EFFECT OF RED WINE AND GRAPE JUICE AGAINST FOODBORNE PATHOGENS AND PROBIOTICS

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

EFFECTS OF RED WINE AND GRAPE JUICE AGAINST FOODBORNE PATHOGENS AND PROBIOTICS

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EFFECT OF RED WINE AND GRAPE JUICE AGAINST FOODBORNE PATHOGENS AND PROBIOTICS

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ABSTRACT

Numerous studies have been documented describing the burgeoning health benefits of red wine consumption, including anti-oxidative, anti-carcinogenic, anti-inflammatory and anti-cardiovascular and antibacterial properties. The inhibitory effects against pathogens may be attributed to the catechin and resveratrol found in red wines. This research was aimed to analyze the effects of red wine and grape juice against foodborne pathogens, *Helicobacter pylori*, *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Salmonella* Typhimurium and *Shigella boydii*, and the probiotic bacteria, *Lactobacillus acidophilus*, *Lactobacillus paracasei*, *Lactobacillus rhamnosus*, and *Bifidobacterium animalis*. All the foodborne pathogens were grown at their respective incubation conditions overnight with an initial concentration of each pathogen being 10^8-10^9 CFU/mL. The probiotics were also grown at their respective anaerobic conditions and the initial number of each probiotic was 10^7 CFU/mL. The different wines, Pinot Noir, Shiraz, and Zinfandel, to name a few, and grape juices were screened against the pathogens using the pour plate technique.

Our work showed, via *in vitro* tests, the antimicrobial activity of specific red wines against various foodborne pathogens. This study also demonstrated that red wines did not drastically affect health beneficial probiotic cultures as they did pathogens. Upon treatment with 40% v/v red wines, the numbers of each pathogen decreased from 10^8-10^9 to 10^4-10^5 CFU/mL, indicating the potent antibacterial property of the wines tested. The inhibitory action of Barton Merlot, Pinot Noir and Shiraz was extremely rapid compared to Zinfandel and Cherry wine. On the other hand, all four probiotic strains tested survived exposure to up to 80% of each red wine, even though the decrease in numbers was significant from the initial 10^7 CFU/mL. The pathogens were inhibited by up to 50-60% red grape juice. This indicates that the alcohol present in wines is not the only factor involved in their bactericidal effect. The inhibitory effect of Tropicana grape juice was extremely rapid against the probiotics tested. All four probiotics tested were significantly different in their inhibitory pattern ($P \leq 0.05$). Since the percentage of alcohol was almost the same in all the red wines tested, Tukey's Studentized Range test showing the presence of interaction effects among the red wines and pathogens and probiotics. The grape juice with the pathogens and probiotics were tested indicating that they were significantly different at $\alpha = 0.05$.

CHAPTER 1

INTRODUCTION

Following a "60 Minutes" television segment on "The French Paradox" in 1991, it became fashionable in the United States to drink wine with meals. The segment attributed the astounding findings of a significant lower rate of coronary heart disease (CHD) among French males as compared to American males. The "paradox" arose when it was noted that the French consume more saturated animal fats that have been connected with an increased incidence of CHD. One possible explanation is that wine is also a staple of the French diet (Renaud and Lorgeril 1992). Since then, numerous studies describing the burgeoning health benefits of red wine consumption, including anti-oxidative, anti-carcinogenic, anti-inflammatory, anti-cardiovascular and antibacterial properties have been documented (Carbó and others 1999; Dolara and others 2005; Goldberg and others 1995; Just and Daeschel 2003; Moretro and Daeschel 2004). Grapes and wines are rich in innumerable phenolic compounds and stilbenes, which possess high antioxidant activity, and several organic acids, such as tartaric and malic acids, which have antimicrobial effects, especially at the low pH of wine (Ikigai and others 1993; Jayaprakasha and others 2003). The amount of these compounds present in red wine usually varies depending on the variety of grapes used and the vinification (Vaquero and others 2007a). Resveratrol (trans-3, 5, 4'-trihydroxystilbene) has been identified as the major active compound of the stilbene phytoalexins (Burns and others 2001). Resveratrol showed an inverse correlation between consumption of red wine and the incidence of cardiovascular diseases. Many studies also elucidated that the consumption

of wine, beer and vodka can have a protective effect against certain foodborne infections (Bellido-Blasco and others 2002; Desenclos and others 1992). The benefits accrue mainly through the additive or synergistic interactions between resveratrol, and its metabolites, with other chemical compounds found in wine or food (Friedman and others 2006). The exact mechanisms responsible for the bactericidal and antimicrobial effects of red wine have not been completely fathomed.

Foodborne diseases cause approximately 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths in the United States each year (CDC, 1999). Foodborne pathogens account for an estimated 14 million illnesses, 60,000 hospitalizations, and 1,800 deaths (Bresee and others 2000). A recent study estimated the annual cost of these illnesses to be $405 million in 2003 (Frenzen and Drake 2005).

Probiotics are viable, defined microorganisms, which confer health benefits when administered in the right amount (Schrezenmeir and De Vrese 2001). There is evidence to believe that probiotics compete with and suppress the growth of undesirable microorganisms in the colon and small intestine and, thus, help stabilize the digestive system (Sanders, 1999).

The specific objectives of this research are:

1) To analyze the pH, acidity and resveratrol content of the red wines and grape juices.
2) To determine the inhibitory effects of different red wines and grape juice against food borne pathogens via agar diffusion and tube dilution tests.
3) To determine the effects of different red wines and grape juice on probiotic bacteria.
4) To investigate the effects of red wine and grape juice polyphenols against pathogens and probiotics.

CHAPTER 2

LITERATURE REVIEW

2.1 Wine

Wine is one of the world's most popular alcoholic beverages. It has been produced and consumed throughout history for cultural, economical, social, religious, and, more recently, health reasons. Wine produced from grapes is constantly being investigated for its health benefits. The increased popularity of wine has resulted in an enormous upsurge in prices of the world's finest wines, a scarce commodity under the best of circumstances. Grapes (*Vitis vinifera*) are one of the world's widely grown fruit crops with an annual production of 58-61 million metric tons (Murthy and others 2002). Grape growing plays a major role in the worldwide fruit production, with an international acreage of approximately 7.8 million hectares (OIV 2002). In the 1980s, there was a high rate of global production of fresh grapes, but due to a reduction in the production surface area, there was a drop in the beginning of the 1990s. However, soon after, the production rate plummeted, due to an increase in output trends, favorable climatic conditions and increase in the partial geographical redistribution of vineyards during this period. America saw its 2005 production reach a record high with 142,6 Mqx (millions of quintals) (OIV 2002).

2.1.1 Wine production

The 1986-1990 time frame was characterized by a net decrease in world viniviticulture production as the yearly annual production went from 3336 Mhl (millions

of hectoliters) to 3042 Mhl, a 8.88% ~ 9%reduction. The next five years witnessed a decrease that amounted to 14%. Beginning in 1995, a reverse trend was observed despite unfavorable climatic conditions, and viniculture production increased by 6.4% in total grape harvest.

2.1.2 Process of wine-making

The steps involved in red wine making process are (Keller 2008):

1. **Crushing and destemming** the grapes to release their juice and pulp. The must obtained is put in a tank to undergo fermentation.
2. **Alcoholic fermentation** is a natural process wherein the yeasts living in the grapes change the sugar contained in the must into alcohol and carbonic gas. This is usually maintained at a temperature around 25 to 30°C and the must is regularly ventilated. Less than 25°C, the wine will not have enough body, above 30°C, the wine will be too tannic. The fermentation process is carried on for 4 to 10 days.
3. **Maceration** is the period when the tannic elements and the color of the skin diffuse in the fermented juice. The contact between the liquid (must) and the solid elements (skin, pips and sometimes stem) will give body and color to the wine. At this stage, complex operations like dissolution, extraction, excretion, diffusion, decoction, and infusion kick in.
4. **Raking** is the step where the wine is separated from the solids or pomace. The wine obtained by raking is called "free run wine".
5. **Malolactic fermentation** is the process during which the malic acid of wine changes into lactic acid and carbonic gas under the action of bacteria living in the

wine. Malic acid is harsh, hence it is changed into lactic acid to make it supple and stable. This fermentation is obtained in a tank during a few weeks at a temperature between 18° and 20°C.

6. **Stabilization:** Wine needs to be clarified and stabilized further to age and improve its quality by putting it in oak casks.

2.1.3 Wine consumption

From the beginning of the 1980s to after the mid 1990s, world wine consumption had a downward plunge and was established as a period of a 10-year sink. This trend soon was reversed and a moderate upraise of 2377Mhl with a + 1% increase was recorded in 2005. Since the 1970s, per capita consumption of wine in the United States has grown from 1.3 gallons to 2.7 gallons per person in 2003. Americans spent more than $850 billion on food and drink in 2002. Overall, the total number of wine drinkers has increased considerably in recent years, too. Only 43% of the population drank wine, while 57% did not, but in 2007, those numbers had reversed, with 57% now drinking wine. Americans drank 3.2 gallons per person in 2007, compared to 2.81 gallons in 2005, and 2.46 in 2000. Per capita consumption has been on a steady rise since 1990, when it was 1.96 gallons (OIV 2002).

2.2 Difference between red and white wine

Red wines are made from black grapes and have a red or blue tint. Most grapes have colorless juice; therefore, to make red wine, the grape skins, which contain nearly all of the grapes' pigmentation, have to remain intact with the juice during all or part of the fermentation process. Tannins are also found in the grape skins, and are transferred

into the wine while the skins are in contact with the juice. Besides the difference in color, the primary difference between red and white wines is the presence of tannins. Found mainly in red wines, tannins provide a dry sensation in the mouth and in the back of the throat. Tannins also help preserve wine, allowing most red wines to be aged longer than white wines (Snyder 2005). Because of its perceived and supported health benefits, red wines' share of the wine market has increased from 18% in 1991 to 39% in 2002, which represents a 129% increase. White wine consumption is decreasing, as well as that of blush and fruit wines (OIV 2002). There are controversies regarding the health benefits of white wine. However, internationally, the United States ranks 34th in per capita wine consumption, just behind Slovakia, Canada and ahead of Latvia (OIV 2002).

2.3 Red wine

Most well designed population studies have demonstrated a 'J curve' relationship between wine consumption and the risk of cardiovascular diseases (Cleophas 1999; Maclure 1993; Marmot 2001). This indicates that people who do not drink alcohol at all and those who consume more than 30 g/day of alcohol (approx. 2.5 standard drinks) have an increased risk of death from all causes, higher blood pressure levels, as well as poorer liver function (Marmot, 2001). On the other hand, moderate alcohol intake (1-2 drinks/day) is associated with a decreased coronary heart disease (CHD) risk in both men and women. Small daily doses seem to have a more protective effect than an equal single daily dose. Moderate doses of alcoholic beverages also have a protective effect in individuals who have already suffered a heart attack, by reducing the risk of subsequent heart attacks. This observed J curve relationship is especially consistent among drinkers

of red wine. This association gained particular attention among the French population, because despite their high intake of saturated fats and other particularly unhealthy foods, their rate of heart disease was relatively low due to their wine intake, a phenomenon known as the French Paradox (Constant 1997). Red wine possesses several potentially heart-healthy mechanisms, including a favorable effect on blood clotting, endothelial function and serum lipids (most notably the ability to raise levels of HDL "good" cholesterol).

The cardio-protective effects observed in red wine drinkers are thought to be attributed at least in part to moderate alcohol intake, but especially due to the polyphenolic compounds red wine possesses, most notably, a flavonoid called resveratrol. Laboratory studies have revealed that resveratrol possesses several cardio-protective actions as well as cancer-protective effects. This may explain why some population studies have found a slight decrease in the risk of some cancers among moderate red wine drinkers.

It is very important to note, however, that higher alcohol consumption significantly increases the risk of many cancers; especially breast cancer in women. Along with smoking and obesity, alcohol consumption is perhaps the most significant lifestyle factor known to increase the risk of cancer, as well as other diseases, such as liver problems. Consequently, 1 to 2 glasses of red wine (with a meal) for men and 1 glass for women, several days of the week, may help to fight heart disease (Snyder 2005). Any more than that however may be doing more harm than good.

2.3.1 Health benefits of red wine consumption

2.3.1.1 Decreased risk of coronary heart disease (CHD)

A cohort study following 6,051 men and 7,234 women, 30-70 years of age for 10-12 years, showed a significant decreased risk for coronary artery disease among wine drinkers. Compared to non-wine drinkers, people who consumed several glasses of wine per day had a 50% reduced risk of death from all causes. The intake of beer or spirits, however, was not associated with risk reduction (Gronbaek and others 1995).

A recent case-control study conducted in Spain also found that a moderate alcohol intake, particularly from red wine, significantly decreased the risk of heart attacks (Fernandez-Jarne and others 2003). The results showed that moderate wine drinkers had a 32% average decreased risk of cardiovascular disease compared to non-drinkers. A J-shaped curve was observed for the relationship between red wine and cardiovascular disease risk, as a very heavy consumption increased the risk in a linear fashion.

2.3.1.2 Protection against atherosclerosis

Alcohol, particularly red wine, has been shown to increase serum concentrations of HDL "good" cholesterol (Serrano-Martinez and others 2004). A multitude of studies has been performed both in animals and humans to examine the relationship between alcohol, wine and particularly red wine in relation to cholesterol levels, atherosclerosis, as well as other risks, and protective effects associated with heart disease (Serrano-Martinez and others 2004). The results from a 3-week study involving pre-menopausal women on the contraceptive pill and post-menopausal women, showed that after wine consumption, the overall HDL "good" cholesterol level was increased in post-

menopausal women, while the LDL "bad" cholesterol levels were reduced in pre-menopausal women as compared with those who received only grape juice (Aviram 1999; Ivanov and others 2001; van der Gaag and others 2000). Another 2-week long study involving 20 healthy males found that red wine, but not white wine, significantly increased HDL levels by 26% and increased plasma apolipoprotein A-I levels by 12% (Lavy and others 1994). Several other human trials have shown that red wine consumption increases the levels of HDL "good" cholesterol (Contaldo and others 1989).

2.3.1.3 Effect on vascular endothelium

Studies, *in vitro*, have found that red wine, but not white wine, has a beneficial effect on vascular smooth muscle cells, thus, having a protective effect by preventing smooth muscle cell proliferation and consequently slowing the development of atherosclerosis (Rosenkranz and others 2002). Moreover, red wine consumption caused significant decreases in fibrinogen, factor VII, plasma C-reactive protein, and oxidized LDL antibody, while causing significant increases in total plasma antioxidant capacity. All of these factors suggest a protective effect of red wine against cardiovascular disease (Avellone and others 2004).

2.3.1.4 Effects on certain cancers

- Although excessive alcohol intake has been shown to increase the risk of various cancers, there is some evidence to suggest that frequent moderate red wine intake may have some cancer preventing effects, due largely to the high level of polyphenols, such as resveratrol and other flavonoids that are found in red wine. Research indicated that red wine reduced the function of proteins in pancreatic cancer cell membranes that are

responsible for pumping chemotherapy out of the cell, making the cells chemo-sensitive and also triggered the production of reactive oxygen species (ROS), which are substances circulating in the human body that have been implicated in a number of diseases (when ROS is increased, cells burn out and die). It also caused apoptosis, which is likely the result of increased ROS and depolarized the mitochondrial membranes, which indicates a decrease in the cell's potential to function (Timothe and others 2007).

