Salmonella Heidelberg Strain Responses to Essential Oil Components

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Abstract

Salmonella are one of the more prominent foodborne pathogens that represent a major health risk to humans. *Salmonella* serovar Heidelberg strains are increasingly becoming an important public health concern, since they have been identified as one of the primary *Salmonella* serovars responsible for human outbreaks. Over the years, *Salmonella* Heidelberg isolates have exhibited higher rates of resistance to multiple antimicrobial agents compared to other *Salmonella* serovars. Essential oils (EOs) have been widely used as alternatives to chemical-based antimicrobials. In the current research, five EOs were screened to determine their antimicrobial activity against 15 *S.* Heidelberg strains from different sources. Oils tested were R(+)-limonene, orange terpenes, cold compressed orange oil, trans-cinnamaldehyde and carvacrol. EOs were stabilized in nutrient broth by adding 0.15% (w/v) agar. Tube dilution assays and minimal inhibitory concentrations (MIC) were determined by observing color changes in samples during exposure to EOs. Carvacrol and *trans*-cinnamaldehyde completely inhibited the growth of *S.* Heidelberg strains, while R(+)-limonene and orange terpenes did not show any inhibitory activity against the strains tested. Cold compressed orange oil only inhibited growth of two of the strains exhibiting an MIC of 1%. All *S.* Heidelberg isolates evaluated exhibited similar responses to the respective EOs. The use of all natural antimicrobials such as specific EOs offers the potential to limit the majority of *S.* Heidelberg isolates that may occur in food production.

Keywords: essential oils, *Salmonella* Heidelberg; antimicrobials

1. Introduction

Foodborne illnesses continue to be one of the primary public health concerns in the United States (U. S.), and it has been estimated that over 1 million Americans contract *Salmonella* each year (Scallan et al., 2011). Annual costs for *Salmonella* control efforts are estimated to be $14.6 billion (Scharff, 2010; Heithoff et al., 2012). S*almonella* is not only a public health concern due to the number of cases per year, but many strains have developed resistance to antimicrobial agents (Kim et al., 2005; Foley & Lynne, 2008; Bajpai, Baek, & Kang, 2012) due to continued therapeutic use of antimicrobials in feed products (Su, Chiu, Chu, & Ou, 2004; Kim et al., 2005).

Salmonella enterica serovar Heidelberg (*S.* Heidelberg) ranks fourth among the top five serovars associated with human infections and is responsible for an estimated 84,000 illnesses in the U. S. annually (CDC, 2008; FDA, 2009; Foley et al., 2011; Han et al., 2011). *Salmonella* Heidelberg is one of the most commonly isolated serovars in the U.S. and Canada from clinical cases of salmonellosis, retail meats and livestock (Zhao et al., 2008; Hur, Jawale, & Lee, 2012). While most *Salmonella* infections are self-limiting and are resolved within a few days, *S.* Heidelberg tends to cause a significantly higher percentage of invasive infections (Vugia et al., 2004; Han et al., 2011). As a result, antimicrobial therapy is often necessary, making antimicrobial resistance a significant concern. Due to the tendency of *S.* Heidelberg to cause severe extra-intestinal infections (Wilmshurst & Sutcliffe, 1995) such as myocarditis and septicemia (Vugia et al., 2004), the occurrence of *S.* Heidelberg multidrug resistance strains is of extreme clinical importance. *Salmonella* Heidelberg strains exhibiting antimicrobial resistance have been isolated from humans, retail meats and food animals (Logue, Sherwood, Olah, Elijah, & Dockter, 2003; Nayak et al., 2004; Kaldhone et al., 2008; Zhao et al., 2008; Lynne, Kaldhone, David, White, & Foley, 2009; Oloya, Doetkott, & Khaitsa, 2009; Han et al., 2011). Studies suggest that poultry-associated *S.* Heidelberg strains harbor IncFIB, IncA/C, IncH2, and IncI1 plasmids, which may contain genes that confer resistance to several antibiotics such as tetracycline, kanamycin, streptomycin, and sulfonamides (Han et al., 2012). Because *S.*

Heidelberg is responsible for causing more invasive infections compared to other serovars, it is important to monitor its prevalence and resistance – novel and unique intervention strategies are a priority to reduce or eliminate its presence.

