579191	
wineMP	0.22777	0.53100	0.429	0.668565	
wineR	-0.11967	0.47494	-0.252	0.801398	
conc30	-2.18356	0.53100	-4.112	6.38e-05	***
conc40	-3.76016	0.53100	-7.081	4.86e-11	***
conc50	-6.15499	0.53100	-11.591	< 2e-16	***
conc60	-8.85643	0.53100	-16.679	< 2e-16	***
speciesLm:wineC	-0.37840	0.75095	-0.504	0.615060	
speciesSa:wineC	0.05952	0.67167	0.089	0.929504	
speciesSd:wineC	-0.14384	0.75095	-0.192	0.848351	
speciesST:wineC	-0.33355	0.78760	-0.424	0.672522	
speciesLm:wineMP	-0.04884	0.78760	-0.062	0.950636	
speciesSa:wineMP	-0.17885	0.67167	-0.266	0.790389	
speciesSd:wineMP	-0.16375	0.71241	-0.230	0.818512	
speciesST:wineMP	-0.42084	0.75095	-0.560	0.576018	
speciesLm:wineR	-0.19324	0.69234	-0.279	0.780538	
speciesSa:wineR	-0.08257	0.61468	-0.134	0.893317	
speciesSd:wineR	-0.13920	0.71241	-0.195	0.845346	
speciesST:wineR	-0.14088	0.71241	-0.198	0.843502	
speciesLm:conc30	1.97601	0.78760	2.509	0.013153	*
speciesSa:conc30	-3.10241	0.69234	-4.481	1.45e-05	***
speciesSd:conc30	-1.24137	0.75095	-1.653	0.100368	
speciesST:conc30	-1.13237	0.78760	-1.438	0.152549	
speciesLm:conc40	3.26570	0.78760	4.146	5.58e-05	***
speciesSa:conc40	-3.89228	0.67167	-5.795	3.77e-08	***
speciesSd:conc40	-1.70227	0.75095	-2.267	0.024803	*

```
speciesST:conc40          -1.79077    0.78760  -2.274 0.024376 *
speciesLm:conc50           2.69524    0.78760   3.422 0.000797 ***
speciesSa:conc50          -2.61253    0.67167  -3.890 0.000149 ***
speciesSd:conc50          -3.12458    0.75095  -4.161 5.27e-05 ***
speciesST:conc50          -2.89777    0.78760  -3.679 0.000324 ***
speciesLm:conc60           3.00681    0.78760   3.818 0.000195 ***
speciesSa:conc60           0.06469    0.67167   0.096 0.923399
speciesSd:conc60          -0.42314    0.75095  -0.563 0.573939
speciesST:conc60          -0.14787    0.78760  -0.188 0.851324
wineC:conc30               1.39655    0.78760   1.773 0.078192 .
wineMP:conc30             -1.33454    0.78760  -1.694 0.092217 .
wineR:conc30              -0.80277    0.75095  -1.069 0.286750
wineC:conc40               0.43510    0.78760   0.552 0.581456
wineMP:conc40             -5.81290    0.78760  -7.380 9.40e-12 ***
wineR:conc40              -2.60250    0.75095  -3.466 0.000687 ***
wineC:conc50              -1.00463    0.78760  -1.276 0.204049
wineMP:conc50             -3.48055    0.78760  -4.419 1.87e-05 ***
wineR:conc50              -3.02218    0.75095  -4.024 8.95e-05 ***
wineC:conc60               0.27725    0.78760   0.352 0.725311
wineMP:conc60             -0.77910    0.78760  -0.989 0.324124
wineR:conc60              -0.51970    0.75095  -0.692 0.489949
speciesLm:wineC:conc30  -1.28518    1.08823  -1.181 0.239444
speciesSa:wineC:conc30   1.59520    1.00751   1.583 0.115414
speciesSd:wineC:conc30  -0.87958    1.11384  -0.790 0.430934
speciesST:wineC:conc30   0.62473    1.13887   0.549 0.584111
speciesLm:wineMP:conc30  0.78066    1.13887   0.685 0.494089
speciesSa:wineMP:conc30 -1.60075    0.99341  -1.611 0.109161
speciesSd:wineMP:conc30  1.25765    1.08823   1.156 0.249612
speciesST:wineMP:conc30 -2.95595    1.11384  -2.654 0.008799 **
speciesLm:wineR:conc30   0.85165    1.07520   0.792 0.429534
speciesSa:wineR:conc30   0.70767    0.95580   0.740 0.460195
speciesSd:wineR:conc30   1.34926    1.08823   1.240 0.216923
speciesST:wineR:conc30   0.48554    1.08823   0.446 0.656101
speciesLm:wineC:conc40  -0.09681    1.08823  -0.089 0.929227
speciesSa:wineC:conc40   2.13988    0.97912   2.186 0.030372 *
speciesSd:wineC:conc40   1.68959    1.11384   1.517 0.131355
speciesST:wineC:conc40   0.63363    1.13887   0.556 0.578772
speciesLm:wineMP:conc40  1.53479    1.13887   1.348 0.179765
speciesSa:wineMP:conc40  4.58065    0.97912   4.678 6.31e-06 ***
speciesSd:wineMP:conc40  1.84370    1.08823   1.694 0.092259 .
speciesST:wineMP:conc40  2.35363    1.11384   2.113 0.036217 *
speciesLm:wineR:conc40   1.03716    1.07520   0.965 0.336257
speciesSa:wineR:conc40   1.54616    0.94094   1.643 0.102393
speciesSd:wineR:conc40  -1.04382    1.08823  -0.959 0.338978
speciesST:wineR:conc40  -0.78929    1.08823  -0.725 0.469378
speciesLm:wineC:conc50   3.31142    1.08823   3.043 0.002758 **
```

```
speciesSa:wineC:conc50   1.87448   0.97912   1.914 0.057429 .
speciesSd:wineC:conc50   2.26199   1.11384   2.031 0.044007 *
speciesST:wineC:conc50   3.29686   1.13887   2.895 0.004349 **
speciesLm:wineMP:conc50  2.04695   1.13887   1.797 0.074253 .
speciesSa:wineMP:conc50  3.28811   0.97912   3.358 0.000990 ***
speciesSd:wineMP:conc50  3.38718   1.06200   3.189 0.001729 **
speciesST:wineMP:conc50  3.52310   1.11384   3.163 0.001883 **
speciesLm:wineR:conc50   2.30867   1.07520   2.147 0.033352 *
speciesSa:wineR:conc50   3.12494   0.94094   3.321 0.001121 **
speciesSd:wineR:conc50   3.19300   1.08823   2.934 0.003860 **
speciesST:wineR:conc50   3.13222   1.08823   2.878 0.004571 **
speciesLm:wineC:conc60   1.96666   1.08823   1.807 0.072695 .
speciesSa:wineC:conc60   0.52440   0.97912   0.536 0.593028
speciesSd:wineC:conc60   0.85181   1.11384   0.765 0.445600
speciesST:wineC:conc60   1.21761   1.13887   1.069 0.286693
speciesLm:wineMP:conc60 -2.42201   1.13887  -2.127 0.035052 *
speciesSa:wineMP:conc60  0.61090   0.97912   0.624 0.533606
speciesSd:wineMP:conc60  0.62704   1.08823   0.576 0.565328
speciesST:wineMP:conc60  0.77320   1.11384   0.694 0.488623
speciesLm:wineR:conc60  -1.54899   1.07520  -1.441 0.151727
speciesSa:wineR:conc60   0.60267   0.94094   0.640 0.522808
speciesSd:wineR:conc60   0.69053   1.08823   0.635 0.526674
speciesST:wineR:conc60   0.58129   1.08823   0.534 0.594007
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Residual standard error: 0.5817 on 153 degrees of freedom
Multiple R-squared: 0.9857,   Adjusted R-squared: 0.9764
F-statistic: 106.5 on 99 and 153 DF, p-value: < 2.2e-16

anova (fwinemodel)

Analysis of Variance Table

Response: cfu

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
species	4	527.91	131.98	390.0541	< 2.2e-16	***
wine	3	115.44	38.48	113.7245	< 2.2e-16	***
conc	4	2499.19	624.80	1846.5663	< 2.2e-16	***
species:wine	12	12.29	1.02	3.0274	0.0007701	***
species:conc	16	211.57	13.22	39.0813	< 2.2e-16	***
wine:conc	12	106.44	8.87	26.2161	< 2.2e-16	***
species:wine:conc	48	95.77	2.00	5.8969	< 2.2e-16	***
Residuals	153	51.77	0.34			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

