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Use of alamarBlue as an Indicator of Microbial Growth in Turbid Solutions for Antimicrobial Evaluation

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USE OF ALAMARBLUE AS AN INDICATOR OF MICROBIAL GROWTH IN
TURBID SOLUTIONS FOR ANTIMICROBIAL EVALUATION

A Thesis Presented

by

PRECIOUS HENSHAW

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TURBID SOLUTIONS FOR ANTIMICROBIAL EVALUATION

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ABSTRACT

USE OF ALAMARBLUE AS AN INDICATOR OF MICROBIAL GROWTH IN
TURBID SOLUTIONS FOR ANTIMICROBIAL EVALUATION

SEPTEMBER 2018

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The use of antibiotics in animal feed is a large cause of concern due to bacterial resistance, which occurs when bacteria change after exposure to antibiotics and become less affected by the drug. Consequently, the desire to find a replacement for these antibiotics has garnered interest in both farmers and consumers. The ideal substance would display antimicrobial activity without promoting bacterial resistance and would still function as a growth promoter in animals. One antimicrobial that fits this criterion is carvacrol, a compound found in oregano extract. Carvacrol exhibits antimicrobial activity in a number of bacteria, including *E.coli O157:H7* and *S.* enteritidis. In this study, the effectiveness of carvacrol nanoemulsions against *E.coli* and *S.* enteritidis growth in a micro-broth dilution assay was tested, as well as the use of alamarBlue^{TM} dye reagent as a bacterial viability indicator. The carvacrol nanoemulsion was diluted in TSB to obtain 2000, 1000, 500, 250, 125, and 62.5 ppm, then aliquots of 100 μl of each dilution were added to a 96-well microtiter plate. Test group wells were inoculated with 100 μl of microorganism while control cells remained uninoculated, and the plate was incubated for 24h at 37 °C. After incubation, 10μl of alamarBlue^{TM} was added to each well, and the

microtiter plate was incubated for one hour. After one hour, the color changes were

analyzed both visually and via fluorescence. Additionally, a traditional plating assay in

which samples were plated on tryptic soy agar was done alongside the microtiter plate

assay. It was found that the minimum inhibitory concentration of carvacrol needed to

inhibit *E. coli* growth was 500ppm, and to inhibit *S.* enteritidis growth was 800ppm.

These results show that carvacrol may be suitable as a possible replacement for antibiotic

in animal feed in the future.

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CHAPTER 1

LITERATURE REVIEW

Antibiotics are substances that either kill or inhibit the growth of microorganisms. Antibiotics can be either bactericidal (causes death in microorganisms) or bacteriostatic (prevents microorganisms from growing). Antibiotics are often added to animal feed mainly to speed up weight gain in cows, chicken, pigs, and other meat-producing animals (Li, 2017). The addition of antibiotics as antimicrobial growth promoters (AGP) increases animal growth rate by between 2-4%. Antibiotics are also used to prevent bacterial infections in animals. Diseases such as necrotic enteritis in poultry and dysentery in pigs is prevented by the use of AGP in animals beginning at a young age. Although the use of AGP has decreased the incidences of these infections in animals, improving feed and hygiene without the addition of AGP has been shown to combat disease just as well (Bogaard et al., 1999). 80% of the antibiotics sold in the United States are used for animal agriculture, and this number is expected to increase within the next 15 years (Martin et al., 2015).

One of the largest concerns with the increased use of antibiotics in animal agriculture is the prevalence of antibiotic-resistant microorganisms. This resistance occurs as a result of natural selection, which favors microorganisms with resistant genes. These resistant microorganisms prevail and are able to resist the effects of antibiotics, rendering those antibiotics less effective (Alliance for the Prudent Use of Antibiotics, 2014). This becomes especially dangerous when these resistant microorganisms are transferred to humans. This can occur through the consumption of meat that is undercooked, through exposure to animals and their feces, or from contaminated

surfaces. When antibiotics are less effective at killing microorganisms, it becomes more difficult to treat disease. Not only does this lead to increased healthcare costs, but it can also lead to serious complications or even death in infected individuals (Chattopadhyay, 2014).