Aromatic plants and their extracts have been examined for their effectiveness in food safety and preservation applications (Fisher & Phillips, 2008). Essential oils, for example, exhibit antimicrobial properties that may make them suitable alternatives to antibiotics (Chaves et al., 2008). These potential attributes and an increasing demand for natural medicinal treatment options have brought attention to the use of EOs as potential antimicrobial alternatives (Fisher & Phillips, 2008; Solórzano-Santos & Miranda-Novales, 2012; Rivera Calo, Crandall, O'Bryan, & Ricke, 2015a). Essential oils derived as by-products in citrus processing have been screened for antimicrobial properties against common foodborne pathogens, and several of these compounds have exhibited antimicrobial properties (Dabbah, Edwards, & Moats, 1970; Rivera Calo et al., 2015a).

Alali et al. (2013) evaluated the use and application of various EOs in broiler drinking water to reduce ceca colonization and fecal shedding of *S.* Heidelberg. Although the EOs evaluated were not a sufficient means to limit *S.* Heidelberg isolates in ceca or reduce shedding, these authors reported a reduction of *S.* Heidelberg colonization in the crops of seeders as well as an increased weight gain in birds that were administered the EOs (Alali et al., 2013). This research was conducted to determine the efficacy of several EOs on *S.* Heidelberg isolates that originated from different sources.

2. Materials and Methods

2.1 Bacterial Strains Used in This Study

Fifteen different *S.* Heidelberg strains were evaluated (Table 1). Strains were isolated from multiple sources such as, turkey and ground turkey, poultry products, cattle, swine, poultry and poultry egg houses, and humans.

Table 1. *Salmonella* Heidelberg strains used in this study

Strain	Source	Reference
ARI-14	poultry products	In this study
SL 476	ground turkey	Fricke et al., 2011
SL 486	human	Fricke et al., 2011
692	chicken egg house	Lynne et al., 2009
945	human	Han et al., 2011
114	cattle	Lynne et al., 2009
163	turkey	Kaldhone et al., 2008
136	swine	Lynne et al., 2009
1148	human	Han et al., 2012
824	turkey	Kaldhone et al., 2008
130	chicken	Lynne et al., 2009
937	human	Han et al., 2011
118	cattle	Lynne et al., 2009
144	swine	Lynne et al., 2009
146	swine	Lynne et al., 2009

2.2 DNA Extraction

One colony of each *S.* Heidelberg strain was inoculated in 5 mL Luria Bertani (LB) broth and incubated at 37 °C, 190 rpm for 16 h in a C76 Water Bath Shaker (New Brunswick Scientific, Edison, NJ, USA). A one mL aliquot of bacterial cells was transferred to a microcentrifuge tube, and samples were centrifuged at $14,000 \times g$ for 10 min and the corresponding supernatants were discarded. Genomic DNA was extracted with the Qiagen DNeasy Blood Tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. The isolated genomic DNA concentration and purity were measured using a NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE,

USA) and DNA samples were subsequently stored at -20 °C.