```
sink("H:/fwineTukey.txt") #Used to capture all output
TukeyHSD(aov(cfu~ species + wine + conc + species:wine +
species:conc + wine:conc), ordered=TRUE)
```

 Tukey multiple comparisons of means
   95% family-wise confidence level
   factor levels have been ordered

Fit: aov(formula = cfu ~ species + wine + conc + species:wine + species:conc +
wine:conc + species:wine:conc)

$species

	diff	lwr	upr	p adj
ST-Sa	0.7776436	0.4709943	1.0842930	0.0000000
Sd-Sa	0.9843501	0.6800377	1.2886626	0.0000000
Ec-Sa	1.8150330	1.5083836	2.1216824	0.0000000
Lm-Sa	4.0997108	3.8039025	4.3955191	0.0000000
Sd-ST	0.2067065	-0.1416614	0.5550744	0.4755935
Ec-ST	1.0373894	0.6869782	1.3878005	0.0000000
Lm-ST	3.3220672	2.9811028	3.6630315	0.0000000
Ec-Sd	0.8306829	0.4823150	1.1790508	0.0000000
Lm-Sd	3.1153607	2.7764965	3.4542249	0.0000000
Lm-Ec	2.2846778	1.9437134	2.6256422	0.0000000

$wine

	diff	lwr	upr	p adj
R-MP	0.6524999	0.3853721	0.9196277	0.0000000
B-MP	1.3207815	1.0493772	1.5921858	0.0000000
C-MP	1.7799958	1.5118428	2.0481488	0.0000000
B-R	0.6682816	0.3989392	0.9376240	0.0000000
C-R	1.1274959	0.8614300	1.3935618	0.0000000
C-B	0.4592143	0.1888551	0.7295735	0.0001124

$conc

	diff	lwr	upr	p adj
50-60	0.7921925	0.469401	1.114984	0
40-60	2.3451996	2.020782	2.669617	0
30-60	4.9931388	4.665288	5.320990	0
0-60	8.3330464	8.021468	8.644625	0
40-50	1.5530071	1.230216	1.875799	0
30-50	4.2009463	3.874705	4.527188	0
0-50	7.5408539	7.230969	7.850739	0
30-40	2.6479392	2.320088	2.975790	0
0-40	5.9878468	5.676268	6.299425	0
0-30	3.3399077	3.024756	3.655059	0

111

Output for interactions is not included.

A-2. R data for Fruit Wine exposure analysis for all types of fruit wine

```
fwine = read.table("H:/fwineeclm.txt",header=T) #data doesn't
include 70% conc or other species
attach (fwine)
fwine    #outputs all data that was read in R as a table
```

Data not included

```
species = factor(Species)
wine = factor(Wine)
conc = factor(Conc)   #This could probably be changed to ordered.
results = Result + 1
cfu=log10(results)
pairs (fwine)
```