2.3.1.5 Antibacterial activity

Although it has often been stated that the United States has the safest food supply in the world, there are approximately 76 million illnesses and 325,000 hospitalizations estimated to occur annually (Mead and others 1999). *Escherichia coli* O157:H7 and non-typhoidal *Salmonella* are the potent foodborne pathogens (Mead and others 1999). One main reason for the capability of the above-mentioned pathogens to cause diseases is through their ability to resist low pH, and a high acid environment (Peterson and others 1989). There are many outbreaks reported from consuming unpasteurized fruit juices and these have been linked to acid resistant pathogens. Numerous *E. coli* O157:H7 and *Salmonella* outbreaks have been linked to apple juice and orange juice consumption (Parish and others 1997) and due to the frequency of such outbreaks, it has been determined that the consumption of unpasteurized fruit juices can be a threat to human health (FDA 1998).

Grape juice and wine also have a low pH and high acid content and could easily act as vectors for *E. coli* O157:H7 and *Salmonella*. Bacteria, when exposed to environmental stress, may respond and adapt to new conditions. However, wine is

unique as it also has a high alcohol content in addition to high amounts of organic acids and low pH. All these factors together contribute to wine's antibacterial property. This has been confirmed by innumerable studies where various foodborne pathogens were exposed to wine, and the results indicate the reduction of their viable counts in the presence of wine. Numerous studies have been conducted that demonstrate the antibacterial property of wine against a notable amount of relevant food-borne pathogenic bacteria (Fernandes and others 2007; Moretro and Daeschel 2004; Sugita-Konishi and others 2001; Weisse and others 1995). Various *in vitro* studies indicated that the potency of wine as an antibacterial agent was higher than a given ethanol concentration and was, in fact, due to a combination of ethanol and organic acids (tartaric, malic, lactic and acetic) (Just and Daeschel 2003; Weisse and others 1995). Reports indicate that the consumption of red wine had a protective effect during foodborne outbreaks of *Salmonella* Enteritidis (Bellido-Blasco and others 2002) and hepatitis A (Desenclos and others 1992). Alcohol consumption also has a protective effect against *Helicobacter pylori* (Brenner and others 2001), which is a major cause of stomach ulcers. Various *in vitro* studies indicate that viable counts of *Enterobacteriaceae* are more rapidly reduced when treated with wine than other alcoholic beverages (Harding and Maidment 1996; Weisse and others 1995). Weisse and others (1995) showed that wine was able to reduce the viable counts of *S*. Enteritidis, *Shigella sonnei*, and *E. coli* by 5 to 6 logs after a 20-min exposure. Other studies show a 5 to 6 log reduction in viable counts of *Salmonella* sp. and *E. coli* after exposure to wine for 5 to 30 min and 20 to 60 min, respectively (Harding and Maidment 1996; Just and Daeschel 2003). Carneiro and others (2007) focused their study on the activity of red wine against the important foodborne pathogen,

Campylobacter jejuni. Undiluted red wine was found to rapidly inactivate *C. jejuni* and further inactivation data were obtained from experiments performed in red wine diluted with water. Their experiments also indicated that the two components in wine, namely ethanol and certain organic acids, act synergistically. Red wine was found to be anti-*Campylobacter*, which suggested that ingestion of wine during a meal may lower the risk of infection by this organism. Moretro and Daeschel (2004), when testing different combinations of ethanol, organic acids and acidity, found that a mixture of 0.15% of malic acid, 0.6% of tartaric acid, 15% of ethanol, and pH 3.0 had the strongest bactericidal effect and suggested that all these compounds acted synergistically and represented the major components for the bactericidal effect of wine. Drinking white wine with raw oysters can help prevent diarrhea and this was reconfirmed by studies conducted by Desenclos and others (1992). These authors studied the effects of an oyster-borne hepatitis A exposed to beverages with alcohol concentrations of >10% which had resulted in a reduced rate of illness. Just and Daeschel (2003) evaluated the survival of *E. coli* O157:H7 and *Salmonella* Typhimurium in a model stomach system. Bacteria were inactivated in both red and white wine within 60 min, but survived up to 16 days in grape juice. When a model stomach system was designed, wine had little effect on *E. coli* O157:H7 survival, whereas *Salmonella* was undetectable after 120 min. A nonvolatile wine fraction containing acids was more powerful in killing *Salmonella* than a volatile wine fraction containing alcohol, thereby, suggesting that the antibacterial activity of wine is acid dependent (Just and Daeschel 2003). Moretro and Daeschel (2004) studied the effects of red and white wines without added sulfite against wild type strains and sigma mutants of foodborne pathogens, like *E. coli* O157:H7, *S.*

Typhimurium, *Staphylococcus aureus* and *Listeria monocytogenes*. They deduced that the wines had bactericidal activity against all strains, with red wine being more potent. *S.* Typhimurium was most sensitive with a 6-log reduction and *S. aureus* was the least sensitive to the wines tested. Mutants having the gene encoding the alternative sigma factor disrupted were more sensitive to wine than their wild-type counterparts. The principal organic acids in wine, namely malic and tartaric acids, in conjunction with ethanol, exerted an immense effect on cell viability in either natural wine or constructed model wine. Weisse and others (1995) reported that red and white wines are as potent as bismuth salicylate against several bacteria that are responsible for traveler's diarrhea. They also demonstrated that diluted alcohol did not induce any significant reduction in colony counts. Sugita-Konishi and others (2001) examined the antibacterial activity of red and white wines against three potential entero-pathogenic bacteria, *S.* Enteritidis, *E. coli* O157:H7 and *Vibrio parahaemolyticus*, *in vitro*. They also identified that the evaporated fraction present in wine had antibacterial activity and examined the ability of wine and the fraction to protect against infection and concluded that there is little evidence for the use of wine as a digestive aid *in vivo*. Daglia and others (2007) studied the antimicrobial action of commercial red and white wines against oral streptococci responsible for caries development and against *Streptococcus pyogenes* responsible for pharyngitis. The compounds responsible for the former activities were succinic, malic, lactic, tartaric, citric and acetic acids. Wine polyphenols, however, exerted no effect against oral streptococci or *S. pyogenes*. Fernandes and others (2007) designed a model stomach, containing a food matrix and a synthetic gastric fluid, and studied the bactericidal effect of ingested wine on *Listeria innocua*. The influence of ethanol and

organic acids, wine constituents with known antimicrobial properties, was also investigated. Their study showed that ethanol had a higher bactericidal effect than a mixture of the main wine organic acids. When the organic acids were tested separately, malic and lactic acids had the strongest effect. The synergy of ethanol with the organic acids suggested that the ingestion of wine during a meal might reduce the quantity of *Listeria* persisting further in the alimentary tract. Murray and others (2002) associated alcohol consumption with a significant decrease in *H. pylori* infection. Another research indicated that modest consumption of wine and beer protects against *H. pylori* infection (Daroch and others 2001). There are some key phenolic phytochemicals in grape that have antimicrobial properties and inhibit the bacteria that cause common types of food poisoning (Akiyama and others, 2001; Weisse and others 1995). It has already been stated that the antimicrobial agent in wine is a polyphenol, resveratrol, produced during the fermentation process. This is active in the acidic environment and may be linked to inhibition of *H. pylori* (Daroch and others 2001; Murray and others 2002). Studies incorporating wine as a food additive in the form of marinades and other similar treatments provide further evidence of its protective role (Friedman and others 2006).

It is known that many naturally occurring compounds found in dietary and medicinal plants, herbs and fruit extracts, possess antimicrobial activities (Kouassi and Shelef 1998; Larson and others 1996). Lin and others (2005) aimed at determining the potential of phenolic phytochemical-enriched wine and vodka to inhibit *H. pylori* in laboratory media. Their study also indicated that raspberry, cinnamon and peppermint-enriched wines had the highest antimicrobial activity. The results indicated that the synergistic contribution of phenolics and the antioxidant activity might be more

important for inhibition than any specific phenolic concentration. This study clearly demonstrates the feasibility of the use of plant extracts as antimicrobial ingredients in alcoholic beverages. Such phenolic profiles also have the added benefit of enhancing host tissue and cellular response through enhanced antioxidant activity (Shetty and Wahlqvist 2004). Studies incorporating wine as a food additive in the form of marinades and other similar treatments provide further evidence of its protective role (Friedman and others 2006). They developed wine formulations containing plant essential oils and oil compounds effective against the foodborne pathogenic bacteria *E. coli* O157:H7 and *Salmonella enterica*. The results showed that wines containing essential oils/oil compounds, added or extracted from oregano or thyme leaves could be used to reduce pathogens in food and other environment.

2.4 Resveratrol (*trans*-3,5,49-trihydroxystilbene)

Among other polyphenolic flavonoids, resveratrol is thought to be, at least in part, responsible for the possible anti-cancer effect of red wine. Resveratrol has been found in at least 72 plant species (distributed in 31 genera and 12 families), a number of which are components of the human diet, such as mulberries, grapes (Langcake and Pryce 1976; Sanders and others 2000), red wine (Siemann and Creasy 1992) and peanuts (Ibern-Gomez and others 2000; Schneider and others 2003). In grapes, especially when exposed to fungal infections (Dercks and Creasy 1989), resveratrol is exclusively synthesized in the skins when the fruits are fresh. Resveratrol concentrations increase significantly during wine fermentation when yeast is added. Consequently, the resveratrol concentration of wine is much higher than that found in fresh grapes and grape juice.

Fresh grape skin contains about 50 to 100 µg of resveratrol per gram, whereas the concentration in red wine is in the range of 1.5 to 3 mg/liter (Siemann and Creasy 1992). Because grape skins are not fermented in the production process of white wines, only red wines contain considerable amounts of resveratrol. Resveratrol, which consists of 2 aromatic rings joined by a methylene bridge, is a member of the stilbene family. It is a parent molecule of viniferins, a family of phytoalexin polymers that prevent the progression of fungal infections. Resveratrol exists as cis- and trans- isomeric forms with trans to cis isomerization facilitated by UV exposure (Fig 1). The stilbene has been the focus of a number of studies investigating its beneficial effects on neurological, hepatic and cardiovascular diseases.

2.4.1 Synthesis, occurrence and content of resveratrol in plants and wine

Synthesis of resveratrol in plants can be induced by microbial infections, ultraviolet radiation and exposure to ozone. Resveratrol is synthesized in the leafy epidermis and skin of grapes, but not in the flesh. It is formed via a condensation reaction between 3 molecules of malonyl CoA and 1 molecule of 4-coumaroyl CoA (Soleas and others 1997). Resveratrol synthase facilitates this condensation reaction, which also produces four molecules of CO_2. One of the richest sources of this compound is *Polygonum cuspidatum*, a weed that is used in traditional Chinese and Japanese medicines (Langcake and Pryce 1976). The primary dietary sources of resveratrol in the human diet are peanuts, peanut butter, grapes, and wine. Resveratrol was first identified in grapevines (*V. vinifera*) in 1976 by Langcake and Pryce. Climate, wilting conditions, and amount of fungal (*Botrytis cinerea*) infection influence the concentration of

resveratrol in grape skin. Grape variety also affects resveratrol concentration, with higher concentrations observed in red grape varieties compared to white (Sieman and Creasy 1992). Due to its presence in grapes, it is no surprise that resveratrol is also found in wines.

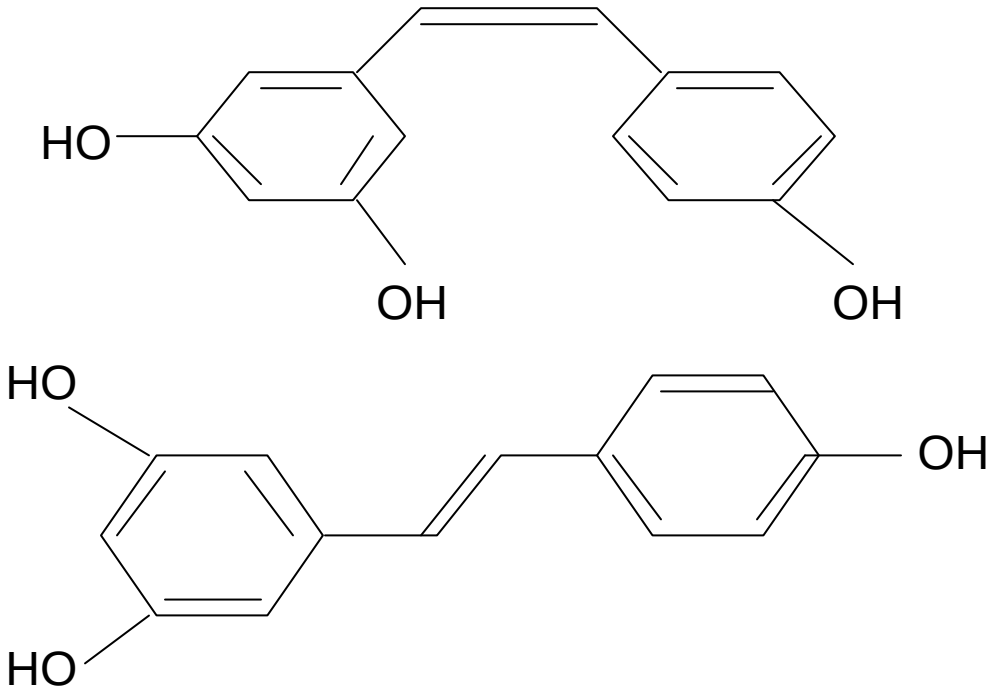


Figure 1. **Cis (top) and trans (bottom) isomers of resveratrol** (Sieman and Creasy 1992).

2.4.2 Health benefits of resveratrol

2.4.2.1 Antioxidant activity of resveratrol

Many compounds with aromatic groups are able to function as antioxidants by forming stable radicals via resonance structures, thereby preventing continued oxidation. Resveratrol contains 2 aromatic groups and has been shown to have a higher hydroxyl radical-scavenging capacity than propyl gallate, vitamin C, and vitamin E. A positive correlation has been established between the antioxidant power of wine and resveratrol content (Alonso and others 2002). The antioxidant activity of resveratrol may also be associated with protection against the progression of atherosclerosis. The oxidation of low-density lipoproteins (LDL) is an important event in the development of this disease.

2.4.2.2 Cardiovascular health and resveratrol

The incidence of coronary heart disease in the French population is low despite their high fat consumption and the heavy use of tobacco products. This observation is referred to as the "French Paradox," and may be associated with increased dietary consumption of red wine. Red wine is one of the few dietary sources of resveratrol and it is believed that this compound is responsible, in part, for the positive cardiovascular effects associated with moderate wine consumption (Constant 1997). The most accepted mechanism of cardioprotection by resveratrol is the inhibition of platelet aggregation (Bhat and others 2001). Studies indicate that the non-alcoholic components of red wine, notably the polyphenolic flavonoids, can exert protective effects on cardiovascular disease by inhibiting the formation of atherosclerotic plaques in a number of ways (Belleville 2002; Leikert and others 2002). Other studies have also shown that red wine

and purple grape juice can enhance platelet and endothelial production of nitric oxide (Fitzpatrick and others 1993; Freedman and others 2001). It was concluded that the reduction in cardiovascular risk by moderate long-term red wine intake is due to a synergistic effect of both the alcohol and the non-alcoholic polyphenol components found in red wine (Naissides and others 2004; Rifici and others 1999, 2002).