2.3 S. Heidelberg Confirmation by PCR

A conventional PCR assay was conducted with a MJ Mini Personal Thermal Cycler (Bio-Rad, Hercules, CA, USA). *Salmonella* Heidelberg specific primers were generated based on the type II restriction enzyme methylase subunit sequences of *S*. Heidelberg (Bronowski & Winstanley, 2009), and upon evaluation of the specificity for all 15 strains, each was determined to belong to the serovar Heidelberg (Table 2). Additionally, exclusivity assays were performed on different *Salmonella* serovars as well as other non-*Salmonella*. The total reaction (25 µL volume) was composed of 1 µL of template DNA, 1 µL of each primer (IDT, Coralville, IA, USA), 12.5 µL of SYBR Green (Cambrex Bioscience, Walkersville, MD, USA), and 9.5 µL of DNase-RNase free water. The PCR conditions consisted of pre-denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s, extension at 72 °C for 30 s, and a final extension cycle at 72 °C for 5 min. PCR products were confirmed on a 1.5% agarose gel and visualized on a transilluminator (Bio-Rad, Hercules).

Table 2. *Salmonella* Heidelberg specific primer pair used in this study

Primer	Sequence	Product	Reference
SH_SHP-2f	5'-GCATA GTTCC AAAGC ACGTT-3'	180	This study
SH_SHP-1r	5'-GCTCA ACATA AGGGA AGCAA-3'	180	This study

2.4 Essential Oils and Cultures

Each of the EOs evaluated (R(+)-limonene, orange terpenes, cold compressed orange oil, trans-cinnamaldehyde and carvacrol) were obtained from Sigma Aldrich (St. Louis, MO, USA). Fifteen *S*. Heidelberg strains from different sources were evaluated (Table 1). One colony of each strain was inoculated into Luria-Bertani (LB) broth and incubated at 42 °C, 190 rpm for 18 h. In addition, analyses were performed on *S*. Heidelberg strains incubated at 37 °C, 190 rpm for 18 h.

2.5 Modified Tube Dilution Assay

To maintain the EOs in a homogeneous mixture, 0.15% agar was added to nutrient broth (NB), boiled for 1 min and subsequently autoclaved. Once the media cooled to room temperature, triphenyl tetrazolium chloride (TTC) (1 %) was added to act as a growth indicator. As previously described by O'Bryan et al. (2008), serial dilutions were made by placing 10 mL of the NB with 0.15% agar (NBA) in the first tube and 5 mL in the remaining tubes for a total of four tubes. One hundred µL of the essential oil was added to the 1st (10 mL) tube for an initial 1% concentration. To perform serial dilutions, 5 mL of the emulsion was transferred to the next tube, and the procedure was repeated for a total of four dilutions. Five mL were removed from the last tube and discarded so that all tubes consisted of equal volumes (5 mL). All tubes were inoculated with 50 µL of an overnight culture of each *S*. Heidelberg strain, and the tubes were subsequently incubated for 24 h at 37 °C. A change in color from light yellow to pink/red indicated growth. Minimum inhibitory concentrations were determined as the lowest concentration of EOs that showed no color change in the medium, which was considered representative of the absence of detectable bacterial growth.

3. Statistical Analysis

MIC tests were repeated as three independent trials. Each mean ± standard deviation was determined using JMP Pro Software Version 11.0 (SAS Institute Inc., Cary, NC).

4. Results and Discussion

Salmonella Heidelberg specific primers (Table 2) were used to confirm that all 15 strains evaluated belong to *S*. Heidelberg (Figure 1). Park and Ricke (2015) developed a multiplex PCR assay to simultaneously detect *Salmonella* serovars Heidelberg, Enteriditis, and Typhimurium. The *S*. Heidelberg primers were generated for this study similar to Park and Ricke (2015), which involves BLAST alignment(s) and subsequent inclusivity and exclusivity assays for primer specificity confirmation. In addition to the potential detection applications, the *S*. Heidelberg specific primers used in this study suggest that although these isolates originated from different environments, there is a certain level of genetic uniformity among these *S*. Heidelberg isolates.