```
fwinemodel=lm(cfu ~species + wine + conc + species:wine +
species:conc + wine:conc + species:wine:conc)
```

112
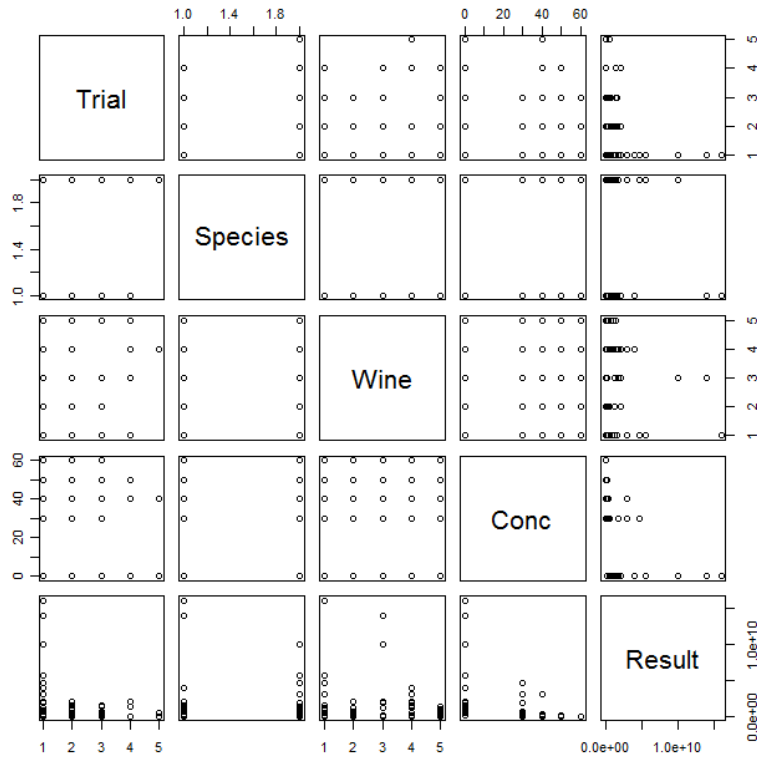
summary (fwinemodel)

Call:
lm(formula = cfu ~ species + wine + conc + species:wine + species:conc +
  wine:conc + species:wine:conc)

Residuals:
   Min   1Q Median   3Q   Max
-1.5663 -0.2256 0.0000 0.1976 2.2782

Coefficients:
	Estimate	Std. Error	t value	Pr(>|t|)	
(Intercept)	9.49581	0.40639	23.366	< 2e-16	***
speciesLm	-0.13515	0.64255	-0.210	0.834020	
wineC	-0.29511	0.64255	-0.459	0.647462	
wineMP	0.22777	0.64255	0.354	0.724045	
wineR	-0.11967	0.57472	-0.208	0.835653	
wineSJP	-0.45398	0.53760	-0.844	0.401292	
conc30	-2.18356	0.64255	-3.398	0.001122	**
conc40	-3.76016	0.64255	-5.852	1.43e-07	***
conc50	-6.15499	0.64255	-9.579	2.32e-14	***
conc60	-8.85643	0.64255	-13.783	< 2e-16	***
speciesLm:wineC	-0.37840	0.90871	-0.416	0.678380	
speciesLm:wineMP	-0.04884	0.95306	-0.051	0.959277	
speciesLm:wineR	-0.19324	0.83779	-0.231	0.818257	
speciesLm:wineSJP	-0.04780	0.83779	-0.057	0.954665	
speciesLm:conc30	1.97601	0.95306	2.073	0.041823	*
speciesLm:conc40	3.26570	0.95306	3.427	0.001027	**
speciesLm:conc50	2.69524	0.95306	2.828	0.006104	**
speciesLm:conc60	3.00681	0.95306	3.155	0.002367	**
wineC:conc30	1.39655	0.95306	1.465	0.147308	
wineMP:conc30	-1.33454	0.95306	-1.400	0.165849	
wineR:conc30	-0.80277	0.90871	-0.883	0.380032	
wineSJP:conc30	-0.57443	0.83779	-0.686	0.495196	
wineC:conc40	0.43510	0.95306	0.457	0.649423	
wineMP:conc40	-5.81290	0.95306	-6.099	5.23e-08	***
wineR:conc40	-2.60250	0.90871	-2.864	0.005517	**
wineSJP:conc40	-0.40881	0.83779	-0.488	0.627096	
wineC:conc50	-1.00463	0.95306	-1.054	0.295460	
wineMP:conc50	-3.48055	0.95306	-3.652	0.000498	***
wineR:conc50	-3.02218	0.90871	-3.326	0.001407	**
wineSJP:conc50	-1.65972	0.83779	-1.981	0.051513	.
wineC:conc60	0.27725	0.95306	0.291	0.771985	
wineMP:conc60	-0.77910	0.95306	-0.817	0.416431	
wineR:conc60	-0.51970	0.90871	-0.572	0.569212	
wineSJP:conc60	-0.18540	0.83779	-0.221	0.825506	

speciesLm:wineC:conc30 -1.28518   1.31684 -0.976 0.332448
speciesLm:wineMP:conc30   0.78066   1.37812   0.566 0.572891
speciesLm:wineR:conc30   0.85165   1.30107   0.655 0.514883
speciesLm:wineSJP:conc30 0.50955   1.23598   0.412 0.681406
speciesLm:wineC:conc40   -0.09681   1.31684 -0.074 0.941603
speciesLm:wineMP:conc40   1.53479   1.37812   1.114 0.269224
speciesLm:wineR:conc40   1.03716   1.30107   0.797 0.428056
speciesLm:wineSJP:conc40 0.17329   1.23598   0.140 0.888903
speciesLm:wineC:conc50   3.31142   1.31684   2.515 0.014215 *
speciesLm:wineMP:conc50   2.04695   1.37812   1.485 0.141949
speciesLm:wineR:conc50   2.30867   1.30107   1.774 0.080338 .
speciesLm:wineSJP:conc50 1.10484   1.23598   0.894 0.374438
speciesLm:wineC:conc60   1.96666   1.31684   1.493 0.139809
speciesLm:wineMP:conc60 -2.42201   1.37812 -1.757 0.083210 .
speciesLm:wineR:conc60   -1.54899   1.30107 -1.191 0.237853
speciesLm:wineSJP:conc60 0.45247   1.23598   0.366 0.715408

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.7039 on 70 degrees of freedom
Multiple R-squared: 0.9743,   Adjusted R-squared: 0.9563
F-statistic: 54.08 on 49 and 70 DF, p-value: < 2.2e-16

anova (fwinemodel)

Analysis of Variance Table

Response: cfu
               Df Sum Sq Mean Sq F value   Pr(>F)
species   1 152.45 152.446 307.6924 < 2.2e-16 ***
wine   4 62.05 15.512 31.3091 6.011e-15 ***
conc   4 937.98 234.494 473.2952 < 2.2e-16 ***
species:wine   4   0.95   0.237   0.4793 0.7508038
species:conc   4 76.93 19.232 38.8164 < 2.2e-16 ***
wine:conc   16 58.42   3.651   7.3699 1.137e-09 ***
species:wine:conc 16 24.18   1.511   3.0498 0.0006598 ***
Residuals   70 34.68   0.495

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

*TukeyHSD(aov(cfu~species+wine+conc+species*wine+species*conc+wine*conc+species*wine*conc), ordered=TRUE)*

  Tukey multiple comparisons of means
    95% family-wise confidence level
    factor levels have been ordered

114

Fit: aov(formula = cfu ~ species + wine + conc + species * wine + species * conc + wine * conc + species * wine * conc)

$species
```
      diff    lwr    upr p adj
Lm-Ec 2.255479 1.99903 2.511928    0
```

$wine
```
         diff      lwr      upr      p adj
R-MP   1.03757899  0.4349685 1.6401895 0.0000770
SJP-MP 1.21473488  0.6494458 1.7800240 0.0000007
C-MP   2.03007798  1.4387851 2.6213708 0.0000000
B-MP   2.08840650  1.4725938 2.7042192 0.0000000
SJP-R  0.17715589 -0.3652614 0.7195731 0.8903854
C-R    0.99249899  0.4230326 1.5619654 0.0000618
B-R    1.05082751  0.4559410 1.6457141 0.0000482
C-SJP  0.81534310  0.2855277 1.3451585 0.0004916
B-SJP  0.87367162  0.3166237 1.4307195 0.0003675
B-C    0.05832852 -0.5250906 0.6417476 0.9986227
```

$conc
```
       diff     lwr      upr p adj
50-60 1.955132 1.3739225 2.536341    0
40-60 4.233095 3.6518855 4.814304    0
30-60 6.201606 5.6203968 6.782815    0
0-60  7.585915 7.0312594 8.140571    0
40-50 2.277963 1.6967538 2.859172    0
30-50 4.246474 3.6652650 4.827683    0
0-50  5.630784 5.0761277 6.185439    0
30-40 1.968511 1.3873020 2.549720    0
0-40  3.352821 2.7981647 3.907476    0
0-30  1.384309 0.8296535 1.938965    0
```

Output for interactions is not included.

A-3. R data for dealcoholized fruit wine exposure analysis