2.4.2.3 Anticarcinogenic activity

Resveratrol protects against cancer by minimizing DNA mutations that lead to cancer, thereby, inducing cell death in cancer cells and blocking formation of new blood vessels (Timothe and others 2007). A well studied mechanism is the inhibition of expression of genes that code for the cyclooxygenases (COX-1 and COX-2), which are implicated in many cancers, as well as the expression of the gene for another cancer-related enzyme called ornithine decarboxylase. Another mechanism is the suppression of angiogenesis (growth of new blood vessels). The aforementioned enzymes promote angiogenesis, hence its suppression may be due in part to its inhibition of the enzymes by resveratrol. Yet another mechanism involves the modulation of various drug-metabolizing enzymes and prevention of the activation of certain carcinogenic compounds while simultaneously enhancing the body's capacity to excrete other harmful compounds (Wolter and others 2002).

2.4.2.4 Anti-inflammatory function

The anti-inflammatory activity of resveratrol is partly through its inhibition of cyclooxygenase and partly through its activity in detoxifying or slowing the production of harmful superoxide radicals. Inflammation processes are mediated by prostaglandins

(PGs). Inhibition of PG activity may be partially due to the chemopreventative and cardioprotective effects of resveratrol (Richard and others 2005).

2.4.2.5 Resveratrol and its anti-aging property

Over the last 20 years, medical scientists in the U.S. and in France, Japan and China confirmed that a low calorie, nutritionally balanced diet not only contributes significantly to sustained good health, but also increases longevity in mice, rats, primates, yeast, round worms and fruit flies. These observations led scientists to search for "longevity genes." Once triggered by environmental cues, the longevity genes "switch on" and induce defensive changes at the cellular level, such as slowing metabolism and enhancing cellular respiration to help the body adapt to a more beneficial survival program. Researchers have found a family of genes, called sirtuins, produced by almost all life forms - from single celled organisms, to plants and mammals - during times of stress, such as famine (or caloric restriction). Sirtuins (silent information regulator proteins) are known to act as guardian genes that protect cells and enhance cellular survival. The human sirtuin, SIRT-1, for example, has been shown to suppress the p53 enzyme system normally involved in suppressing tumor growth and instigating cell death (apoptosis). By suppressing p53 activity, SIRT-1 prevents the cycle of premature aging and apoptosis normally induced when cellular DNA is damaged or stressed, thus giving cells enough time to repair any damage and prevent unnecessary cell death. A second sirtuin found in yeast, SIR2, has also been shown to become activated when placed under stress. SIR2 has been shown to increase DNA stability and speed cellular repairs, while increasing total cell lifespan. Essentially, sirtuins buy cells time to repair damage

(Seung-Hoi and Montming 2006; Sinclair and Guarente 2006). Caloric restriction triggers the activation of sirtuins, one of the mechanisms by which caloric restriction extends lifespan. The combination of resveratrol and SIRT1 stimulates a number of stress-modifying and life-extending processes including apoptosis, immune defense mechanisms, neuronal protection and metabolic optimization in liver, muscle and fat cells. Resveratrol has other actions, including stimulation of ATP production in the mitochondria of mice and modulation of insulin growth factor 1 (IGF-1), improving insulin sensitivity, mitigating against obesity and minimizing the development of fatty livers in mice fed a high-fat diet (Howitz and others 2003).

2.4.2.6 Antibacterial activity of resveratrol

Interest in phenolic compounds of grapes and wines increased in recent years because of their potential human health benefits (Caillet and others 2006; Frankel and others 1995; Zafrilla and others 2003). Vaquero and others (2007a) studied the influence of phenolic compounds from wines on the growth of *L. monocytogenes.* The consumption of wine with meals may protect our health against some foodborne organisms. Vaquero and others (2007b) also investigated the antimicrobial properties of pure phenolic compounds and polyphenols of different wines against pathogens and concluded that resveratrol is the key polyphenol involved in the antibacterial property.

2.5 Other polyphenols of interest in red wine

Other than resveratrol and quercetin, another active compound of red wine called delphinidin has also shown beneficial effects by preserving endothelium integrity, the alteration of which leads to diseases including cardiovascular diseases, such as

atherosclerosis, and is often associated with cancers (Martin and others 2003). Further investigations have found that red wines' ability to inhibit oxidation of lipoproteins is due to the non-alcoholic components, namely its polyphenols (A study involving 21 male subjects taking de-alcoholized red wine extract or quercetin (another polyphenol flavonoid found in red wine) supplements showed that both could decrease LDL oxidation after 2 weeks (Chopra and others 2000). Furthermore, total plasma antioxidant capacity has also been shown to be increased by alcohol-free red wine, but not white wine (Serafini and others 1998). The anti-platelet activity of wine is explained not only by its alcohol content, but also by the polyphenolic components, which occur most heavily in red wine. Furthermore, wine phenolics increase vitamin E levels while decreasing the oxidation of platelets submitted to oxidative stress (Boneu 1989). Resveratrol and quercetin, two of the polyphenols found in red wine, have been shown to inhibit TF activity in a dose dependant fashion (Di Santo and others 2003).

2.6 Grape juice

Grapes (*V. vinifera)* have an approximate annual production of 58 million metric tones (FAO 1997). Grape seeds are highly rich in monomeric phenolic compounds, like catechins, epicatechins, epicatechin-3-O-gallate and dimeric, trimeric and tetrameric procyanidins, all of which are antimutagenic and antiviral agents (Saito and others 1998). The health benefits of catechins and procyanidins have led to the use of grape seed extract as a dietary supplement (Soleas and others 1997). Phenolic compounds extracted from 12 different varieties of grapes showed antioxidant activity towards LDL oxidation *in vitro* (Jayaprakash and others 2001; Mayer and others 1997). The antibacterial and

antioxidant activities of grape seed extracts were investigated (Jayaprakash and others 2003). Grape juice and skin and seed extracts of *V. vinifera* var. Ribier black table grapes were found to be highly inhibitory towards *L. monocytogenes* (Rhodes and others 2006). They also found that this grape was active against all *Listeria* species but not against *Bacillus sereus, Salmonella* Menston, *E .coli, S. aureus* or *Yersinia enterocolitica*. It has been noted that grape juice is less effective against bacteria than wine, although the content of organic acids are similar (Harding and Maidment 1996; Just and Daeschel 2003). Grape juice with an addition of 10% industrial methylated spirit (IMS) is reported to have a much stronger effect against *E. coli* O157:H7, than grape juice and IMS that were tested individually (Harding and Maidment 1996).

2.7 Probiotics

Probiotics are live microorganisms that are similar to beneficial microorganisms found in the human gut. They are also called "friendly bacteria" or "good bacteria." The word “probiotics” was initially used as an antonym of the word “antibiotic”. It is derived from a Greek word meaning “for life” (Hamilton-Miller and others 2003).

The genus *Lactobacillus* belongs to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, family *Lactobacillaceae* and its closest relatives are the genera *Paralactobacillus* and *Pediococcus* (Garrity and others 2004). This is the most numerous genus, comprising 106 described species. *Lactobacillus acidophilus*, *L. salivarius*, *L. casei*, *L. plantarum*, *L. fermentum*, *L. reuteri* and *L. brevis* have been the most common *Lactobacillus* species isolated from the human intestine (Mitsuoka 1992). The functional

properties and safety of particular strains of *L. casei*, *L. rhamnosus*, *L. acidophilus*, and *L. johnsonii* have been extensively studied and well documented.

Bifidobacteria were first isolated from feces of breast-fed neonates. These rod-shaped, non-gas producing and anaerobic organisms were named *Bifidobacterium bifidus* due to their bifurcated morphology. They are generally characterized as Gram-positive, non-spore forming, non-motile and catalase-negative anaerobes with a special metabolic pathway, which allows them to produce acetic acid in addition to lactic acid in the molar ratio of 3:2. Due to their fastidious nature, these bacteria are often difficult to isolate and grow in the laboratory (Lee and others 1999). The taxonomy of bifidobacteria has changed continuously since they were first isolated. They had been initially assigned to the genera *Bacillus*, *Bacteroides*, *Nocardia*, *Lactobacillus* and *Corynebacterium*, before being recognized as a separate genus in 1974. Due to their high (>50 mol%) G+C content, bifidobacteria are phylogenetically assigned to the Actinomycete division of Gram-positive bacteria (Holt and others 1994).

Leuconostoc mesenteroides and *Sporolactobacillus inulins* are lactic acid bacteria (LAB) that confer many health benefits (Holzapfel and others 1998). Certain non-LAB, such as *Saccharomyces cerevisiae* and *S. boulardii* exhibit probiotic-like benefits and have been used in pharmaceutical applications (Holzapfel and others 2001).

2.7.1 Importance of probiotics

Probiotics have been documented to alleviate lactose intolerance symptoms and prevent and reduce diarrheal symptoms. The prevention and management of allergies is another area in which probiotics may potentially exert their beneficial role

(Yazdanbakhsh and others 2002). A recent study also indicated that early consumption of probiotic preparations containing *Lactobacillus* GG may reduce prevalence of atopic eczema later in life (Gueimonde and others 2006). Antigenotoxicity, antimutagenicity and anti-carcinogenicity are important potential functional properties of probiotics, which have received much attention recently. Some epidemiological researches have emphasized that probiotic intake may be related to a reduced colon cancer incidence (Hirayama and Rafter 2000) and experimental studies showed the ability of the lactobacilli and bifidobacteria to decrease the genotoxic activity of certain chemical compounds (Tavan and others 2002) and increase the antimutagenic activity during growth in selected media (Lo and others 2004). It is well established that diets rich in saturated fat or cholesterol would increase the serum cholesterol level, which is one of the major risk factors for coronary heart diseases. Mann and Spoerry (1974) were the first to observe a decrease in serum cholesterol levels in men fed large quantities (8.33 L man^{-1} day^{-1}) of milk fermented with *Lactobacillus*. As they suggested, this was possibly due to the production of hydroxymethyl-glutarate by probiotic bacteria, which was reported to inhibit hydroxymethylglutaryl-CoA reductases required for the synthesis of cholesterol. Probiotic cultures produce a wide range of antibacterial compounds, including organic acids, such as lactic and acetic acids, hydrogen peroxide, bacteriocins, various low-molecular-mass peptides, and antifungal peptides/proteins, fatty acids, phenyllactic acid, and OH-phenyllactic acid. Lactic and acetic acids are the main organic acids produced during the growth of probiotics, and their pH-lowering effect in the gastrointestinal tract has a bacteriacidal or bacteriostatic effect. Low-molecular-mass compounds, such as lactic acid, have been reported to be inhibitory towards Gram-

negative pathogenic bacteria like *H. pylori* (Alakomi and others 2000). Several *in vitro* studies on cell models of inflammatory bowel disease (IBD) have shown the ability of certain probiotic strains, such as *L. rhamnosus* GG, to modulate the immune system by down-regulating TNF-α-induced IL-8 production (Zhang and others 2005). Studies also suggest that the immune system might be beneficially affected in the presence of probiotics through the action of recognition receptors expressed on the surface of epithelial cells (Isolauri and others 2001). Probiotics may also influence the immune interactions by suppressing the growth and attachment of potential pathogens, thereby acting as inhibitory agents against foodborne pathogens.

2.8 Foodborne diseases

The Centers for Disease Control and Prevention estimates that, in the U.S. alone, foodborne pathogens are responsible for 76 million illnesses every year. Of the people affected by those illnesses, 300,000 are hospitalized and more than 5,000 die. These widespread outbreaks of food-borne illnesses are attributed, in part, to the fast-paced distribution of foods across the nation. Foodborne pathogens account for an estimated 14 million illnesses, 60,000 hospitalizations, and 1,800 deaths (Bresee and others 2000). *Salmonella* is one of the most common causative agents of intestinal infections in the U.S. In 2005, a total of 16,614 laboratory-confirmed cases of infections in the FoodNet surveillance areas were identified, in descending order, as *Salmonella* (6,471), *Campylobacter* (5,655), *Shigella* (2,078), *Yersinia* (159), and *Listeria* (135) (CDC 2005). The CDC estimates that 73,000 cases of *E. coli* O157:H7 infections occur annually in the U.S. A recent study estimated the annual cost of these illnesses to be $405 million in

2003 (Frenzen and Drake 2005). The annual cost of foodborne illnesses caused by the four most common bacterial pathogens alone (*Salmonella*, *Shigella*, *Campylobacter* and *E. coli*) has been estimated at $6.9 billion. In addition, *L. monocytogenes* is estimated to cause approximately 1,600 cases of listeriosis annually, resulting in 415 deaths (Farber and Peterkin 1991). *H. pylori* regularly colonizes the human stomach and may lead to the onset of various gastric-related diseases (Dunn and others 1997). Most patients with duodenal ulcer can be cured by killing *H. pylori* with antibiotics (Moayyedi and others 2000). The lifetime risk of infection with *H. pylori* is ~50% among people in industrialized countries, and 90% in developing countries.

2.8.1 Important foodborne pathogens

2.8.1.1 *Salmonella*

Salmonella serotypes continue to be a threat to food safety in United States. Members of *S. enterica* account for more than 99% of human salmonellosis (Ahmer and others 1999). It is estimated that from 2 to 4 million cases of salmonellosis occur in the U.S. annually. Infections are commonly acquired by animal to human transmission through consumption of undercooked food products derived from livestock or domestic fowl. The incidence of salmonellosis appears to be rising in both in the U.S. and other industrialized nations. *S.* Enteritidis isolations from humans have shown a dramatic rise in the past decade, particularly in the northeastern United States (6-fold or more), and the increase in human infections is spreading south and west, with sporadic outbreaks in other regions. *S.* Typhi and the paratyphoid bacteria normally cause septicemia and produce typhoid or typhoid-like fever in humans. Other forms of salmonellosis generally

produce milder symptoms. Studies suggest that the ability of *S*. Typhimurium to elicit severe polymononuclear leukocytes (PMN) infiltration in the intestinal mucosa is vital for causing disease and contributes to fluid loss during diarrhea (Wood and others 1998). The *Salmonella*–specific virulence determinant for causing diarrhea and PMN influx in the intestinal mucosa is the invasion–associated type III secretion system (TTSS1) (Watson and others 1998). *Salmonella*–specific virulence determinants contributing to the ability to survive and multiply in macrophages include genes that confer resistance to macrophage killing mechanisms (Roudier and others 1990).