Minimum inhibitory concentrations were determined by observing tubes for any color change to pink or red (Table 3). For the *S*. Heidelberg strains grown at 42 °C, results indicate that *trans*-cinnamaldehyde and carvacrol

exhibited MICs of 131 μg/mL and 122 μg/mL, respectively, by completely inhibiting the growth of each *S.* Heidelberg strain, while orange terpenes and R(+)-limonene did not exhibit any inhibitory activity against any of these strains. Cold compressed orange oil only inhibited growth of two of the strains (945 and 114), with MIC's of 843 μg/mL. No relationship was observed between the source of the strain and the EOs tested. Similar results were obtained when strains were grown at 37 °C, although under this condition cold compressed orange oil showed no antimicrobial activity for all strains, while at 42 °C it inhibited strains 945 and 114 with an MIC of 843 μg/mL.

Table 3. Minimum inhibitory concentrations (μg/mL) of five EOs against *Salmonella* Heidelberg isolates

Isolate	Essential oils				
	carvacrol	R(+)-limonene	orange terpenes	orange oil	*trans*-cinnamaldehyde
SL 486	122 ± 0	No effect	No effect	No effect	131 ± 0
SL 476	122 ± 0	No effect	No effect	No effect	131 ± 0
ARI-14	122 ± 0	No effect	No effect	No effect	131 ± 0
692	122 ± 0	No effect	No effect	No effect	131 ± 0
945	122 ± 0	No effect	No effect	843 ± 0	131 ± 0
114	122 ± 0	No effect	No effect	843 ± 0	131 ± 0
163	122 ± 0	No effect	No effect	No effect	131 ± 0
136	122 ± 0	No effect	No effect	No effect	131 ± 0
1148	122 ± 0	No effect	No effect	No effect	131 ± 0
824	122 ± 0	No effect	No effect	No effect	131 ± 0
130	122 ± 0	No effect	No effect	No effect	131 ± 0
937	122 ± 0	No effect	No effect	No effect	131 ± 0
118	122 ± 0	No effect	No effect	No effect	131 ± 0
144	122 ± 0	No effect	No effect	No effect	131 ± 0
146	122 ± 0	No effect	No effect	No effect	131 ± 0

There are several indications that there may be differences in responses among *Salmonella* serovars and in some cases even among strains. This is critical to consider since overall recommendations for antimicrobial application to *Salmonella* can sometimes be made based on the responses of only a few serovars and/or strains. The growth responses of *S.* Typhimurium grown in spent media generated from several of the *S.* Heidelberg isolates used in this study have been evaluated (Rivera Calo, Park, Baker, & Ricke, 2015b). The spent media from twelve of the fifteen *S.* Heidelberg isolates evaluated in this study decreased the growth of *S.* Typhimurium (ATCC 14028), which suggests that *S.* Heidelberg isolates may compete in some manner with other *Salmonella* serovars (Rivera Calo et al., 2015b). Additionally, studies have shown the existence of differences between serovars as well as strains within the same serovar of *Salmonella enterica* can occur for certain antimicrobials. In their research, González et al. (2012) compared *hilA* gene expression in response to acid stress between different *Salmonella* serovars and strains. Results showed that there are serovar and strain differences in virulence gene expression and acid tolerance; regulation of *hilA* showed to be serovar and strain dependent as well as dependent on acid type (González et al., 2012). In an extensive genome analysis comparison between UK-1 and other *S.* Typhimurium strains, Luo et al. (2012) reported that virulence factors pertaining to one strain might increase or decrease virulence when present in a different strain. Shah et al. (2011) reported that isolates from *S.* Enteritidis that have been recovered from poultry or poultry environment are not equally pathogenic, nor do they have similar invasiveness.