```
driedwine = read.table("H:/driedwines.txt",header=T)
attach (driedwine)
driedwine    #outputs all data that was read in R as a table
```

Data output not included.

```
species = factor(Species)
wine = factor(Wine)
```

```
conc = factor(Conc)   #This could probably be changed to ordered.
results = Result+1
cfu=log10(results)

driedwinemodel=lm(cfu ~species + wine + conc + species*wine +
species*conc + wine*conc + species*wine*conc)

summary (driedwinemodel)
```

Call:
lm(formula = cfu ~ species + wine + conc + species * wine + species *
    conc + wine * conc + species * wine * conc)

Residuals:
    Min   1Q   Median   3Q   Max
-1.30207 -0.05897 0.00000 0.05188 1.25454

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)	
(Intercept)	9.612784	0.341865	28.119	< 2e-16	***
speciesLm	-0.640050	0.483470	-1.324	0.192089	
speciesSa	-1.035420	0.483470	-2.142	0.037551	*
speciesSd	-0.699099	0.483470	-1.446	0.154956	
speciesST	-0.263299	0.483470	-0.545	0.588658	
wineD_C	-0.015111	0.483470	-0.031	0.975201	
wineD_P	-0.012591	0.441346	-0.029	0.977365	
wineD_R	0.046792	0.441346	0.106	0.916026	
conc60	-2.969331	0.483470	-6.142	1.77e-07	***
speciesLm:wineD_C	0.151119	0.683730	0.221	0.826054	
speciesSa:wineD_C	0.196609	0.683730	0.288	0.774981	
speciesSd:wineD_C	-0.006515	0.683730	-0.010	0.992439	
speciesST:wineD_C	0.085052	0.654622	0.130	0.897192	
speciesLm:wineD_P	0.009366	0.654622	0.014	0.988646	
speciesSa:wineD_P	0.213378	0.654622	0.326	0.745936	
speciesSd:wineD_P	-0.014933	0.654622	-0.023	0.981899	
speciesST:wineD_P	-0.007412	0.654622	-0.011	0.991015	
speciesLm:wineD_R	-0.056664	0.654622	-0.087	0.931397	
speciesSa:wineD_R	-0.117316	0.654622	-0.179	0.858558	
speciesSd:wineD_R	-0.041683	0.654622	-0.064	0.949505	
speciesST:wineD_R	-0.074551	0.654622	-0.114	0.909825	
speciesLm:conc60	-0.371466	0.683730	-0.543	0.589551	
speciesSa:conc60	-5.201576	0.683730	-7.608	1.13e-09	***
speciesSd:conc60	-0.159023	0.683730	-0.233	0.817118	
speciesST:conc60	-0.920090	0.683730	-1.346	0.184997	
wineD_C:conc60	-0.199073	0.683730	-0.291	0.772240	
wineD_P:conc60	-4.977464	0.624157	-7.975	3.22e-10	***
wineD_R:conc60	-1.847325	0.624157	-2.960	0.004854	**