2.8.1.2 *Escherichia coli* O157:H7

Enterohemorrhagic *E. coli* (EHEC) O157:H7 was recognized as a cause of enteric disease following two outbreaks of hemorrhagic colitis in 1982 associated with undercooked hamburgers served at fast food restaurants. Numerous food- and water-borne outbreaks linked to EHEC have been documented, and it is estimated that the O157:H7 serotype is responsible for more than 73,000 cases of illness and 61 deaths each year in the U.S., while non-O157 EHEC cause approximately 37,000 and 30 deaths each year (Mead and others 1999). The production of Shiga toxins, intestinal colonization and production of enteropathogenic *E. coli*-like attaching and effacing lesions are major virulence factors associated with EHEC strains (LeBlanc 2003). Symptoms include hemorrhage, colitis and possibly hemolytic uremic syndrome, and possible contaminants are ground beef and raw milk. Thorough cooking of meat and avoidance of cross contamination may help prevent disease.

2.8.1.3 *Listeria monocytogenes*

L. monocytogenes, an emerging pathogen since the 1970s, causes an estimated 2,500 cases of serious illness and 500 deaths per year in the U.S. (Batt 1999). Listeriosis is a major health concern due to the severity of the disease, high mortality rate and opportunistic nature of the pathogen. *L. monocytogenes* primarily affects immuno-compromised patients, including the young, the elderly and pregnant individuals. In addition to being a deadly pathogen, it is well suited to growth and survival in food. *Listeria* cells initially adhere to intestinal enterocytes and penetrate the intestinal wall. The host cells, in defense, trap the bacteria in phagosomes. Listeriolysin O (LLO) lyses the phagosome, releasing *L. monocytogenes* into the host cell's cytosol. Following multiplication in the host cytoplasm, *L. monocytogenes* travels across the host cell and upon actin polymerization and vacuole lysis, the bacteria infect the neighboring cells (Marco and others 1997). Possible symptoms include meningitis, sepsticemia, miscarriage, and the possible contaminants are vegetables, milk, cheese, meat, seafood. Purchasing pasteurized dairy products and cooking foods properly, avoiding cross-contamination and use of sanitary practices may help prevent listeriosis. *L. monocytogenes* is considered a foodborne pathogen, and is also recognized as one of the leading causes of death due to bacterial food poisoning. Listerial pathogenesis is a complex process and various bacterial and host factors are pivotal in its infection process. Understanding how environmental factors like those encountered in food or potential host, influence virulence gene expression is critical in unraveling the detailed pathogenic mechanisms.

2.8.1.4 *Shigella*

Diarrheal diseases are the leading worldwide cause of death among children. The World Health Organization estimates that 5 million deaths occur annually from diarrheal disease and shigellae are responsible for 10% of these mortalities (Kotloff and others 1999). Data from the 1998 FoodNet surveillance network found that *Campylobacter*, *Salmonella* and *Shigella* were the most common isolates from patients with gastroenterititis (CDC 1999). In a highly orchestrated movement, *Shigella* species invade the host by using their own genetic machinery and exploit the host inflammatory response to facilitate the invasion process. After ingestion, shigellae transit the acidic environment of the stomach and ultimately attach to target cells (LeBrec and others 1964). Entry is initially via engulfment by M cells, and attachment of the bacteria to the host cell induces the formation of filopodia and finally the microfilament arrangement eventually leads to the engulfment of the bacterial cell by pseudopods, allowing the entry into the host cell (Gao and Kwaik 2000). Common symptoms include abdominal pain, cramps, diarrhea, fever, vomiting, blood, and pus, and possible contaminants include salads, raw vegetables, dairy products, and poultry. Proper washing and sanitizing techniques can help prevent the disease.

CHAPTER 3

MATERIAL AND METHODS

3.1 Wines and grape juices

Red wines that are commonly consumed in the U.S. and purple and white grape juices were purchased from local supermarkets and stored at 5°C until used (within 30 min of opening). A listing of the wines and juices used in this study is shown in Table 3.1. A White Zinfandel and one white grape juice were included in experiments for comparison.

Table 3.1 List of red wines and grape juices used in this study.

Wine	Grape juice
Merlot 1	Tropicana Purple grape juice
Merlot 2	Welch's Purple grape juice
Shiraz	Welch's white grape juice
Cabernet Sauvignon	
Pinot Noir	
Tempranillo 2004	
Grenache Shiraz	
Chambourcin	
Zinfandel & Carignane 2000	
Cherry wine	
Hybrid and American blend	
Red Zinfandel	
Blush Zinfandel	
White Zinfandel	

3.2 Chemical analyses

3.2.1 Analyses of pH and titratable acidity

The wines and grape juices were analyzed for pH and titratable acidity (TA). TA measures the total amount of protons available in a juice or wine, and is expressed as g/L tartaric acid equivalent. A 10 mL aliquot of each wine mixed with 90 mL distilled water was titrated against 0.1 N NaOH (Fisher Scientific, Fair Lawn, NJ, U.S.A.) to a pH endpoint of 8.2. All pH and TA determinations were replicated twice.

3.2.2 High performance liquid chromatography (HPLC) analyses

3.2.2.1 Chemicals

Methanol, water, o-phosphoric acid (H_3PO_4), acetonitrile (all HPLC grade), and sodium chloride were purchased from Fisher Scientific Co. (Fair Lawn, NJ, U.S.A.). Trans-resveratrol was procured from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

3.2.2.2 Instrumentation

The resveratrol in each wine was extracted by HPLC and the concentration determined using isocratic HPLC separation with UV detection. A Perkin-Elmer, series 4 HPLC (Norwalk, CT, U.S.A.) equipped with a Rheodyne loop injector (20 µL) and an LC 90 UV spectrophotometric detector, set at 306 nm, was used. The column was a L*I.D. 25 cm x 4.6 mm, 5 µm particle size Supelcosil™ LC – 18 HPLC column (Supelco Inc, Bellefonte, PA, U.S.A.). Samples were injected into the column filled with the stationary reverse phase and the separation was carried out at room temperature. The isocratic elution at a flow rate of 1.0 mL/min used the mobile phase

of 25% acetonitrile, 0.1% H3PO4 and NaCl (c = 5mmol/l) in demineralized water. A Varian Star Chromatography Work Station Processing Software (Walnut Creek, CA, U.S.A.) was used for data acquisition and reprocessing. After each analysis, the column was rinsed with mobile phase for 10 min (Hanzlikova and others 2004; Intarapichet and Gruen 2000).

3.2.2.3 Standards and method validation

A stock solution of 250 µg/mL of *trans*-resveratrol was prepared in mobile phase and stored in the dark at –20ºC. *Trans*-resveratrol standard solutions of 0.01, 0.1, 0.25, 0.5, 0.75, 1.0, 2.5 and 5.0 µg/mL were prepared for the external standard calibration curve. Recovery of the method was determined in replicates of six using the 0.25, and 2.5 µg/mL of *trans*-resveratrol standard solution. The recovery of *trans*-resveratrol was also determined from spiked wine samples using the 0.5 and 2.5 µg/mL standard solutions.

The analytical parameters considered were precision, linearity, selectivity, sensitivity and recovery:

Precision. The precision of the analytical method was determined by analyzing eight aliquots of a homogenous wine sample, with and without spiking of a *trans*-resveratrol standard.

Linearity. A calibration curve of *trans*-resveratrol determined the linearity of the method. Integrated peak areas were plotted against the concentrations of the standard solutions.

Selectivity. The t*rans*-resveratrol was quantified at an absorbance of 306 nm, according to Juan and others (1999) and Trela and WaterHouse (1996).

Recovery. Two different concentrations of *trans*-resveratrol standard solutions and spiking two different varieties of wine samples with standard solutions (0.25 ppm and 2.5 ppm) were used to deduce the recovery percentage.

3.2.2.4 Sample preparation

Thirteen red and one white wines of various vintages and varieties, and two purple grape juices and one white grape juice (Table 3.1) were purchased from local stores. One milliliter of the wine sample was transferred into reaction vials, and dried under nitrogen gas in the dark to prevent the isomerization of *trans* to *cis* form. The dried residue was dissolved in 1 mL mobile phase and filtered through a 0.45 μm nylon syringe filter. The filtrate was collected in an amber vial and analyzed by HPLC. Twenty microliters of the filtrate was injected into the HPLC column. Three replicates of each wine sample were prepared for HPLC measurement. One milliliter of the grape sample was dried under nitrogen gas and dissolved in 1 mL mobile phase and filtered, and the filtrate was analyzed by HPLC. Three replicates of each grape juice were prepared for HPLC measurement.

3.3 Bacterial cultures and growth conditions

Isolates of *Helicobacter pylori*, *Listeria monocytogenes EGD*, *Escherichia coli* O157:H7 35150, *Salmonella* Typhimurium and *Shigella boydii* were from the culture collection of the University of Missouri Food Microbiology Laboratory. The probiotics tested in this study included different strains of lactobacilli and bifidobacteria, namely *L. paracasei*, *L. acidophilus* LA-2, *L. rhamnosus* GG and *Bifidobacterium animalis* from the same culture collection. *E. coli* O157:H7, *Salmonella* and *Shigella* were propagated

in tryptic soy broth supplemented with 5% yeast extract (TSBY). *L. monocytogenes* was grown in brain heart infusion (BHI) broth, and *H. pylori* in Brucella broth supplemented with 5% fetal bovine serum. All pathogenic cultures, except for *H. pylori*, were aerobically incubated at 37^{o}C. *H. pylori* was incubated at 37^{o}C under microaerophilic conditions using CampyPak™ gas generators. All probiotic cultures were propagated in Lactobacilli MRS agar and incubated anaerobically at 37^{o}C. All microbial media and reagents were purchased from Difco Labs. (Benton, Dickinson & Co, Sparks, MD, U.S.A.)

3.4 Preliminary studies

All the foodborne pathogens were grown at their respective incubation conditions overnight with an initial concentration of each pathogen being 10^{8}-10^{9} CFU/mL. Respective agar media plates were prepared and diluted samples of each pathogen were pour-plated using a varied concentration of wine ranging from 10-100% v/v, and incubated at their respective temperatures. Upon incubation, the different plates were examined for the survival rates of each pathogen in the presence of different wine concentrations. This preliminary step allowed us to narrow down the wine concentration that will be used for the following agar diffusion and plate count techniques. Concentrations ranging from 10-40% wine (v/v) gave too many colonies to count (TNTC) whereas the wine concentrations from 40-80% gave us countable plates. Wine concentrations of 100% killed all bacteria. This study, therefore, utilized the wine concentration range of 40-100% (v/v) for the following experiments to check for the effects of red wine against the foodborne pathogens.

The same procedure was followed for the growth of probiotics and its effects against red wine and grape juice. The initial number of each probiotic was 10^7 CFU/mL.

3.5 Agar diffusion assay

An agar well diffusion assay was performed to determine if the organisms were susceptible to different beverages. Freshly grown cells of each pathogen to be tested, except for *L. monocytogenes* and *H. pylori*, were spread-plated onto tryptic soy agar (TSA). BHI agar was used for *L. monocytogenes*, and blood agar for *H. pylori*. Wells were aseptically cut out of each agar plate with a sterile metal cork borer of 8 mm diameter. The five wells in each plate were filled with 100 µl of different percentages of each red wine and grape juice to be tested (middle well was filled with sterile peptone buffer as the control). Percentages ranging from 10% - 80% (v/v) of the beverages in peptone water were tested. The plates were accordingly incubated at 37°C and observed after 24 h. The diameter of inhibition zones surrounding each well was measured. The experiment was repeated. Concentrations of the selected wines and grape juices that gave the most inhibitory effects on the tested pathogens were analyzed as described below.

3.6 Minimum inhibitory and lethal concentrations

The minimum inhibitory concentration (MIC) of each selected wine was considered as the maximum dilution that inhibited the growth of a microorganism tested, but not necessarily kills the organism. The minimum lethal concentration (MLC) is defined as the maximum dilution (minimum concentration) that killed the test organism. MIC/MLC values were determined by the tube dilution and agar dilution methods. The different wines and grape juices were added to broth media specific for each pathogen, as

outlined above, to final concentrations in the range of 10% to 100% (v/v), with a final volume of 10 ml per tube. Controls were broth media with no wine or grape juice added. Each tube was inoculated with 20 µl of pathogenic or probiotic strain, incubated accordingly at 37°C and scored for growth by streaking on appropriate agar plates and pour-plating in agar media. The MIC was determined as the lowest concentration of red wine or grape juice that inhibited growth in the tube but not completely on the agar plate, while the MLC was determined as the minimum concentration that completely inhibited growth in the tube as well as on the plate. All experiments were replicated three times.

3.7 Total polyphenol activity

The wine samples were dried under nitrogen in the dark and the dried residue rehydrated and used to treat all the test bacteria as described above. The dried wine samples were rehydrated with the mobile phase (acetonitrile) and respective broth media were added to it to make the total volume of 4mL. Each tube was inoculated with 10 µl of pathogenic or probiotic strain, incubated accordingly at 37°C and scored for growth by streaking on appropriate agar plates and pour-plating in agar media.

3.8 Microscopic evaluation of cells after exposure to wine

One pathogen and one probiotic were tested in this step to examine the effect of wine on these microorganisms. A Fisher Micromaster microscope (Fisher Scienctific, NJ, U.S.A.) connected to a computer with Micron Software (Westover Scientific, Seattle, WA, U.S.A.) installed was used to make digital images of the slides. A 40% wine concentration (Merlot 2 which had the highest resveratrol concentration) was prepared and inoculated with 20 µL *E. coli* O157:H7 and a silimar approach was taken to prepare

another 40% wine concentration with 20 µL *B. animalis*, and incubated at 35°C aerobically and 37°C anaerobically respectively. After incubation, a gram stain of each was prepared and the slide was examined. Gram stain controls were also made for comparison.

3.9 Statistical analysis

Results of microbiological tests were transformed into log values plus one count and all data were analyzed as a Randomized Complete Block design. Differences were determined using Fisher's Protected Least Significant Difference (LSD) with $P \leq 0.05$.

CHAPTER 4

RESULTS

4.1 Chemical and HPLC analyses

Table 4.1 shows the pH, TA, alcohol content and *trans*-resveratrol content of each wine and grape juice tested. The pH of the wines and grape juices ranged from 2.5 – 3.7, which demonstrated their acidic property. The alcohol content of the wines, as stated on each bottle label, ranged from 10-14%, and their TA values closely resembled one another. The effects of the HPLC conditions and sample preparation procedures were evaluated to develop a rapid, specific and precise method for measuring *trans*-resveratrol in different wines and grape juices. According to Juan and others (1999), methanol gave the best recovery and resulted in the highest chromatographic resolution for *trans*-resveratrol determination. Hence, methanol was used as the solvent for standard solutions and wine sample preparation.

Precision. The precision, expressed as the relative standard deviation (% RSD) was 3.65%.

Linearity. The calibration obtained was linear with a correlation coefficient of 0.9998.

Selectivity. The *trans*-resveratrol was well resolved and free from interfering peaks (data not shown).

Recovery. The recovery percentage was deduced and it ranged from 91.43 – 102.24% with an average recovery of 96.83%.

4.1.1 *Trans*-resveratrol concentrations

The amounts of *trans*-resveratrol varied considerably for different vintages and varieties of the beverages, ranging from none detected in Tropicana grape juice (<0.1) to 3.5384 µg/mL in Merlot 2 (Table 4.1). The drier red wines, namely Pinot Noir, Shiraz, Tempranillo 2004 and Grenache Shiraz, the higher *t*-resveratrol content compared to their sweeter red wine counterparts, Merlot 1, Blush and Cherry table wines. This can be attributed to the fact that the drier the red wine, the higher the flavonoid (polyphenol) boost (Full of Health, 2008). Figure 4.1 shows the chromatogram of some of the selected red wines in the study and indicates the varied *t*-resveratrol content in each of them.

