1 Addition of phytochemical-rich plant extracts mitigate the antimicrobial
2 activity of essential oil/wine mixtures against *Escherichia coli* O157:H7 but
3 not against *Salmonella enterica*

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Abstract

Marinades for preparing raw meats for cooking are frequently made of wine and herbs. We simulated several formulations of potential antimicrobial marinades with these components and other food compatible/food derived extracts. Red wine formulations containing essential oils from oregano or thyme, or their primary active components carvacrol and thymol, respectively, and a mixture of plant extract powders from phytochemical-rich apple skin, green tea, and olive, were evaluated for inhibitory activity against the foodborne pathogens *Escherichia coli* O157:H7 and *Salmonella enterica*. Red wine alone exhibited low activity, as did the plant extract suspended in the wine. Surprisingly, the high activity of oregano or thyme essential oils in red wine was reduced in *E. coli*, but not in *Salmonella*, by addition of the plant extract. This study shows that essential oils in red wine can be an effective antimicrobial in food, however the possibility exists that phytochemicals, added to the treatment solution or natively present in the food itself, could adversely impact the antimicrobial activity and should be addressed with future studies.

1. Introduction

Food mores are deeply ingrained and difficult to change, even when the potential benefits are obvious. For broader acceptance, it is better to build upon existing food preparation techniques. An opportunity exists in the process of food preparation for the use of value-added marinades. Marination before cooking is a common culinary technique used to create tenderness and enhance flavor in foods, particularly in meats. Raw meats are frequently contaminated with foodborne bacteria which can cause illness. The U.S. Food Safety Inspection Service reported that for a survey period in 2012, the percent positive rate for *Salmonella* on chicken parts at the end of the process line was 26.3% (Food Safety Inspection Service, 2012). By a similar survey in 2014-2015, the positive rate for *Salmonella* was 3.92% and for *E. coli* O157:H7 1.07% in beef and veal processed carcasses (Food Safety Inspection Service, 2015). To prevent illness, not only must meat be cooked to a sufficient temperature to kill bacteria, but the bacteria need to be kept from cross-contaminating other foods in the kitchen setting, especially with uncooked fruits and vegetables. Addition of food-compatible antimicrobials to marinades have the potential to mitigate the health risks associated with foodborne bacteria in raw meat by both decreasing the bacterial load present in the marinated food, and by lessening the spread of bacteria onto kitchen surfaces and subsequently to other foods. Wines and herbs are common components of marinades and are also prospective food-compatible antimicrobials.

In previous studies we described the antimicrobial activities of a series of wine recipes containing natural food-compatible antimicrobials, and suggested that some of the

formulations might be suitable as antimicrobial wine marinades (Friedman, Henika, & Levin, 2015; Friedman, Henika, Levin, & Mandrell, 2006, 2007). The reported antimicrobial effect of wines may be due to its content of organic acids, alcohol, phenolic compounds, or sulfites, as well as its low pH (Waite & Daeschel, 2007). It is likely a combination of these factors (Boban, et al., 2010; Møretrø & Daeschel, 2004). Wine marinades applied to a variety of meats have been reported to be effective against pathogenic bacteria (Díez & Patarata, 2013; Isohanni, Alter, Saris, & Lyhs, 2010; Linares, Garrido, Martins, & Patarata, 2013; Nisiotou, Chorianopoulos, Gounadaki, Panagou, & Nychas, 2013; Rhoades, Kargiotou, Katsanidis, & Koutsoumanis, 2013). Wine is a versatile ingredient, as it is itself a source of antimicrobial components, as well as a food-compatible vehicle for other naturally-derived antimicrobials. The alcohol content of wine can contribute toward solubilizing the essential oils for use in foods; a common challenge encountered when using essential oils (Friedman, et al., 2006).