```
speciesLm:wineD_C:conc60  0.244402   0.966940   0.253 0.801581
speciesSa:wineD_C:conc60  1.335085   0.966940   1.381 0.174034
speciesSd:wineD_C:conc60 -0.030537   0.966940  -0.032 0.974943
speciesST:wineD_C:conc60 -1.822037   0.925775  -1.968 0.055099 .
speciesLm:wineD_P:conc60  4.170484   0.925775   4.505 4.53e-05 ***
speciesSa:wineD_P:conc60  4.370220   0.925775   4.721 2.23e-05 ***
speciesSd:wineD_P:conc60 -0.629828   0.925775  -0.680 0.499709
speciesST:wineD_P:conc60 -0.224037   0.925775  -0.242 0.809857
speciesLm:wineD_R:conc60  0.304477   0.925775   0.329 0.743733
speciesSa:wineD_R:conc60  1.511392   0.925775   1.633 0.109387
speciesSd:wineD_R:conc60 -3.671081   0.925775  -3.965 0.000254 ***
speciesST:wineD_R:conc60 -3.584980   0.925775  -3.872 0.000339 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.4835 on 46 degrees of freedom
Multiple R-squared: 0.9897,    Adjusted R-squared: 0.981
F-statistic: 113.5 on 39 and 46 DF,  p-value: < 2.2e-16
```

anova (driedwinemodel)

Analysis of Variance Table

```
Response: cfu
               Df Sum Sq Mean Sq   F value    Pr(>F)
species          4  71.28   17.82   76.2378 < 2.2e-16 ***
wine             3  52.65   17.55   75.0813 < 2.2e-16 ***
conc             1 767.75  767.75 3284.5797 < 2.2e-16 ***
species:wine    12  24.40    2.03    8.6985 2.561e-08 ***
species:conc     4  43.68   10.92   46.7204 1.226e-15 ***
wine:conc        3  51.26   17.09   73.0992 < 2.2e-16 ***
species:wine:conc 12 23.65    1.97    8.4327 4.009e-08 ***
Residuals       46  10.75    0.23
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

TukeyHSD(aov(cfu~species+wine+conc+species*wine+species*conc+wine
*conc+species*wine*conc), ordered=TRUE)

```
 Tukey multiple comparisons of means
   95% family-wise confidence level
   factor levels have been ordered
```

Fit: aov(formula = cfu ~ species + wine + conc + species * wine + species * conc + wine * conc + species * wine * conc)

117

$species
```
          diff       lwr       upr     p adj
ST-Sa 1.30513380  0.8335434 1.7767242 0.0000000
Sd-Sa 1.32512909  0.8398666 1.8103916 0.0000000
Lm-Sa 2.45104626  1.9657837 2.9363088 0.0000000
Ec-Sa 2.49777601  2.0374155 2.9581365 0.0000000
Sd-ST 0.01999529 -0.4515951 0.4915857 0.9999507
Lm-ST 1.14591246  0.6743220 1.6175029 0.0000001
Ec-ST 1.19264221  0.7467167 1.6385677 0.0000000
Lm-Sd 1.12591717  0.6406546 1.6111797 0.0000004
Ec-Sd 1.17264692  0.7122865 1.6330074 0.0000000
Ec-Lm 0.04672975 -0.4136307 0.5070902 0.9984367
```

$wine
```
          diff        lwr        upr      p adj
D_R-D_P 0.34188346 -0.04667048 0.7304374 0.1026323
D_C-D_P 1.67168744  1.28313351 2.0602414 0.0000000
D_B-D_P 1.74691641  1.34876711 2.1450657 0.0000000
D_C-D_R 1.32980399  0.94125005 1.7183579 0.0000000
D_B-D_R 1.40503295  1.00688365 1.8031823 0.0000000
D_B-D_C 0.07522897 -0.32292034 0.4733783 0.9578091
```

$conc
```
      diff    lwr    upr p adj
0-60 5.97572 5.76584 6.1856     0
```

Output for interactions is not included.

REFERENCES

Akagawa M, Shigemitsu T, Suyama K. 2003. Production of hydrogen peroxide by polyphenols and polyphenol-rich beverages under quasi-physiological conditions. Biosci Biotech Bioch 67(12):2632-40.

Arima H, Ashida H, Danno G. 2002. Rutin-enhanced antibacterial activities of flavonoids against *Bacillus cereus* and *Salmonella enteritidis*. Biosci Biotechnol Biochem 66(5):1009-14.

Babich H, Stotzky G. 1978. Influence of pH on inhibition of bacteria, fungi, and coliphages by bisulfite and sulfite. Environ Res 15:405-17.

Barker C, Park SF. 2001. Sensitization of *Listeria monocytogenes* to low pH, organic acids, and osmotic stress by ethanol. Appl Environ Microbiol 67(4):1594-600.

Battcock M, Azam-Ali S. 1998. Fermented fruits and vegetables: a global perspective. Rome, Italy: Food and Agriculture Organization of the United Nations. 108 p.

Benoit RE, Starkey RL. 1968. Inhibition of decomposition of cellulose and some other carbohydrates by tannin. Soil Sci 105:291-6.