Table 4.1 pH, titratable acidity, and alcohol and t-resveratrol contents of red wine and grape juice.

Red wine	**Variety**	**pH**	**Titratable acidity (%)**	***t*-Resveratrol (μg/mL)**	**Alcohol (%)**
Merlot 1	Merlot	3.62 ± 0.025	6.375 ± 0.041	0.2724 ± 0.017	13
Merlot 2	Merlot	3.48 ± 0.03	5.475 ± 0.19	3.5384 ± 0.078	13
Shiraz	Shiraz	3.63 ± 0.072	5.925 ± 0.239	2.2745 ± 0.014	13.5
Cabernet Sauvignon	Cabernet Sauvignon	3.77 ± 0.0529	5.775 ± 0.040	0.2781 ± 0.007	13
Pinot Noir	Pinot Noir	3.58 ± 0.025	6.60 ± 0.321	2.2745 ± 0.014	12
Tempranillo 2004	Tempranillo	3.52 ± 0.152	6.225 ± 0.098	1.0683 ± 0.0049	14
Grenache Shiraz	Grenache 60% & Shiraz 40%	3.46 ± 0.052	6.525 ± 0.098	1.8632 ± 0.072	13
Chambourcin	Chambourcin	3.36 ± 0.06	7.125 ± 0.125	0.3979 ± 0.0260	14

Zinfandel & Carignane	Zinfandel & Carignane	3.72 ± 0.02	6.075 ± 0.230	0.5136 ± 0.004	13.5
Cherry wine	Cherry wine	3.58 ± 0.01	13.125 ± 0.064	0.1275 ± 0.0265	12
Hybrid and American	Hybrid and American blend	2.33 ± 0.026	6.675 ± 0.117	0.1373 ± 0.001	12
Red Zinfandel	Red Zinfandel	2.86 ± 0.026	6.225 ± 0.055	0.4949 ± 0.007	13.9
Blush Zinfandel	Blush Zinfandel	3.25 ± 0.015	7.875 ± 0.105	0.1826 ± 0.002	9
White Zinfandel	White Zinfandel	2.31 ± 0.015	8.550 ± 0.125	0.0770 ± 0.007	9
Tropicana Grape juice	-	2.49 ± 0.075	6.075 ± 0.072	<0.1	-
Welch's 100% grape juice	-	2.62 ± 0.113	6.150 ± 0.115	0.0932 ± 0.016	-

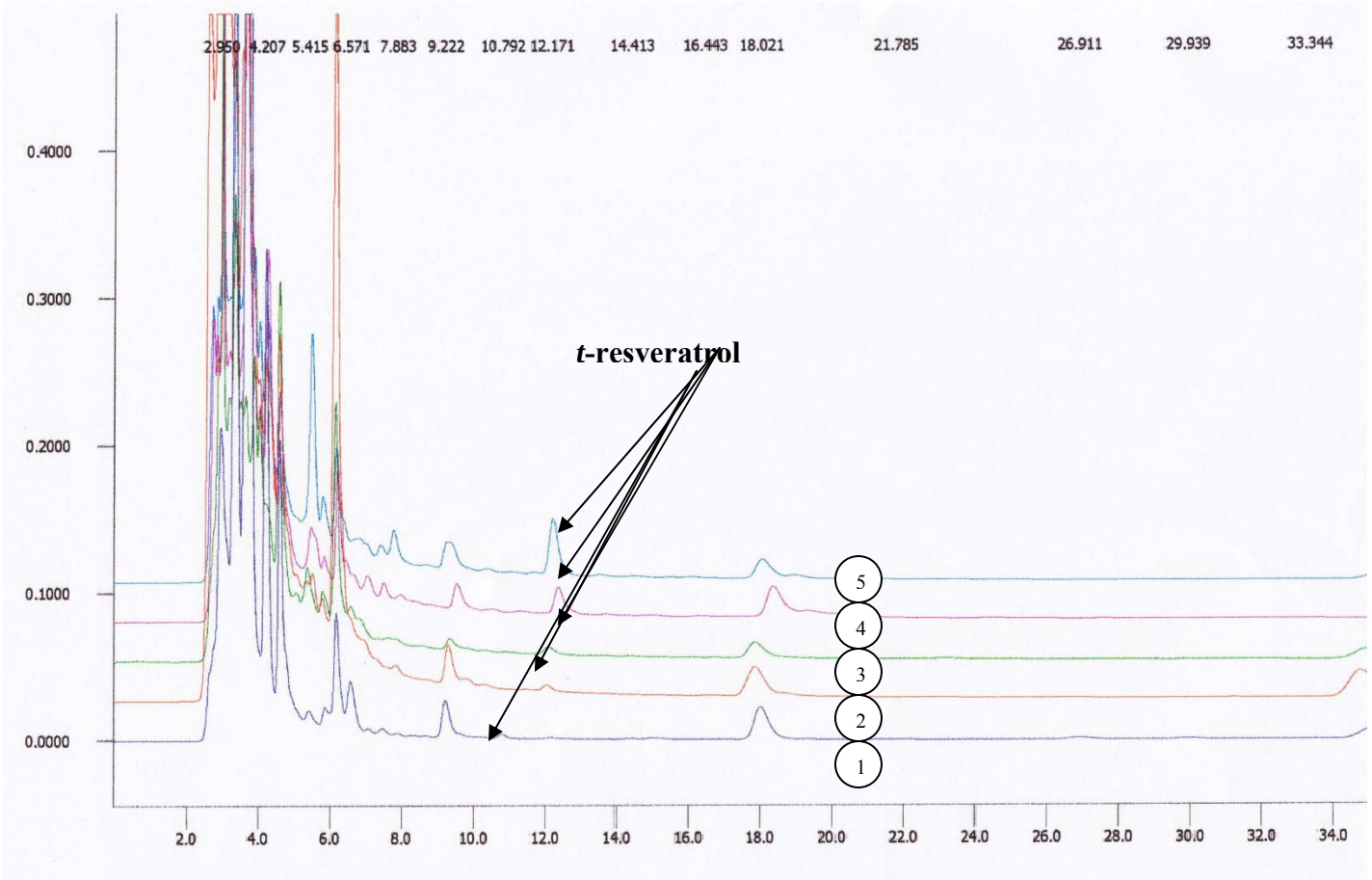


Figure 4.1 **Resveratrol content in Merlot 2 (5), Chambourcin (4), Zinfandel & Carignane (3) Grenache Shiraz (2), and Cherry wine (1).**

4.2 Inhibition pattern of red wines against pathogens and probiotics

The red wines, Merlot 1 and Merlot 2, Pinot Noir, Shiraz, Cabernet Sauvignon and Zinfandel, at 20%-50% concentrations (v/v), were tested using the well diffusion test to determine minimum inhibitory concentrations against pathogens and probiotics. The concentration of wine that conferred inhibition to *Salmonella* Typhimurium, *Listeria monocytogenes*, *and Escherichia coli* O157:H7 was found to be 40-50%. *Helicobacter pylori* and *Shigella boydii* grew slightly at 50% wine concentration. The red wines were then screened against the pathogens using the tube dilution test where growth of the pathogens was inhibited by 40-80% wine concentrations. All the pathogens were completely inhibited by 80% of each red wine, except Blush Zinfandel and Cherry wine. Cabernet, Merlot 2 and Pinot Noir at 80% resulted in a 1 mm diameter zone of inhibition of *H. pylori*, *E. coli* O157:H7, *S.* Typhimurium and *S. boydii*, whereas Blush Zinfandel and Cherry wine showed no inhibition for any of these pathogens. *H. pylori* was the most susceptible among all the pathogens tested, followed by *E. coli* O157:H7, *S.* Typhimurium, *S. boydii* and *L. monocytogenes* (0.6-1.2 mm diameter) (Table 4.2). Cabernet and Merlot 2 were the most potent while Blush Zinfandel was the least bactericidal even though the pH of the former was 3.8 and 3.6, respectively, while that of the latter was 3.3. In contrast, all the probiotic bacteria tested grew in the presence of 40-80% wine concentrations (Table 4.2) without any discernible zone of inhibition around each well, indicating a higher tolerance to red wine than the foodborne pathogens tested. The results of this study show that certain red wines do have antimicrobial properties against foodborne pathogens while not detrimentally affecting health beneficial probiotic

bacteria. The low inhibitory pattern of Blush Zinfandel and Cherry wine can be explained again by the fact that these two wines had very low levels of *t*-resveratrol (Table 4.1), indicating that alcohol in itself was not the only antibacterial agent because these two wines had relatively high alcohol contents of 9% and 12%, respectively.

Table 4.2[1] Inhibition pattern of red wines against pathogens[2] and probiotics[3].

Red Wine (40% v/v)	ST	EC	LM	SB	HP	Probiotics
Merlot	++	++	+	++	++	-
Pinot Noir	++	++	+	++	++	-
Blush Zinfandel	-	-	-	-	-	-
Cabernet	++	+	+	++	++	-
Shiraz	++	++	+	++	++	-
Tempranillo 2004	+	+	+	+	+	-
Grenache Shiraz	+	+	+	++	++	-
Chambourcin	++	++	+	++	++	-
Merlot 2	++	++	+	+	++	-
Zinfandel & Carignane	+	+	+	+	+	-
Cherry wine	-	-	-	-	-	-

[1] - = no inhibition; + inhibition = <0.6 mm; ++ inhibition = 0.7-1.0 mm.

[2] Pathogens tested were: ST = *S.* Typhimurium; EC = *E. coli* O157:H7; LM = *L. monocytogenes*; SB = *S. boydii*; HP = *H. pylori*

[3] Probiotics tested included *B. longum*, *L. acidophilus*, *L. paracasei* and *L. rhamnosus*.

4.3 Antibacterial activity of red wine

4.3.1 Effects against pathogens

All the red wines tested were effective against the pathogens at the lowest concentration of 40%. Higher concentrations of these wines completely killed the pathogens. Figure 4.2a shows the antibacterial effect of 40% v/v red wines against pathogens. Upon treatment with 40% v/v red wines, the numbers of each pathogen decreased from an initial concentration of 10^8-10^9 CFU/mL to 10^4-10^5 CFU/mL. Chambourcin and Pinot Noir were the most antibacterial against the pathogens tested, with final counts of 10^3 CFU/mL compared to the remaining wines, namely Cabernet, Merlot 1 and Zinfandel & Carignane 2000 (Figure 4.2a). The inhibitory activity of Cabernet against *E. coli* O157:H7 and *S. boydii* was similar but significantly different ($P \leq 0.05$) against *S.* Typhimurium, *L. monocytogenes* and *H. pylori*. The 5 log reduction of the former four pathogens, when treated with Pinot Noir, was significantly lower than that of *H. pylori* (Figure 4.2a) which was reduced by 3 log CFU. At 40% v/v concentration, Chambourcin completely killed *S.* Typhimurium and *H. pylori* as can be discerned from the zero count. The bactericidal pattern for *E. coli* O157:H7 and *H. pylori* were not significantly different from each other when treated with the different red wines, except for Pinot Noir, Zinfandel & Carignane 2000 and Chambourcin. There was no significant difference in inhibition ($P \geq 0.05$) among *S. boydii*, *S.* Typhimurium and *L. monocytogenes* as seen with Cabernet and Merlot 1 in Fig 4.2a. Shiraz, Merlot 2 and Tempranillo are drier red wines that had lower counts compared to Blush Zinfandel and Cherry wine, which may explain why their inhibition activity significantly differed from the former drier red wines. There was a 5 log reduction of the former three wines against

all the pathogens tested compared to a 3-4 log reduction by Cherry and Blush Zinfandel (Figure 4.2b). This also demonstrates the fact that drier the red wine, the greater the flavonoid boost and the greater the bactericidal capability. Overall, the inhibitory action of Merlot 2, Chambourcin, and Pinot Noir were large compared to that of Cabernet, Merlot 1, Blush Zinfandel and Cherry wine. Shiraz, Tempranillo 2004 and Zinfandel & Carignane 2000 showed intermediate potential. These results can be attributed, in part, to the low levels of polyphenols present in Cabernet, Merlot 1, Blush Zinfandel and Cherry wine. The bactericidal pattern of all the pathogens tested was not significantly different from each other when treated with Cabernet, Merlot 1, Blush Zinfandel and Cherry wine ($P \geq 0.05$). The antibacterial activity of Shiraz, Tempranillo 2004 and Zinfandel & Carignane 2000 also showed no discernible difference among the pathogens tested. The tested pathogens were highly sensitive to Pinot Noir and Chambourcin, as can be detected from Fig 4.2a. This fact can be largely due to the high levels of polyphenols present in these two dry red wines. However the inhibitory pattern of *H.pylori* when treated with Pinot Noir is unclear. White Zinfandel used in the experiment, as a control was not effective in reducing the counts of the pathogens tested.