Previous research has shown that different EOs exhibit antimicrobial activity pre- and post-harvest on *Salmonella*. The antimicrobial effect of EOs has been reported to be concentration dependent (Sivropoulou et al. 1996). Ravishankar et al. (2010) reported that at a 0.2% concentration, antimicrobials, carvacrol and cinnamaldehyde completely inactivated the antibiotic-resistant and -susceptible isolates of *S. enterica*. Zhou et al. (2007) investigated the antimicrobial activity of cinnamaldehyde, thymol and carvacrol individually and in

combination against *S.* Typhimurium, and reported the lowest concentrations of cinnamaldehyde, thymol and carvacrol inhibiting the growth of *S.* Typhimurium significantly were 200, 400 and 400 mg/L, respectively. When combined, cinnamaldehyde/thymol, cinnamaldehyde/carvacrol and thymol/carvacrol demonstrated that the concentration of cinnamaldehyde, thymol and carvacrol could be decreased from 200, 400 and 400 mg/L to 100, 100 and 100 mg/L, respectively (Zhou et al., 2007). In this study we used cinnamaldehyde in its *trans* isomer, which is the form that is present as a major component of bark extract of cinnamon (Kollanoor Johny et al., 2008).

The MIC method has previously been used to evaluate the effectiveness of orange EOs against different *Salmonella* serovars (Enteritidis, Seftenberg, Tennessee, Kentucky, Heidelberg, Montevideo, Michigan, Stanley, among others) (O'Bryan, Crandall, Chalova, & Ricke, 2008). These authors reported that three out of the seven citrus EOs evaluated (orange terpenes, d-limonene, and terpenes purified fractions from orange essence) exhibited antimicrobial activity, and that there was not a significant difference in the responses among the *Salmonella* serovars evaluated (O'Bryan et al., 2008). However, orange terpenes and d-limonene exhibited the most antimicrobial activity with a MIC of 1% for each serovar, and terpenes from orange essence exhibited MIC range from 0.125 to 0.5% (O'Bryan et al., 2008). In the current study, no effect was observed for any of the *S.* Heidelberg isolates subjected to orange terpenes. These contrasting observations may be due to the fact that O'Bryan et al. (2008) evaluated commercial essential oils and fractions (single fold d-limonene), which may contain additional antimicrobial properties that were not present in the essential oils evaluated in the current study. Additionally, despite the fact that limonene is one of the more well-known and characterized of the EOs from citrus products (Dabbah et al., 1970; Caccioni, Guizzardi, Biondi, Renda, & Ruberto, 1998) which can exert potent, broad-spectrum antimicrobial activity (Di Pasqua, Hoskins, Betts, & Mauriello, 2006), this essential oil, in the form of R(+)-limonene did not exhibit any antimicrobial activity in this study.

Essential oils have also been studied to have antimicrobial activity against *Salmonella* when used post-harvest. In their research, Alali et al. (2013) determined the effect of non-pharmaceuticals (a blend of organic acids, a blend of EOs, lactic acids, and a combination of levulinic acid and sodium dodecyl sulfate) on weight gain, feed conversion ratio, mortality of broilers and their ability to reduce colonization and fecal shedding of *S.* Heidelberg. Their results showed that the broilers that received the EOs had significantly increased weight gain and mortalities were lower compared to other treatments. *Salmonella* Heidelberg contamination in crops was significantly lower in challenged and unchallenged broilers that received EOs and lactic acids in drinking water, when compared to other treatments (Alali et al., 2013). Results of our study further supports that some EOs could be used as antimicrobial agents against the foodborne pathogen *Salmonella*. Further studies will be needed to confirm the antimicrobial effect of these EOs *in vivo* and which combinations represent optimal antimicrobial activity.

Hoffmann et al. (2014) compared the genomes of 44 S. Heidelberg strains, and identified at least 273 genes that varied among these strains. Uniform responses to various EOs were observed for each *S.* Heidelberg isolate in this study, which suggests that the inhibitory mechanism(s) of EOs result from a broad inhibitory action such as potentially a membrane effect. Additionally, the genetic diversity among *S.* Heidelberg isolates may not play a role in the efficacy of survival when exposed to EOs. Additional studies on *S.* Heidelberg and EOs may be necessary to identify the most appropriate pre- and post-harvest applications of essential oil for limiting *Salmonella* spp. in the food supply.

Acknowledgments

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