Boban N, Tonkic M, Budimir D, Modun D, Sutlovic D, Punda-Polic V, Boban M. 2010. Antimicrobial effects of wine: Separating the role of polyphenols, pH, Ethanol, and other wine components. J Food Sci 75(5):M322-6.

Britz TJ, Tracey RP. 1990. The combination effect of pH, SO2 ethanol and temperature on the growth of *Leuconostoc oenos.*" J Applied Bacteriol 68(1):23-31.

Brooks L, McCloskey L, McKesson D, Sylvan M. 2008. Adams-Harbertson protein precipitation-based wine tannin method found invalid. J AOAC Int 91(5):1090-4.

Carneiro A, Couto JA, Mena C, Queiroz J, Hogg T. 2008. Activity of wine against *Campylobacter jejuni*. Food Control 19(8):800-5.

Center for Disease Control and Prevention. 2010. CDC Media Relations - Press Release: December 15, 2010. Atlanta, GA: Center for Disease Control and Prevention. Available from: www.cdc.gov/media/pressrel/2010/r101215.html. Accessed October 17, 2011.

Chinnam N, Dadi PK, Sabri SA, Ahmad M, Kabir MA, Ahmad Z. 2010. Dietary bioflavonoids inhibit *Escherichia coli* ATP synthase in a differential manner. Int J Biol Macromol 46(5):478-86.

Daglia M, Papetti A, Grisoli P, Aceti C, Dacarro C, Gazzani G. 2007. Antibacterial activity of red and white wine against oral *Streptococci*. J Agric Food Chem 55(13):5038-42.

Dai J, Patel JD, Mumper RJ. 2007. Characterization of blackberry extract and its antiproliferative and anti-inflammatory properties. J Med Food 10(2):258-65.

Daroch F, Hoeneisen M, Gonzalez CL, Kawaguchi F, Salgado F, Solar H, Garcia A. 2001. *In vitro* antibacterial activity of Chilean red wines against *Helicobacter pylori*. Microbios 104(408):79-85.

Delehanty JB, Johnson BJ, Hickey TE, Pons T, Ligler FS. 2007. Binding and neutralization of lipopolysaccharides by plant proanthocyanidins. J Nat Prod 70(11):1718-24.

Department of the Treasury, Alcohol and Tobacco Tax and Trade Bureau (U.S.). 2011a. Statistical Report-Beer. Tax and Trade Bureau Documents. TTB S5110-12-2010. 1 p. Available from DOT, Washington, DC.

Department of the Treasury, Alcohol and Tobacco Tax and Trade Bureau (U.S.). 2011b. Statistical Report-Wine. Tax and Trade Bureau Documents. TTB TTB S5120-12-2010. 2p. Available from DOT, Washington, DC.

Doell L. 2010. Eggs, beef and produce: the top 10 food recalls of 2010. AOL Daily Finance. New York, NY. Available from: http://www.dailyfinance.com/2010/12/13/eggs-beef-and-produce-the-top-10-food-recalls-of-2010/. Accessed October 17, 2011.

Flynn D. 2010. *E. coli* meat recalls remain low in 2010. Food Safety News. Seattle, WA. Available from: http://www.foodsafetynews.com/2010/12/e-coli-recalls-up-about-one-third-over-09-but-still-low/. Accessed October 17, 2011.

Frankel EN, Waterhouse AL, Teissedre PL. 1995. Principal phenolic phytochemicals in selected California wines and their antioxidant activity in inhibiting oxidation of human low-density lipoproteins. J Agric Food Chem 43(4):890-4.

Friedman LA, Kimball AW. 1986. Coronary heart disease mortality and alcohol consumption in Framingham. Am J Epidemiol 124(3):481-9.

Friedman M, Henika PR, Levin CE, Mandrell RE. 2006. Antimicrobial wine formulations active against the foodborne pathogens *Escherichia coli* O157:H7 and *Salmonella enterica*. J Food Sci 71(7):M245-51.

Friedman M, Henika PR, Levin CE, Mandrell RE. 2007. Recipes for antimicrobial wine marinades against *Bacillus cereus*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella enterica*. J Food Sci 72(6):M207-13.

Gañan M, Martínez-Rodríguez AJ, Carrascosa AV. 2009. Antimicrobial activity of phenolic compounds of wine against *Campylobacter jejuni*. Food Control 20(8):739-42.

Gronbaek M, Deis A, Sorensen TIA, Becker U, Schnohr P, Jensen G. 1995. Mortality associated with moderate intakes of wine, beer, or spirits. Brit Med J 310(6988):1165-9.

Hada LS, Kakiuchi N, Hattori M, Namba T. 1989. Identification of antibacterial principles against *Streptococcus mutans* and inhibitory principles against glucosyltransferase from the seed of *Areca catechu* L. Phytother Res 3(4):140-4.

Harbertson JF, Picciotto EA, Adams DO. 2005. Phenolic and anthocyanin assay for use with spectrophotometer. University of California-Davis. Davis, CA. Available from: http://wineserver.ucdavis.edu/adams/tannin/totalassay.pdf. Accessed February 18, 2010.

Harding C, Maidment C. 1996. An investigation into the anti-bacterial effects of wine and other beverages. J Biol Educ 30(4):237.

Harman, D. 1956. Aging: a theory based on free radical and radiation chemistry. J Gerontol 11(3):298-300.

Harman, D. 1992. Role of free radicals in aging and disease. Ann NY Acad Sci 673(1):126–41.

Heinonen IM, Lehtonen PJ, Hopia AI. 1998. Antioxidant activity of berry and fruit wines and liquors. J Agric Food Chem 46:25-31.

Hollman PCH, Cassidy A, Comte B, Heinonen M, Richelle M, Richling E, Serafini M, Scalbert A, Sies H, Vidry S. 2011. The biological relevance of direct antioxidant effects of polyphenols for cardiovascular health in humans is not established. J Nutr 141(5):989S-1009S.