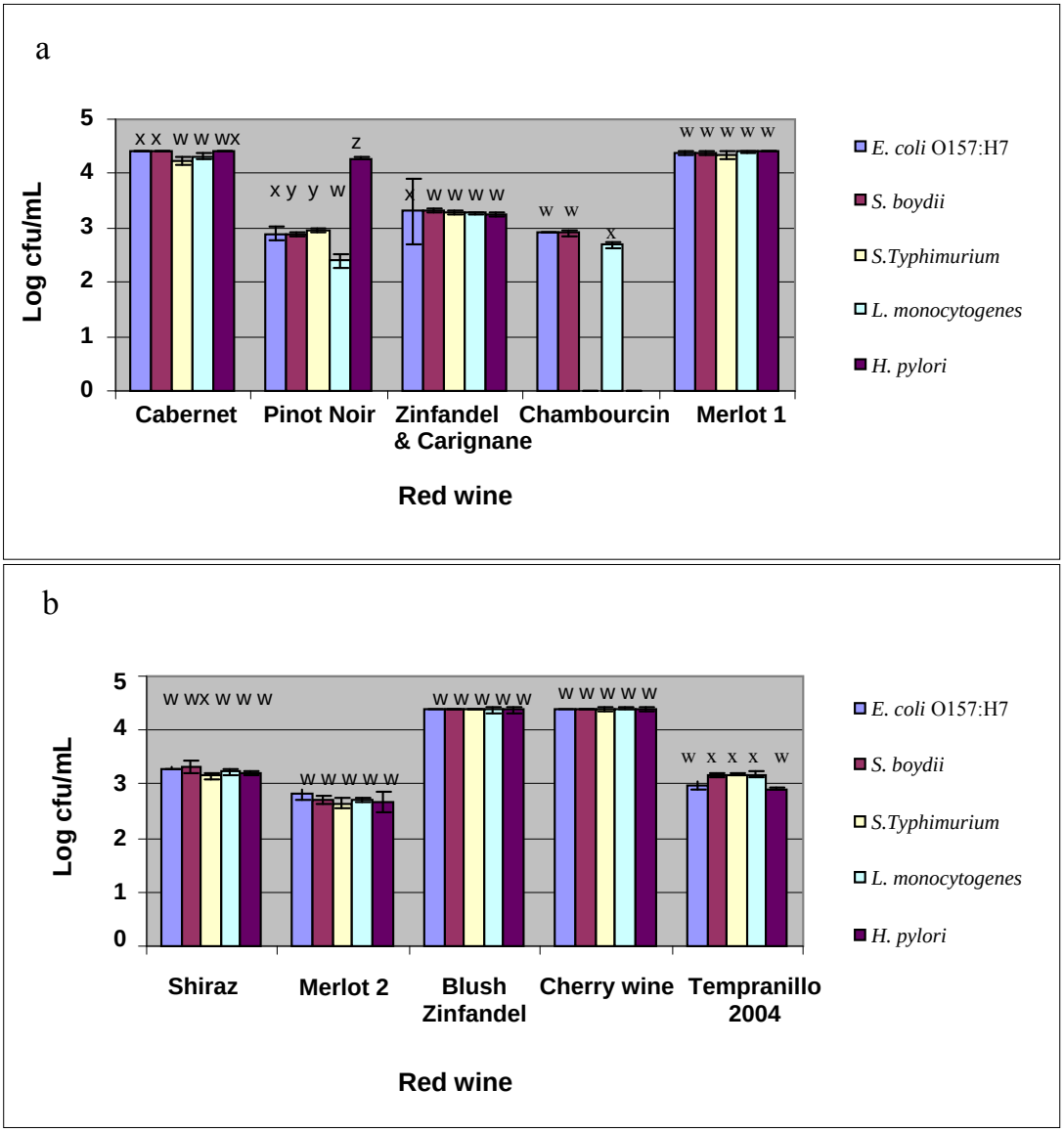
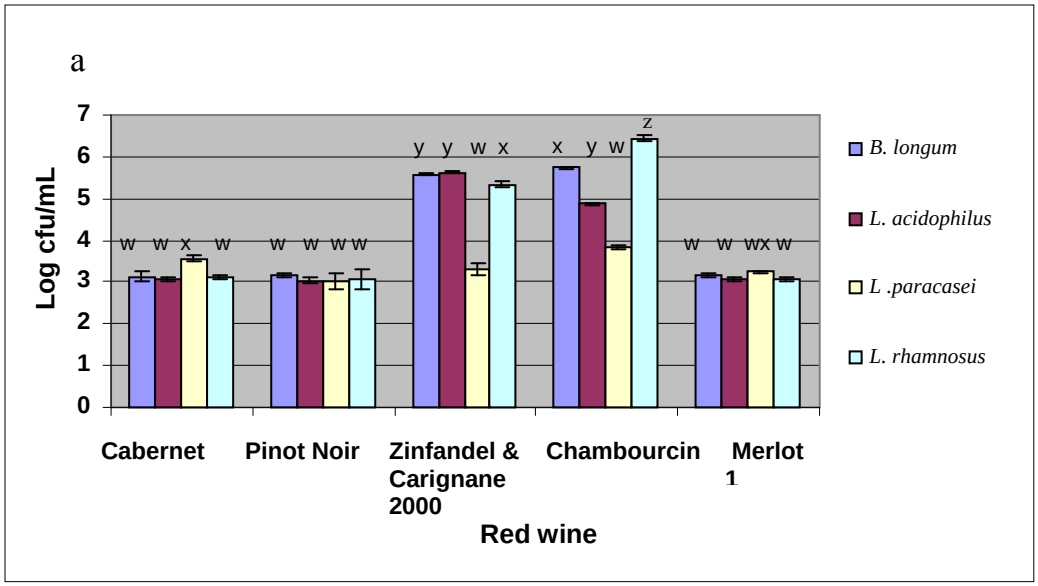


Figure 4.2 **Antibacterial activity of red wines at 40% concentration against foodborne pathogens. The initial numbers of each pathogen (control) were 10^8-10^9 CFU/mL. Means with the same letters are not significantly different at $P \leq 0.05$.**

4.3.2 Effects against probiotics

All four probiotic strains tested survived exposure of up to 80% of each red wine, even though the decrease in their numbers was prominent from the initial 10^7 CFU/mL (Figure 4.3a, b). *L. acidophilus* even survived in the presence of 100% Pinot Noir, albeit the number was reduced by 6 log CFU/mL. The inhibitory action of Cabernet, Pinot Noir and Merlot 1 were generally similar against the four probiotics (Figure 4.2c). *B. longum* and *L. rhamnosus* were more resistant than the other two lactobacilli against Chambourcin and Zinfandel & Carignane 2000. *L. acidophilus*, among the probiotics tested, was most resistant to Shiraz, followed by Zinfandel & Carignane 2000, Merlot 2 and Blush Zinfandel. Overall, *L. paracasei* appeared to be the least resistant to the red wines among all the probiotics tested and Cherry wine and Blush Zinfandel were the least bactericidal wines tested against the probiotics as were the case with the pathogens (Figure 4.3b). The bactericidal pattern of the probiotics tested was not significantly differently from each other when treated with Cabernet, Pinot Noir and Merlot 1 ($P \geq 0.05$).



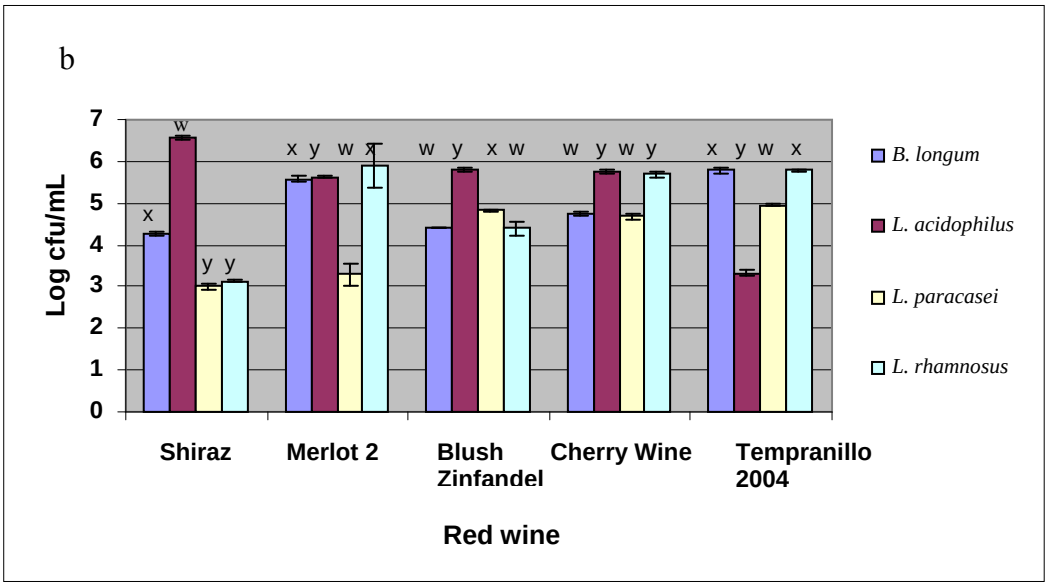


Figure 4.3 **Antibacterial activity of 80% red wines against probiotics. The initial number of each probiotic (control) was 10^7 CFU/mL. Means with the same letters are not significantly different at $P \leq 0.05$.**

4.4 Antibacterial activity of grape juice

4.4.1. Effects against pathogens and probiotics

As shown in Figure 4.4a, the pathogens were inhibited by up to 50-60% purple grape juice. Concentrations higher than these totally killed all the pathogens and probiotics tested. This indicated that the alcohol present in red wines was not the only factor involved in their bactericidal effect. All the pathogens tested survived in the presence of 80% white grape juice with a reduction of 3 log CFU/mL in their numbers. Inhibition by 50% grape juices was similar among *E. coli* O157:H7, *S. boydii*, *S.* Typhimurium and *L. monocytogenes*, but significantly different from that of *H. pylori* for Tropicana and Welch's white. Tropicana purple and Welch's white grape juice did not have much of an inhibitory effect on *H. pylori* (Fig 4.4a). The probiotics were inhibited by 50% purple grape juice with a reduction of 1-4 log CFU/mL in numbers (Figure 4.4b) However there appears to be a discernible rapid inhibitory effect of Tropicana purple grape juice against the probiotics (Fig 4.4b) with *L. rhamnosus* being the most significantly sensitive among the probiotics to all the grape juices tested ($P \leq 0.05$).

4.5 Total polyphenol activity

The dried residues when treated with the different pathogens showed zero counts on the respective agar plates denoting the complete inhibition of the pathogens.

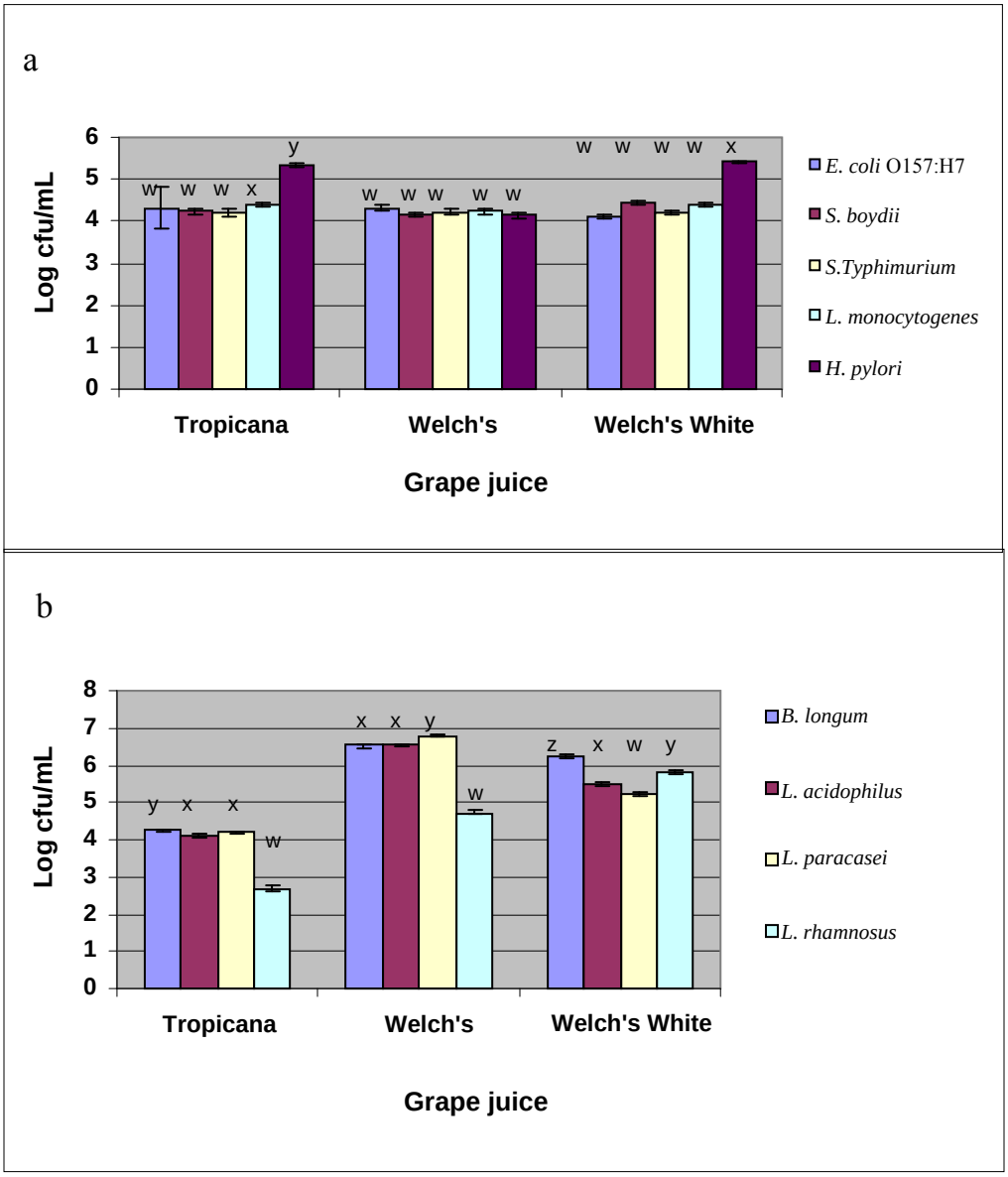


Figure 4.4 **Antibacterial activity of 50% grape juice against pathogens and probiotics. The initial number of each pathogen (control) was 10^8-10^9 CFU/mL and each probiotic was 10^7 CFU/mL. Means with the same letters are not significantly different at $P \leq 0.05$.**

4.6 Microscopic examination

Images of the treated and untreated *B. animalis* and treated and untreated *E. coli* are shown in Figure 4.5. Comparison of the images of the control and treated bacteria demonstrated more cell debris in the treated images, which is indicative of cell death. However, the images of the treated probiotic and treated pathogen differed in the amount of debris. This is believed to be due to the probiotics' higher tolerance to wine.

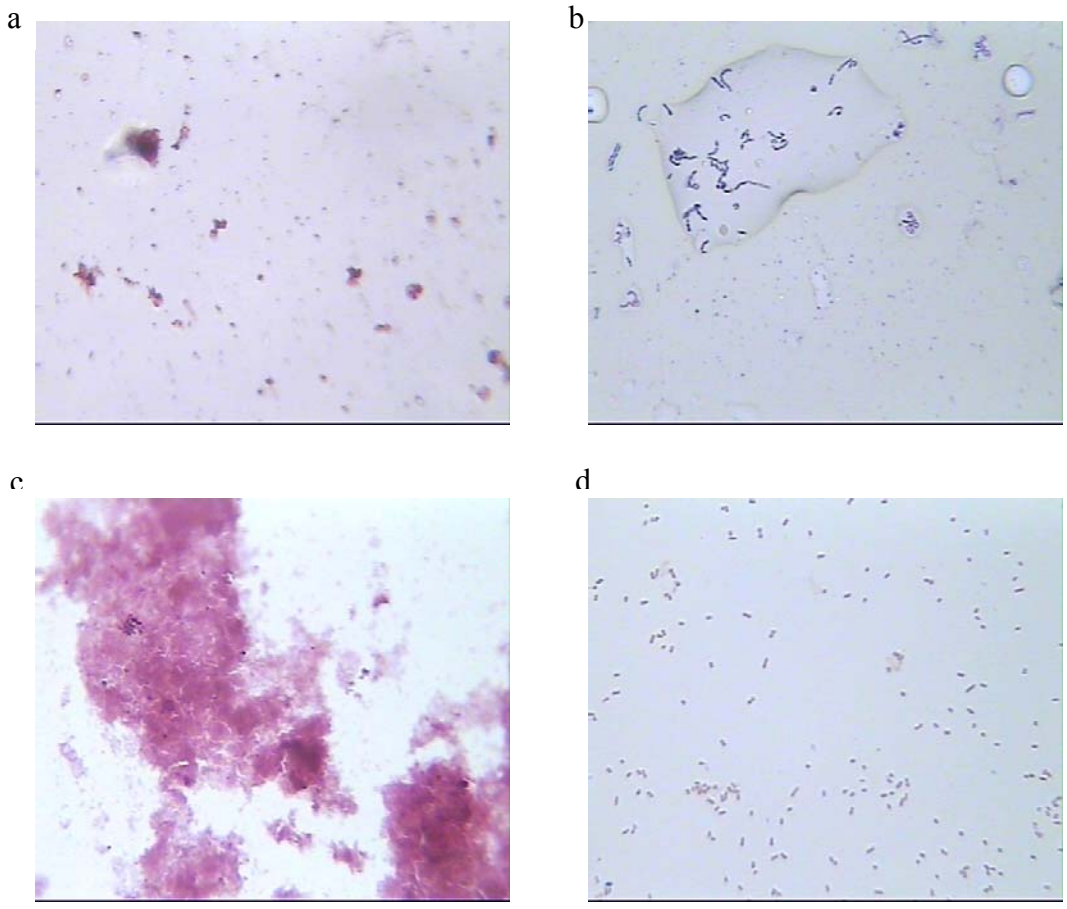


Figure 4.5 **Microscopic images of *B. animalis* treated with 40% Merlot 2 (a), untreated *B. animalis* control, *E. coli* O157:H7 treated with 40% Merlot 2 (c) and untreated *E. coli* O157:H7 control (d).**

CHAPTER 5

DISCUSSION

Numerous studies have been conducted that demonstrate the antibacterial property of wine against a notable amount of relevant food-borne pathogenic bacteria (Fernandes and others 2007; Moretro and Daeschel 2004; Sugita-Konishi and others 2001; Weisse and others 1995). Studies incorporating wine as a food additive in the form of marinades and other similar treatments provide further evidence of its protective role (Friedman and others 2006). Various *in vitro* studies indicated that the potency of wine as an antibacterial agent was higher than a given ethanol concentration and was, in fact, due to a combination of ethanol and organic acids (tartaric, malic, lactic and acetic) (Just and Daeschel 2003; Weisse and others 1995).