Essential oils (EO), many of them derived from the same herbs found in traditional marinades, are often potent antimicrobials, however due to the strong flavor imparted by these products, their use in foods has been limited. But with marinades, usually only a portion of the marinade transfers to the food and the remainder is discarded. Thus stronger concentrations can be used than would be organoleptically acceptable if the whole product was consumed. In a previous study we found that the essential oil compounds cinnamaldehyde from cinnamon, and thymol and carvacrol from both thyme and oregano, were the three most active compounds against all of the following bacteria: *E. coli* RM1484, *S. enterica* RM1309, *C. jejuni* RM1221, *L. monocytogenes* RM2199, *L. monocytogenes* RM2388 (Friedman, Henika, & Mandrell, 2002).

Many of phenolic-rich phytochemicals have also shown antimicrobial activity (Coppo & Marchese, 2014; Daglia, 2012; Friedman, Henika, & Levin, 2013). Apple skin, olives, and green tea are all foods that contain high levels of phytochemicals. Apple skin contains quercetin, procyanidins, catechins, phloridzin, phlorizin, epicatechin, rutin, and chlorogenic acid (Apple PolyTM, 2010). Olive pulp juice contains hydroxytyrosol, oleuropein, tyrosol, oleuropein aglycone, and gallic acid (Soni, Burdock, Christian, Bitler, & Crea, 2006). Green tea contains flavan-3-ols, including (+)-catechin, (+)-gallocatechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin 3-gallate, and (-)-epigallocatechin 3-gallate, as well as the flavonols, kaempferol, myricetin, and quercetin (U.S. Department of Agriculture: Agricultural Research Service, 2015).

The objective of the present study was to evaluate the bactericidal activity of a red wine in combination with high-phenolic plant extracts (apple skin, olive, and green tea) and the bactericidal essential oils from oregano and thyme (flavor-compatible with typical marinades), and their bioactive compounds carvacrol and thymol, respectively, against *E. coli* O157:H7 and *Salmonella enterica*.

2. Materials and methods

2.1. Test compounds

Oregano and thyme plant essential oils (EO) were obtained from Lhasa Karnak Herb Co., Berkeley, CA. Carvacrol and thymol were obtained from Sigma Chemical Co., St. Louis, MO. The commercial olive extract powder (Hidrox-12[®]; 12% olive phenolics of which hydroxytyrosol is ~6% total), prepared by freeze-drying the juice of organic California-grown olives, was a gift of the manufacturer, CreAgri, Inc., Hayward, CA.

The commercial apple skin extract powder (Apple Poly[®]; 82% polyphenols) was obtained from Apple Poly LLC, Morrill NE. Green tea extract, freeze-dried steeped green tea, was prepared as described previously (Friedman, et al., 2015). The red wine used in these studies was Beringer (St. Helena, CA) Pinot noir, vintage 2012 (13.9% alcohol; pH 3.6). The wine was obtained from a local store.

2.2. Preparation of test formulations

The essential oils and their components were from oregano (active component: carvacrol) or thyme (active component: thymol). The wine buffer used for dilutions was 12% wine, pH 3.6. This wine buffer did not kill the bacteria in our assay. Olive powder (0.02%), green tea (0.02%) and apple skin (0.1%) extracts were dissolved in wine. The essential oils (EO) or EO components were first diluted 1:10 in 95% ethanol, then 1:100 into either wine or wine containing plant extracts for a final 0.1% EO concentration.

2.3. Bactericidal assay

Escherichia coli O157:H7 (strain SEA18B88 - isolated from apple juice by the FDA) and *Salmonella enterica* serovar Hadar (strain MH139 - isolated from ground turkey) were used in this study. Broth suspensions with a bacterial load of $\sim 10^9$ CFU/ml were obtained by 24 h growth (37°C, 200 rpm) in Lucia-Bertani media (LB) (Difco, Sparks, MD). The broth was vortexed and 1 ml of the bacterial suspension was added to a 1.9 ml plastic snap cap tube. The bacteria were pelleted by centrifugation in a microfuge (15,800 g) for 2 min. This pellet was the starting point for the bactericidal (BA50) test.