Ikigai H, Nakae T, Hara Y, Shimamura T. 1993. Bactericidal catechins damage the lipid bilayer. Biochim Biophys Acta 1147:132-6.

Ingram LO. 1981. Mechanism of lysis of *Escherichia coli* by ethanol and other chaotropic agents. J Bacteriol 146(1):331-6.

Ingram LO, Buttke TM. 1985. Effects of alcohols on micro-organisms. In: Rose AH, Tempest DW, editors. Advances in Microbial Physiology. New York, NY: Academic Press. Volume 25:253-300.

Isohanni P, Alter T, Saris P, Lyhs U. 2010. Wines as possible meat marinade ingredients possess antimicrobial potential against *Campylobacter*. Poultry Sci 89:2704-10.

Jackson RS. 1994. Wine Science: Principles and Applications,1st ed. San Diego, California: Academic Press. 475 p.

Jagtap UB, Waghmare SR, Lokhande VH, Suprasanna P, Bapat VA. 2011. Preparation and evaluation of antioxidant capacity of Jackfruit (*Artocarpus heterophyllus* Lam.) wine and its protective role against radiation induced DNA damage. Ind Crop Prod 34(3):1595-1601.

Jarvis B. 1996. Cider, perry, fruit wines and other alcoholic fruit beverages. In: Arthey D, Ashhurst PR, editors. Fruit Processing. 1st ed. Bishopbriggs, Glasgow: Blackie Academic and Professional. p. 97-134.

Jordan SL, Glover J, Malcolm L, Thomson-Carter FM, Booth IR, Park SF. 1999. Augmentation of killing Escherichia coli O157 by combinations of lactate, ethanol, and low-pH conditions. Appl Environ Microb 65(3):1308-11.

Kakiuchi N, Hattori M, Nishizawa M, Yamagishi T, Okuda T, Namba T. 1986. Studies on dental caries prevention by traditional medicines. VIII. Inhibitory effect of various tannins on glucan synthesis by glucosyltransferase from *Streptococcus mutans*. Chem Pharm Bull 34(2):720-5.

Lin YT, Vattem D, Labbe RG, Shetty K. 2005. Enhancement of antioxidant activity and inhibition of *Helicobacter pylori* by phenolic phytochemical-enriched alcoholic beverages. Process Biochem 40:2059-65.

Liu C, Chen R, Su YC. 2006. Bactericidal effects of wine on *Vibrio parahaemolyticus* in oysters. J Food Prot 69(8):1823-8.

Lou Z, Wang H, Zhu S, Ma C, Wang Z. 2011. Antibacterial activity and mechanism of action of chlorogenic acid. J Food Sci 72:M398-403.

Lyr H. 1961. Hemmungsanalytische untersuchungen an einigen Ektoenzymen holzzerstorender Pilze. Enzymologia 23:231-48.

Lyr H. 1965. On the toxicity of oxidized polyphenols. Phytopathol Z 52:229-40.

Mandalari G, Bennett RN, Bisignano G, Trombetta D, Saija A, Faulds CB, Gasson MJ, Narbad A. 2007. Antimicrobial activity of flavonoids extracted from bergamot (*Citrus bergamia* Risso) peel, a byproduct of the essential oil industry. J Appl Microbiol 103(6):2056-64.

Mandalari G, Bisignano C, D'Arrigo M, Ginestra G, Arena A, Tomaino A, Wickham MSJ. 2010. Antimicrobial potential of polyphenols extracted from almond skins. Lett Appl Microbiol 51(1):83-9.

Marimon JM, Bujanda L, Gutierrez-Stampa MA, Cosme A, Arenas JI. 1998. *In vitro* bactericidal effect of wine against *Helicobacter pylori*. Am J Gastroenterol 93(8):1392.

Mason AJ, Gasnier C, Kichler A, Prévost G, Aunis D, Metz-Boutigue M, Bechinger B. 2006. Enhanced membrane disruption and antibiotic action against pathogenic bacteria by designed histidine-rich peptides at acidic pH. Antimicrob Agents Ch 50(10):3305-11.

McLean RJC, Pierson LS, Fuqua C. 2004. A simple screening protocol for the identification of quorum signal antagonists. J Microbiol Meth 58(3):351-60.

MFK Research. 2007. The impact of wine, grapes and grape products on the American economy: family businesses building value. St. Helena, California. p. 32.

Miller MB, Bassler BL. 2001. Quorum sensing in bacteria. Ann Rev Microbiol 55(1):165-99.

Mole S, Waterman PG. 1987. A critical analysis of techniques for measuring tannins in ecological studies. Oecologia 72(1):148-56.

Møretrø T, Daeschel MA. 2004. Wine is bactericidal to foodborne pathogens. J Food Sci 69(9):M251-7.

Nohynek LJ, Alakomi HL, Kähkönen MP, Heinonen M, Helander IM, Oksman-Caldentey KM, Puupponen-Pimiä RH. 2006. Berry phenolics: Antimicrobial properties and mechanisms of action against severe human pathogens. Nutr Cancer 54(1):18-32.

Olsson ME, Andersson CS, Oredsson S, Berglund RH, Gustavsson KE. 2006. Antioxidant levels and inhibition of cancer cell proliferation *in vitro* by extracts from organically and conventionally cultivated strawberries. J Agric Food Chem 54(4):1248-55.

Pahor M, Guralnik JM, Havlik RJ, Carbonin P, Salive ME, Ferrucci L, Corti MC, Hennekens CH. 1996. Alcohol consumption and risk of deep venous thrombosis and pulmonary embolism in older persons. J Am Geriatr Soc 44(9):1030-7.