This study demonstrated that red wine could reduce the number of food-borne pathogens effectively and also showed its effect on probiotics. Just and Daeschel (2003) showed similar results when they treated *E. coli* O157:H7 and *Salmonella* spp. with Chardonnay and Pinot Noir. Weisse and others (1995) significantly reduced the number of *E. coli* O157:H7, *Salmonella* serotype Typhimurium and *Shigella sonnei* using red and white wine from 10^6 CFU units/mL to undetectable concentrations within 20 min of exposure. Our results reaffirmed these results as 40% v/v red wine concentration was sufficient to lower the pathogen numbers from 10^9 to 10^4 CFU units/mL. Moretro and Daeschel (2004) detected that red wine was the most potent against all foodborne pathogens tested compared to white wines without added sulfites. Typically, a 3-6 log reduction in viable cell counts is expected after 10 min of exposure. Another study indicated that red wine led to a 4-5 fold reduction of the initial population of *Listeria*

innocua in a model stomach system (Fernandes and others 2007). Our study found that red wine inhibited *L. monocytogenes*, widening the scope of red wine as an antilisterial agent. Many have investigated the impact of phenolic compounds on *H. pylori* inhibition. Mahady and Pendland (2000) and Mahady and others (2003) determined the MIC 50 value of 12.5 μg/mL of resveratrol against *H. pylori* strains using an agar disk diffusion assay, while Chan (2002) determined the MIC of resveratrol against *S. aureus*, *Enterococcus faecalis* and *Pseudomonas aeruginosa* to be 171 to 342 μg/mL by a broth dilution assay. Murray and others (2002) indicated that modest consumption of wine and beer protects against *H. plori* infection by facilitating the eradication of the organism. Additionally, Friedman and others (2006) developed wine formulations containing plant essential oils and oil compounds that were significantly effective against *E. coli* O157:H7 and *Salmonella enterica*. According to Waite and Daeschel (2007), *S. aureus* was more resistant to wine treatment than was *E. coli* O157:H7. Our experiments demonstrated different inhibition patterns for the pathogens tested against different red wines. All five species showed similar susceptibility to red wine. Another reason for not being able to detect the most tolerant and sensitive pathogen in the study can also be attributed to the fact that wine effectiveness in the inactivation of bacteria is strongly dependant on the specific wine composition as reaffirmed by Fernandes and others (2007).

White and Blush wines tested in our study did not significantly inhibit the pathogens tested. This was in contrast to the findings of Weisse and others (1995) that demonstrated that the dilutions of white wine reduced the number of the test organisms more rapidly than the red wine dilutions. However, Lin and others (2005) enhanced the antioxidant activity and inhibition rate of *H. pylori* by phenolic phytochemical-enriched

alcoholic beverages, in particular wine and vodka. Their study indicated that peppermint-enriched white wine was the most potent, followed by cinnamon-enriched white wine which had the second highest antimicrobial activity compared to other phytochemical-enriched wines namely, green tea- and raspberry-enriched white wines. Fernandes and others (2007) also tested white and red wines and found a difference in their inactivation kinetics.

Probiotics, which are considered the good bacteria, were also exposed to red wine and grape juice in this study. Our work is the first to report the antibacterial effects of wine and grape juice against both foodborne pathogens and probiotics. Probiotics were observed to survive at higher concentrations of red wine. However, the cause of this difference is unclear. Physiological characteristics of ten palm-wine yeast isolates obtained from nine localities in four provinces in southern Cameroon were assessed using sensitivity to chloramphenicol, tolerance to acetic acid, ethanol tolerance, osmotolerance, as well as protein and alcohol dehydrogenase (ADH) polymorphism. None of these isolates was sensitive to 30 μg/ml chloramphenicol, and all were non-tolerant to 1% acetic acid. Results indicated that 80% of the strains were tolerant to ethanol and were able to grow in 15% alcohol solution (Beechem and others 2007). A study showed that *Pediococcus halophilus* and *Leuconostoc paramesenteroides* could grow in 10% NaCl added broth medium (Ohhira and others 1988).

Grape seeds are rich in monomeric phenolic compounds, like catechins and epicatechins, and have been reported to have numerous antiviral and antimutagenic properties by various investigators (Jayaprakasha and others 2003; Saito and others 1998). Jayaprakasha and others (2003) reported that all the grape seed extracts were

antibacterial against *Bacillus spp.*, *S. aureus*, *E. coli* and *Pseudomonas aeruginosa.* Rhodes and others (2006) investigated the antilisterial activity of grape juice and grape extracts derived from *V. vinifera* variety Ribier and also documented that grape juice was inactive against *Bacillus cereus*, *Salmonella* Menston, *E. coli* and *S. aureus.* Our study reported that purple grape juice was actively inhibitory against pathogens and probiotics. According to Weisse and others (1995), it is neither the alcohol in wine nor the pH alone that makes it bactericidal, as 10% ethanol was sparsely inhibitory compared with the control. Fernandes and others (2007) also demonstrated that the combination of the acids with ethanol showed a higher antibacterial effect than the mixture of acids and ethanol as separate entities. They suggested that the vital antimicrobial agent in wine seemed to be the polyphenols liberated during wine fermentation. We reaffirmed this fact by conducting the total polyphenolic activity study where the alcohol in the wine was completely purged with liquid nitrogen and used to treat the pathogens. This resulted in complete inhibition of the pathogens. However, an opposite pattern was discerned for the probiotics, which could withstand the total polyphenolic concentrations. Thus, from our study, we concluded that the inhibitory effects can be attributed not just to the alcohol, but the polyphenols, including *t*-resveratrol, along with the pH and acidity that synergistically resulted in the bactericidal property of red wines against the foodborne pathogens tested. The understanding behind the phenomenon that probiotics were more resistant to treatment with wine entails more molecular and quantitative studies that were beyond the scope of our experimental design.

Additional components in wine have been documented to possess antibacterial properties in various literatures. Soleas and others (1997) quantified the levels of

phenolic compounds (*trans* and *cis*- resveratrol, gallic acid, ferulic acid, caffeic acid, *p*-coumaric acid, vanillic acid and gentisic acid) in different red and white wines. According to Moretro and Daeschel (2004), red wine contains a much higher level of *t*-resveratrol compared to white wine. Our data confirm this fact, and also documented the fact that drier red wines with higher flavonoid contents, is directly related to higher bactericidal property of the wine.

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

In conclusion, our work showed, via *in vitro* tests, the antimicrobial activity of specific red wines against various foodborne pathogens. This study also demonstrated that red wines did not drastically affect health beneficial probiotic cultures as they did pathogens. The probiotics could withstand red wines up to 80% v/v whereas the pathogens were completely killed at red wine concentrations of above 40% v/v.

According to Moretro and Daeschel (2004), red wine contains a much higher level of *t*-resveratrol compared to white wine. Our data confirmed this fact and also found that the drier the red wine, the higher the *t*-resveratrol concentration and, the higher the bactericidal property of the wine. Our study clearly showed that Blush and White Zinfandels and Cherry wines were not as potent as the other counterparts in bringing about bactericidal properties against the pathogens. Since the percentage of alcohol was almost the same in all the red wines tested, Tukey's Studentized Range test showing the presence of interaction effects among the red wines and pathogens and probiotics, and among the grape juice with the pathogens and probiotics tested indicated that they were significantly different at $\alpha = 0.05$.

From our study, we also deduced that the inhibitory effects can be attributed not just to the alcohol, but the polyphenols, including *t*-resveratrol, along with the pH and acidity that synergistically resulted in the bactericidal property of red wines against the foodborne pathogens tested.

Additional studies on the understanding behind the phenomenon that probiotics were more resistant to treatment with wine should be conducted that entails more molecular and quantitative studies and will help us to fathom the difference in the inhibitory pattern between the foodborne pathogens and probiotics.

APPENDIX

SAS program for interactions between wine and pathogens

```
options linesize=100 pagesize=70;
data one; infile 'h:book1.csv' dsd firstobs=2 missover;
input path$ wine$ x1 x2 x3 x4 x5 x6;
proc print;
data two; set one;
 cnt=x1; rep=1; det=1; output;
 cnt=x2; rep=1; det=2; output;
 cnt=x3; rep=2; det=1; output;
 cnt=x4; rep=2; det=2; output;
 cnt=x5; rep=3; det=1; output;
 cnt=x6; rep=3; det=2; output;
data two; set two;
lcnt=log10(cnt+1);
proc print;
proc glm; class rep path wine;
model lcnt=rep path wine path*wine;
means path wine path*wine ;
lsmeans path wine path*wine/stderr pdiff;
run;

/*
proc glm; class rep path wine conc;
model lcnt=rep path wine conc path*wine path*conc wine*conc path*wine*conc;
means path wine path*wine path*conc wine*conc path*wine*conc;
lsmeans path wine path*wine path*conc wine*conc path*wine*conc/stderr pdiff;
  */
```

Table 1

Pathogen	Wine	lcnt LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
e.coli	**Barton Merlot**	2.81444142	0.04157710	<.0001	1
e.coli	**Cabernet**	4.39795738	0.04157710	<.0001	2
e.coli	**Campo Viego**	2.96575724	0.04157710	<.0001	3
e.coli	**Chambourcin**	0.00000000	0.04157710	1.0000	4
e.coli	**Cherry wine**	4.39795738	0.04157710	<.0001	5
e.coli	**Kenwood**	3.61258698	0.04157710	<.0001	6

Pathogen	Wine	lcnt LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
e.coli	**Merlot**	4.39312574	0.04157710	<.0001	7
e.coli	**Pinot Noir**	2.64249452	0.04157710	<.0001	8
e.coli	**Shiraz**	3.30033916	0.04157710	<.0001	9
e.coli	**Zinfandel**	4.39795738	0.04157710	<.0001	10
helico	**Barton Merlot**	2.68555929	0.04157710	<.0001	11
helico	**Cabernet**	4.39795738	0.04157710	<.0001	12
helico	**Campo Viego**	2.91235991	0.04157710	<.0001	13
helico	**Chambourcin**	-0.00000000	0.04157710	1.0000	14
helico	**Cherry wine**	4.38180644	0.04157710	<.0001	15
helico	**Kenwood**	3.24992852	0.04157710	<.0001	16
helico	**Merlot**	4.39795738	0.04157710	<.0001	17
helico	**Pinot Noir**	4.27661404	0.04157710	<.0001	18
helico	**Shiraz**	3.20163446	0.04157710	<.0001	19
helico	**Zinfandel**	4.37809389	0.04157710	<.0001	20
lis	**Barton Merlot**	2.70013175	0.04157710	<.0001	21
lis	**Cabernet**	4.31541333	0.04157710	<.0001	22
lis	**Campo Viego**	3.17497807	0.04157710	<.0001	23
lis	**Chambourcin**	2.68609919	0.04157710	<.0001	24
lis	**Cherry wine**	4.39000905	0.04157710	<.0001	25
lis	**Kenwood**	3.27719133	0.04157710	<.0001	26
lis	**Merlot**	4.40616007	0.04157710	<.0001	27
lis	**Pinot Noir**	2.39534293	0.04157710	<.0001	28
lis	**Shiraz**	3.23222760	0.04157710	<.0001	29
lis	**Zinfandel**	4.37847383	0.04157710	<.0001	30
sal	**Barton Merlot**	2.64164931	0.04157710	<.0001	31
sal	**Cabernet**	4.23075748	0.04157710	<.0001	32
sal	**Campo Viego**	3.17532906	0.04157710	<.0001	33
sal	**Chambourcin**	-0.00000000	0.04157710	1.0000	34
sal	**Cherry wine**	4.38870489	0.04157710	<.0001	35

Pathogen	Wine	lcnt LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
sal	**Kenwood**	3.28570009	0.04157710	<.0001	36
sal	**Merlot**	4.34140560	0.04157710	<.0001	37
sal	**Pinot Noir**	2.95593963	0.04157710	<.0001	38
sal	**Shiraz**	3.15224581	0.04157710	<.0001	39
sal	**Zinfandel**	4.39795738	0.04157710	<.0001	40
shig	**Barton Merlot**	2.70261707	0.04157710	<.0001	41
shig	**Cabernet**	4.39795738	0.04157710	<.0001	42
shig	**Campo Viego**	3.17319759	0.04157710	<.0001	43
shig	**Chambourcin**	2.90820170	0.04157710	<.0001	44
shig	**Cherry wine**	4.39795738	0.04157710	<.0001	45
shig	**Kenwood**	3.31207638	0.04157710	<.0001	46
shig	**Merlot**	4.38266978	0.04157710	<.0001	47
shig	**Pinot Noir**	2.90020151	0.04157710	<.0001	48
shig	**Shiraz**	3.30733039	0.04157710	<.0001	49
shig	**Zinfandel**	4.39795738	0.04157710	<.0001	50

SAS program for interaction between wine and probiotics

```
options linesize=100 pagesize=70;
data one; infile 'h:book1.csv' dsd firstobs=2 missover;
input pro$ wine$ x1 x2 x3 x4 x5 x6;
proc print;
data two; set one;
 cnt=x1; rep=1; det=1; output;
 cnt=x2; rep=1; det=2; output;
 cnt=x3; rep=2; det=1; output;
 cnt=x4; rep=2; det=2; output;
 cnt=x5; rep=3; det=1; output;
 cnt=x6; rep=3; det=2; output;
data two; set two;
lcnt=log10(cnt+1);
proc print;
proc glm; class rep pro wine;
model lcnt=rep pro wine pro*wine;
```

```
means pro wine pro*wine ;
lsmeans pro wine pro*wine/stderr pdiff;
run;

/*
proc glm; class rep pro wine conc;
model lcnt=rep pro wine conc pro*wine pro*conc wine*conc pro*wine*conc;
means pro wine pro*wine pro*conc wine*conc pro*wine*conc;
lsmeans pro wine pro*wine pro*conc wine*conc pro*wine*conc/stderr pdiff;
  */
```