We previously used the BA50 test to assess antimicrobial activity of natural substances against pathogenic bacteria (Friedman, et al., 2013; Friedman, et al., 2006,

2007; Friedman, et al., 2002). It is defined as the concentration of antimicrobial substance which kills 50% of the bacteria. Bacteria were suspended by mixing the pellet with 1 ml of wine buffer using a pipette and a 1 ml tip. This suspension ($\sim 10^9$ bacteria/ml) was serially diluted four times by a factor of 10, to $\sim 10^5$ bacteria/ml. The test solutions were prepared in advance by diluting the formulations serially by half with wine buffer for an array of six doses from 100% to 3.125% of formula. Before inoculation the 10^5 bacterial suspension was mixed by vortexing for 5 s. This suspension (50 μ l) was added to 450 μ l of the test solution, which was then mixed by vortexing for another 5 s. The final concentration of bacteria in each dose was thus $\sim 10^4$ CFU/ml and the dose series used for graphing was 90% (i.e. 50 μ l into 450 μ l), 45%, 22.5%, 11.3%, 5.6%, 2.8% and wine buffer. At the end of the incubation period (22 °C, 5 min) each test solution was mixed by vortexing for 5 s followed by application of three individual 15 μ l drops to three of the top grids of a square LB agar plate. The plate was tilted so that the drops could run down the face of the plate. The 90% and the 11.3% doses were plated together (to take advantage of the 6 grids/plate), as were the 45% and 5.6%, and the 22.5% and 2.8% doses. The wine control was plated separately. The plates were dried for at least 10 min and then incubated for 24 h at 37°C.

In order to evaluate the bactericidal vs. the bacteriostatic effect of the formulations, a parallel series of incubated bacteria in test solutions were pelleted by centrifugation in a microfuge (15,800 g) for 2 min and then suspended in wine buffer. After an incubation period (90 min/ 37°C) the bacteria were plated and colonies counted.

2.4. Calculations and data analysis

The incubated plates for the BA50 test contained ~150 CFU per 15 µl streak for the control and for doses which had no effect. CFU were scored for all doses and reported as CFU/15 µl streak. With the aid of SigmaPlot 11 (Systat Software Inc., Chicago, IL) BA50 values were calculated by plotting CFU against the dose and using non-linear regression analysis to find a best fit 3-parameter logistic dose-response curve. The BA50 value was the dose that corresponded to a 50% reduction in CFU and was interpolated from the graph.

The significance of differences between treatments was tested with ANOVA, followed by the Tukey Test (SigmaPlot 11). P values < 0.05 were deemed significant.

3. Results

Table 1 shows that red wine or red wine containing the plant powder mix was inactive (BA50 > 90% of test solution, i.e. no reduction in CFU at the highest dose) against *E. coli* and only slightly active (BA50 approaching 90%, the highest dose) against *Salmonella*. Wine with added EO was highly active against the bacteria; more so in *Salmonella* than *E. coli*. An unexpected finding was that for *E. coli*, activity decreased (BA50 increased from 26 to 41.5%) when the plant powder mix was added to the wine-EO solution, but that activity was restored when the EO component was substituted for the related EO. There was no such effect for *Salmonella*.

Pelleting the bacteria from the test solutions followed by microbial testing showed that the antimicrobial solutions before and after pelleting showed similar activities, suggesting that the antimicrobial activity was bactericidal rather than bacteriostatic (data not shown). The response of the bacteria to either oregano or thyme EO was similar.

4. Discussion

In previous studies we reported our findings on the antibacterial activities, measured using the BA50 assay of a number of formulations containing combinations of food-compatible compounds in wines (Friedman, et al., 2006, 2007), essential oils and their bioactive compounds (Friedman, et al., 2006, 2007), and high-phenolic plant extracts of apple, tea, and olive (Friedman, et al., 2013; Friedman, et al., 2015). Mixtures of wine and EO were highly effective in the BA50 test. The alcohol in the wine improved the solubility of the EO in aqueous solutions, allowing a stronger concentration to be used. It is also presumed that other wine components contribute to the antimicrobial effect as extracted red wine polyphenols isolated by chromatography were highly antimicrobial, even though the natural wine alone had little/no effect (Friedman, et al., 2007). We also reported that polyphenols isolated from white (Chardonnay) wine were less active than those from red wine. Powdered olive juice, olive pomace, and the olive component, hydroxytyrosol, were antimicrobial against *E. coli* and *Salmonella*, whereas apple peel extract was only antimicrobial against *Salmonella* (Friedman, et al., 2013). Green tea extract was not antimicrobial against either bacteria, but its component, epigallocatechin gallate was, suggesting that concentrations of the active component are not generally high enough in the whole tea extract (Friedman, et al., 2013).