Papadopoulou C, Soulti K, Roussis IG. 2005. Potential antimicrobial activity of red and white wine phenolic extracts against strains of *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*. Food Technol Biotechnol 43(1):41-6.

Pinhero RG, Paliyath G. 2001. Antioxidant and calmodulin-inhibitory activities of phenolic components in fruit wines and its biotechnological implications. Food Biotechnol 15(3):179-92.

Puupponen-Pimiä R, Nohynek L, Alakomi H, Oksman-Caldentey K. 2005. The action of berry phenolics against intestinal pathogens. Biofactors 23:243-51.

Rajković MB, Sredović ID. 2009. The determination of titratable acidity and total tannins in red wine. J Agric Sci 54(3):223-46.

Ren D, Sims JJ, Wood TK. 2001. Inhibition of biofilm formation and swarming of Escherichia coli by (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone. Environ Microbiol 3(11):731-6.

Rodrigo R, Miranda A, Vergara L. 2011. Modulation of endogenous antioxidant system by wine polyphenols in human disease. Clinica Chimica Acta 412(5-6):410-24.

Rodriguez Vaquero MJ, Alberto MR, de Nadra MCM. 2007a. Antibacterial effect of phenolic compounds from different wines. Food Control 18(2):93-101.

Rodriguez Vaquero MJ, Alberto MR, Mancadenadra M. 2007b. Influence of phenolic compounds from wines on the growth of *Listeria monocytogenes*. Food Control 18(5):587-93.

Rufián-Henares JA, Morales FJ. 2008. Microtiter plate-based assay for screening antimicrobial activity of melanoidins against *E. coli* and *S. aureus*. Food Chem 111(4):1069-1074.

Sarni-Manchado P, Cheynier V, Moutounet M. 1998. Interactions of grape seed tannins with salivary proteins. J Agric Food Chem 47(1):42-7.

Scalbert A. 1991. Antimicrobial properties of tannins. Phytochem 30(12):3875-83.

Seeram NP, Aviram M, Zhang Y, Henning SM, Feng L, Dreher M, Heber D. 2008. Comparison of antioxidant potency of commonly consumed polyphenol-rich beverages in the United States. J Agric Food Chem 56(4):1415-1422.

Serafini M, Maiani G, Ferro-Luzzi A. 1998. Alcohol-free red wine enhances plasma antioxidant capacity in humans. J Nutr 128(6):1003-7.

Sheth NK, Wisniewski TR, Franson TR. 1988. Survival of enteric pathogens in common beverages: An *in vitro* study. Am J Gastroenterol 83(6):658-60.

Stampfer MJ, Colditz GA, Willett WC, Speizer FE, Hennekens CH. 1988. A prospective study of moderate alcohol consumption and the risk of coronary disease and stroke in women. New Engl J Med 319(5):267-73.

Sugita-Konishi Y, Hara-Kudo Y, Iwamoto T, Kondo K. 2001. Wine has activity against entero-pathogenic bacteria *in vitro* but not *in vivo*. Biosci Biotech Bioch 65(4):954-7.

Suwalak S, Voravuthikunchai SP. 2009. Morphological and ultrastructural changes in the cell structure of enterohaemorrhagic *Escherichia coli* O157:H7 following treatment with *Quercus infectoria* nut galls." J Electron Microsc 58(5): 315-20.

Taguri T, Tanaka T, Kouno I. 2006. Antibacterial spectrum of plant polyphenols and extracts depending upon hydroxyphenyl structure. Biol Pharm Bull 29(11):2226-35.

Tian L, Wang H, Abdallah AM, Prinyawiwatkul W, Xu Z. 2011. Red and white wines inhibit cholesterol oxidation induced by free radicals. J Agric Food Chem 59(12):6453-8.

Truelsen T, Grønbæk M, Schnohr P, Boysen G. 1998. Intake of beer, wine, and spirits and risk of stroke: The Copenhagen City Heart Study. Stroke 29(12):2467-72.

USDA Economics, Statistics and Market Information System. 2009. Value of production for selected fruit and tree nuts, by State, 2006 to date. United States Department of Agriculture. Washington, DC. Available from: http://usda01.library.cornell.edu/usda/ers/89022/2009/Table-A18.xls. Accessed October 15, 2011.

Vattem DA, Mihalik K, Crixell SH, McLean RJ. 2007. Dietary phytochemicals as quorum sensing inhibitors. Fitoterapia 78(4):302-10.

Villarino M, Sandín-Espana P, Melgarejo P, de Cal A. 2011. High chlorogenic and neochlorogenic acid levels in immature peaches reduce *Monilia laxa* infection by interfering with fungal melanin biosynthesis. J Agric Food Chem 59:3205-13.

Waite JG, Daeschel MA. 2007. Contribution of wine components to inactivation of food-borne pathogens. J Food Sci 72(7):M286-91.

Weisse ME, Eberly B, Person DA. 1995. Wine as a digestive aid: Comparative antimicrobial effects of bismuth salicylate and red and white wine. Brit Med J 311(7021):1657-60.

Yi W, Fischer J, Krewer G, Akoh CC. 2005. Phenolic compounds from blueberries can inhibit colon cancer cell proliferation and induce apoptosis. J Agric Food Chem 53(18):7320-9.

Yildirim HK. 2006. Evaluation of colour parameters and antioxidant activities of fruit wines. Int J Food Sci Nutr 57(1/2):47-63.

Zhuang H, Du J, Wang Y. 2011. Antioxidant capacity changes of 3 cultivar Chinese pomegranate (*Punica granatum L.*) juices and corresponding wines. J Food Sci 76(4):C606-11.

Zoecklein BW, Fugelsang KC, Gump BH, Nury FS. 1995. Wine Analysis and Production, 1st ed. New York: Chapman and Hall. 621 p.