Probiotics	Wine	lcnt LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
bifido	**Barton Merlot**	5.51073379	0.06091879	<.0001	1
bifido	**Cabernet**	3.25563151	0.06091879	<.0001	2
bifido	**Campo Viego**	5.69257763	0.06091879	<.0001	3
bifido	**Chambourcin**	5.69360530	0.06091879	<.0001	4
bifido	**Cherry wine**	4.68306407	0.06091879	<.0001	5
bifido	**Kenwood**	5.55143592	0.06091879	<.0001	6
bifido	**Merlot**	3.20700643	0.06091879	<.0001	7
bifido	**Pinot Noir**	3.20624843	0.06091879	<.0001	8
bifido	**Shiraz**	4.24485865	0.06091879	<.0001	9
bifido	**Zinfandel**	4.39795738	0.06091879	<.0001	10
l.acido	**Barton Merlot**	5.78448732	0.06091879	<.0001	11
l.acido	**Cabernet**	3.36444643	0.06091879	<.0001	12
l.acido	**Campo Viego**	5.97304616	0.06091879	<.0001	13
l.acido	**Chambourcin**	6.52422068	0.06091879	<.0001	14
l.acido	**Cherry wine**	4.80271088	0.06091879	<.0001	15
l.acido	**Kenwood**	5.62538223	0.06091879	<.0001	16
l.acido	**Merlot**	3.06893171	0.06091879	<.0001	17
l.acido	**Pinot Noir**	3.13862171	0.06091879	<.0001	18
l.acido	**Shiraz**	3.07018789	0.06091879	<.0001	19
l.acido	**Zinfandel**	5.77112368	0.06091879	<.0001	20
l.para	**Barton Merlot**	3.34531510	0.06091879	<.0001	21

Probiotics	Wine	lcnt LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
l.para	**Cabernet**	3.66142646	0.06091879	<.0001	22
l.para	**Campo Viego**	4.95763648	0.06091879	<.0001	23
l.para	**Chambourcin**	3.88656635	0.06091879	<.0001	24
l.para	**Cherry wine**	4.71252415	0.06091879	<.0001	25
l.para	**Kenwood**	3.46333629	0.06091879	<.0001	26
l.para	**Merlot**	3.25252882	0.06091879	<.0001	27
l.para	**Pinot Noir**	3.25941542	0.06091879	<.0001	28
l.para	**Shiraz**	3.13205632	0.06091879	<.0001	29
l.para	**Zinfandel**	4.78912897	0.06091879	<.0001	30
l.rham	**Barton Merlot**	5.47731647	0.06091879	<.0001	31
l.rham	**Cabernet**	3.14752843	0.06091879	<.0001	32
l.rham	**Campo Viego**	5.83464402	0.06091879	<.0001	33
l.rham	**Chambourcin**	6.47357916	0.06091879	<.0001	34
l.rham	**Cherry wine**	5.75225057	0.06091879	<.0001	35
l.rham	**Kenwood**	5.37743684	0.06091879	<.0001	36
l.rham	**Merlot**	3.13191520	0.06091879	<.0001	37
l.rham	**Pinot Noir**	3.13723354	0.06091879	<.0001	38
l.rham	**Shiraz**	3.16841207	0.06091879	<.0001	39
l.rham	**Zinfandel**	4.52424107	0.06091879	<.0001	40

SAS program for the interaction between pathogens and grape juice

```
PROC IMPORT OUT= WORK.one
        DATAFILE= "U:\Book1viticulture.xls"
        DBMS=EXCEL REPLACE;
    SHEET="Sheet4$";
    GETNAMES=YES;
    MIXED=NO;
    SCANTEXT=YES;
    USEDATE=YES;
    SCANTIME=YES;
RUN;
```

```
data two(drop=f3-f8); set one;
 cnt=f3; rep=1; det=1; output;
 cnt=f4; rep=1; det=2; output;
 cnt=f5; rep=2; det=1; output;
 cnt=f6; rep=2; det=2; output;
 cnt=f7; rep=3; det=1; output;
 cnt=f8; rep=3; det=2; output;
run;

data two;
set two;
combo=cats(pathogens,grape_juice);
lcnt=log10(cnt+1);
run;
proc univariate data=two;
var cnt;
histogram cnt/normal;
run;
proc univariate data=two;
class combo;
var cnt;
histogram cnt/normal;
run;

proc univariate data=two;
var lcnt;
histogram lcnt/normal;
run;
proc univariate data=two;
class combo;
var lcnt;
histogram lcnt/normal;
run;
ods rtf file='U:\pathogens_grape.doc';
proc glm; class rep pathogens grape_juice;
model lcnt=rep pathogens grape_juice pathogens*grape_juice;
means pathogens grape_juice pathogens*grape_juice /tukey lines;
lsmeans pathogens grape_juice pathogens*grape_juice/stderr pdiff;
run;
quit;
ods rtf close;
ods rtf file='U:\pathogengrape_combo.doc';
proc glm; class rep combo;
model lcnt=rep combo;
```

```
means combo /tukey lines;
lsmeans combo/stderr pdiff;
run;
quit;
```

SAS program for interaction between probiotics and grape juice

```
PROC IMPORT OUT= WORK.one
            DATAFILE= "U:\Book1viticulture.xls"
            DBMS=EXCEL REPLACE;
     SHEET="Sheet4$";
     GETNAMES=YES;
     MIXED=NO;
     SCANTEXT=YES;
     USEDATE=YES;
     SCANTIME=YES;
RUN;

data two(drop=f3-f8); set one;
 cnt=f3; rep=1; det=1; output;
 cnt=f4; rep=1; det=2; output;
 cnt=f5; rep=2; det=1; output;
 cnt=f6; rep=2; det=2; output;
 cnt=f7; rep=3; det=1; output;
 cnt=f8; rep=3; det=2; output;
run;

data two;
set two;
combo=cats(pro,grape_juice);
lcnt=log10(cnt+1);
run;
proc univariate data=two;
var cnt;
histogram cnt/normal;
run;
proc univariate data=two;
class combo;
var cnt;
histogram cnt/normal;
run;
```

```
proc univariate data=two;
var lcnt;
histogram lcnt/normal;
run;
proc univariate data=two;
class combo;
var lcnt;
histogram lcnt/normal;
run;
ods rtf file='U:\pro_grape.doc';
proc glm; class rep pro grape_juice;
model lcnt=rep pro grape_juice pro*grape_juice;
means pro grape_juice pro*grape_juice /tukey lines;
lsmeans pro grape_juice pro*grape_juice/stderr pdiff;
run;
quit;
ods rtf close;
ods rtf file='U:\prograpе_combo.doc';
proc glm; class rep combo;
model lcnt=rep combo;
means combo /tukey lines;
lsmeans combo/stderr pdiff;
run;
quit;
```

Table 4 Least Square Means for the combination effect of pathogen* wine with the LSD = 0.116.

	E. coli O157:H7	*S. boydii*	*S.* Typhimurium	*L. monocytogenes*	*H. pylori*
Cabernet Sauvignon	$_{x}$ 4.397 ^{d}	$_{x}$ 4.397 ^{c}	$_{w}$ 4.230 ^{c}	$_{w}$ 4.315 ^{d}	$_{wx}$ 4.397 ^{c}
Pinot Noir	$_{x}$ 2.642 ^{a}	$_{y}$ 2.900 ^{a}	$_{y}$ 2.955 ^{a}	$_{w}$ 2.395 ^{a}	$_{z}$ 4.276 ^{b}
(Kenwood)Zinfandel & Carignane 2000	$_{x}$ 3.612 ^{c}	$_{w}$ 3.312 ^{b}	$_{w}$ 3.285 ^{b}	$_{w}$ 3.277 ^{c}	$_{w}$ 3.249 ^{a}
Chambourcin	$_{w}$ 2.911 ^{b}	$_{w}$ 2.908 ^{a}	0.000	$_{x}$ 2.686 ^{b}	0.000
Merlot 1	$_{w}$ 4.393 ^{d}	$_{w}$ 4.382 ^{c}	$_{w}$ 4.341 ^{c}	$_{w}$ 4.406 ^{d}	$_{w}$ 4.397 ^{c}
Shiraz	$_{w}$ 3.300 ^{c}	$_{wx}$ 3.307 ^{c}	$_{w}$ 3.152 ^{b}	$_{w}$ 3.232 ^{b}	$_{w}$ 3.201 ^{c}
Merlot 2	$_{w}$ 2.814 ^{a}	$_{w}$ 2.702 ^{a}	$_{w}$ 2.641 ^{a}	$_{w}$ 2.700 ^{a}	$_{w}$ 2.685 ^{a}
Blush Zinfandel	$_{w}$ 4.397 ^{d}	$_{w}$ 4.397 ^{d}	$_{w}$ 4.397 ^{c}	$_{w}$ 4.378 ^{c}	$_{w}$ 4.378 ^{d}
Cherry wine	$_{w}$ 4.397 ^{d}	$_{w}$ 4.397 ^{d}	$_{w}$ 4.388 ^{c}	$_{w}$ 4.390 ^{c}	$_{w}$ 4.381 ^{d}
Campo Tempranillo 2004	$_{w}$ 2.965 ^{b}	$_{x}$ 3.173 ^{b}	$_{x}$ 3.175 ^{b}	$_{x}$ 3.174 ^{b}	$_{w}$ 2.912 ^{b}

^{abcd} same letter within the same column shows no significant difference at $P > 0.05$.
$_{wxyz}$ same letter within the same row shows no significant difference at $P > 0.05$.
The data was analyzed as a Randomized Complete Block design in which the treatments were arranged as a 5 by 5 factorial (5 wines by 5 pathogens). Differences were determined using Fisher's Protected Least Significance Difference (LSD) with $P < 0.05$.

Table 5 Least Square Means for the combination effect of probiotics* wine with the LSD = 0.168.

	B. longum	*L. acidophilus*	*L. paracasei*	*L. rhamnosus*
Cabernet Sauvignon	$_{w}$ 3.255 ^{a}	$_{w}$ 3.364 ^{b}	$_{x}$ 3.661^{c}	$_{w}$ 3.147 ^{a}
Pinot Noir	$_{w}$ 3.206 ^{a}	$_{w}$ 3.138 ^{a}	$_{w}$ 3.259 ^{a}	$_{w}$ 3.137^{a}
(Kenwood)Zinfandel & Carignane 2000	$_{y}$ 5.551^{b}	$_{y}$ 5.625 ^{c}	$_{w}$ 3.463 ^{b}	$_{x}$ 5.377 ^{b}
Chambourcin	$_{x}$ 5.693^{b}	$_{y}$ 6.524 ^{d}	$_{w}$ 3.886 ^{d}	$_{y}$ 6.473 ^{c}
Merlot 1	$_{w}$ 3.207 ^{a}	$_{w}$ 3.068 ^{a}	$_{wx}$ 3.252 ^{a}	$_{w}$ 3.131 ^{a}
Shiraz	$_{x}$ 4.244 ^{a}	$_{w}$ 3.070 ^{a}	$_{y}$ 3.132^{a}	$_{y}$ 3.168 ^{a}
Merlot 2	$_{x}$ 5.510 ^{c}	$_{y}$ 5.784 ^{c}	$_{w}$ 3.345 ^{b}	$_{x}$ 5.477 ^{c}
Blush Zinfandel	$_{w}$ 4.397 ^{a}	$_{y}$ 5.771 ^{c}	$_{x}$ 4.789 ^{c}	$_{w}$ 4.524 ^{b}
Cherry wine	$_{w}$ 4.683 ^{b}	$_{y}$ 4.802 ^{b}	$_{w}$ 4.712 ^{c}	$_{y}$ 5.752 ^{d}
Campo /Tempranillo 2004	$_{x}$ 5.692 ^{d}	$_{y}$ 5.973 ^{d}	$_{w}$ 4.957 ^{d}	$_{x}$ 5.834 ^{d}

^{abcd} same letter within the same column shows no significant difference at $P > 0.05$.
$_{wxyz}$ same letter within the same row shows no significant difference at $P > 0.05$.
The data was analyzed as a Randomized Complete Block design in which the treatments were arranged as a 5 by 4 factorial (5 wines by 4 probiotics). Differences were determined using Fisher's Protected Least Significance Difference (LSD) with $P < 0.05$.

Table 6. Least square means for the combination effect of pathogens* grape juice with the LSD = 0.154.

	E. coli O157:H7	*S. boydii*	*S.* Typhimurium	*L. monocytogenes*	*H. pylori*
Tropicana Grape juice	$_{w}$ 4.075 ^{a}	$_{w}$ 4.170 ^{a}	$_{w}$ 4.211 ^{a}	$_{x}$ 4.401 ^{b}	$_{y}$ 5.326 ^{b}
Welch's dark Grape juice	$_{w}$ 4.188 ^{a}	$_{w}$ 4.189 ^{a}	$_{w}$ 4.217 ^{a}	$_{w}$ 4.240 ^{a}	$_{w}$ 4.140 ^{a}
Welch's White Grape juice	$_{w}$ 4.423 ^{b}	$_{w}$ 4.313 ^{a}	$_{w}$ 4.209 ^{a}	$_{w}$ 4.403 ^{b}	$_{x}$ 5.412 ^{b}

^{abcd} same letter within the same column shows no significant difference at $P > 0.05$.
$_{wxyz}$ same letter within the same row shows no significant difference at $P > 0.05$.
The data was analyzed as a Randomized Complete Block design in which the treatments were arranged as a 3 by 5 factorial (3 grape juices by 5 pathogens). Differences were determined using Fisher's Protected Least Significance Difference (LSD) with $P < 0.05$.

Table 7. Least Square Means for the combination effect of probiotics* grape juice with the LSD = 0.0728.

	B. longum	*L. acidophilus*	*L. paracasei*	*L. rhamnosus*
Tropicana Grape juice	$_{y}4.303^{a}$	$_{x}4.145^{a}$	$_{x}4.211^{a}$	$_{w}2.721^{a}$
Welch's dark Grape juice	$_{x}6.509^{c}$	$_{x}6.505^{c}$	$_{y}6.786^{c}$	$_{w}4.694^{b}$
Welch's White Grape juice	$_{z}6.300^{b}$	$_{x}5.500^{b}$	$_{w}5.200^{b}$	$_{y}5.890^{c}$

^{abcd} same letter within the same column shows no significant difference at $P > 0.05$.
$_{wxyz}$ same letter within the same row shows no significant difference at $P > 0.05$.
The data was analyzed as a Randomized Complete Block design in which the treatments were arranged as a 3 by 4 factorial (3 grape juices by 4 probiotics). Differences were determined using Fisher's Protected Least Significance Difference (LSD) with $P < 0.05$.

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