It was our hypothesis that a mixture of several natural antimicrobial powders could improve the efficacy of the wine/EO solutions and possibly act synergistically due to their acting by different mechanisms. Essential oils are thought to inhibit bacteria by changing the fluidity of cell membranes (Gill & Holley, 2006). The phenols in plants and wine inhibit by multiple modes of action, including membrane damage, inhibition of

nucleic acid synthesis, inhibition of metabolism, and inhibition of cell wall and cell membrane synthesis (Cushnie & Lamb, 2011). It seems that under the test conditions of the present study, our hypothesis that these two different categories of antimicrobials might act synergistically was not borne out. Nemereshina et al. found extracts of different species in the Plantaginaceae family were either growth inhibiting or growth-stimulating to *E. coli* depending on the type of polyphenols the extract (Nemereshina, Tinkov, Gritsenko, & Nikonorov, 2015). The high-tannin extracts were correlated with iron-binding, and were growth inhibiting. The high-flavonoid extracts acted as prebiotics and induced growth. Extending their findings to our study, it could be possible that our plant phenolic mix acted as a prebiotic for *E. coli* but not for *Salmonella*, slightly stimulating growth simultaneous to the essential oil exerting an antimicrobial effect. Another possibility is that the phenolic compounds in the powder somehow reduce access of the essential oil to the *E. coli* cell membrane, or somehow stabilize the membrane. We do not know what this mechanism might be. It does not appear to be a solubility problem, as no effect is seen for *Salmonella*.

5. Conclusions

Although several studies have reported that red wine inhibited the growth of bacteria, in this study, treatment with red wine alone was inactive against *E. coli* and only modestly active against *Salmonella* under the test conditions. This is in agreement with a previous study (Friedman, et al., 2015). Addition of the essential oil to the wine however had a strong antimicrobial effect on the bacteria, but further addition of the plant powder mix reduced the efficacy of the wine-EO solution against *E. coli*, but not against

Salmonella. Substitution of the essential oil with its purified main component, carvacrol or thymol, partially restored activity, likely due to the higher concentration of active component. In conclusion, the results of this study complement and extend the previously mentioned studies on antimicrobial wine/plant extract formulations active against foodborne pathogens. Further studies should investigate combinations of phenolic rich extracts and essential oils for additive, synergistic, and antagonistic effects against foodborne pathogenic bacteria.

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Table 1

Calculated BA50 values (% test solution that decreased the bacteria by 50%) for wine-based formulations with essential oil or essential oil component and a 3-plant extract mix. Low values are more antimicrobial.

Component	<i>Escherichia coli</i>	<i>Salmonella</i>
	1484	<i>enterica</i> 1309
Wine	>90 ^a	63 ± 4
Wine + plant extract mix	>90	79 ± 4
Wine + oregano EO	26 ± 2 ¹	11 ± 2 ^{1,2}
Wine + plant extract mix + oregano EO	41.5 ± 0.8 ²	15 ± 0.8 ¹
Wine + plant extract mix + carvacrol	26 ± 6 ¹	11.5 ± 0.001 ^{1,2}
Wine + thyme EO	29 ± 10 ^{1,2}	13.4 ± 0.5 ^{1,2}
Wine + plant extract mix + thyme EO	49 ± 2	10.7 ± 0.4 ^{1,2}
Wine + plant extract mix + thymol	22.9 ± 0.001 ¹	9.4 ± 0.3 ²

^a Within columns, and per bacterial species, shared superscript numbers mean values not significantly different, $p < 0.05$.

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