It is therefore imperative that the use of antibiotics decrease significantly. Substances exist that have a similar effect to antibiotics without causing resistance in microorganisms. One of these substances is carvacrol, a chemical found in oregano extract. Carvacrol exhibits antimicrobial properties, which has been demonstrated in several studies. In *Staphylococcus aureus, Listeria monocytogenes,* and *Escherichia coli* biofilms, carvacrol was shown to reduce the growth of sessile cells by 5 logarithmic cycles (Espina et al., 2017). Another study showed that the combination of pulsed light and carvacrol reduced *E. coli* by 4 log cycles on the surface of contaminated cucumber slices (Taştan et al., 2017). Carvacrol is also shown to be effective as an antimicrobial agent in food packaging applications when used in the lining of packaging films (Luzi et al., 2017). In addition to being an antimicrobial, carvacrol also does not seem to cause resistance in bacteria. In one study, carvacrol was shown to exhibit bactericidal properties in Gram-positive bacteria through the mechanism of membrane damage. Researchers' attempts to isolate carvacrol-resistant mutants were unsuccessful, suggesting that carvacrol did not cause resistance in group-A streptococci (Magi et al. 2015). This makes it a possible and very promising replacement for antibiotics in animal feed.

Carvacrol has been demonstrated to reduce cells numbers by bactericidal action or by inactivating bacterial cells. Due to its hydrophobic nature, carvacrol can be emulsified to increase its effectiveness in water-based products. A nanoemulsion, an emulsion

containing droplets that are 100nm or smaller, can be easily and cost-effectively created by titrating oil extract and water-soluble surfactants into buffer. This spontaneous emulsification stabilizes the carvacrol and makes it suitable for food applications (Chang et al. 2013).

It is important to first take into consideration the effectiveness of antimicrobial treatment. There are several methods used to evaluate the efficacy of antimicrobial treatments. One is by the use of a cell viability test such as a LIVE/DEAD BacLight stain. When cells are stained with LIVE/DEAD BacLight, which is a mixture of green and red fluorescent nucleic acids, they show up under fluorescent light differently depending on the health of the cell. Green stains indicate live cells, while red stains indicate dead cells (Fantner et al., 2010). The greatest advantages of using a LIVE/DEAD stain are their low cost, chemical stability, and their ease to use. The largest disadvantages include having to wait for a long period to get results, and the toxicity of the dye to live cells (Perfetto et al., 2011).

The agar disk-diffusion method is another method to evaluate antimicrobials. In this method, agar plates are inoculated with the microorganism, and filter paper disks soaked in the antimicrobial agent are placed on the surface of the agar. During incubation, the antimicrobial agent releases into the agar, inhibiting the growth of the microorganism surrounding the disk. After incubation, the diameters of the zones of inhibition are measured (Balouiri et al., 2016). The main advantage of this method is the ability to test many different antibiotics at the same time. However, the disk diffusion method does not work well for anaerobic microorganisms (Appleman et. al, 1976).

Thin-layer chromatography bioautography is a method in which the

chromatogram with antimicrobial agent is sprayed with a suspension of the microoroganism. After incubation, tetrazolium salts are added to help visualize the areas which inhibit microbial growth. The salts react with the dehydrogenase present in living cells, allowing for the visibility of the zones of inhibition. One of the advantages of using this method is that it can be used to detect inhibition and antifungal activity of plant pathogens. A disadvantage is that it can be difficult to ensure that the agar and the plate are completely in contact (Dewanjee et al. 2015).

Another popular method is ATP bioluminescence, which measures the adenosine triphosphate produced by living microorganisms. The amount of ATP is quantified by the addition of D-luciferin to cells, which in the presence of ATP, converts to oxyluciferin. The light produced from this reaction is quantified by a luminometer. With this method, the relationship between cell viability and luminescence is linear (Balouiri et al. 2016). Advantages of the ATP bioluminescence assay is that it is an easy method, it is very sensitive, has a low cost, and is rapid - results can be obtained within minutes. The disadvantages include interference from the luminescence of food samples. It is also not a good method for detecting spores due to the low ATP level in spores (AIB 2013).

The Broth Dilution Method, performed by diluting the antimicrobial agent into a growth medium dispensed in either tubes or microtiter plates, is one of the most basic methods of measuring antimicrobial efficiency and the gold standard in minimum inhibitory concentration determination. Tubes or wells containing diluted microbial agent are inoculated with the microorganism and incubated. The minimum inhibitory concentration, or MIC, is the lowest concentration of the antimicrobial agent that inhibits the growth of the microorganism. However, because this inhibition is measured by visual

observation, the microbial agent must be a clear solution. As many antimicrobial agents are turbid, this method alone cannot be used alone to assess MIC, and reagents must be included in broth dilution assays in order to verify the MIC (Balouiri et al. 2016). Advantages to the broth dilution method include the ability to analyze quantitatively as well as the ease of standardizing assays. Disadvantages include the inability to vary the antimicrobial agents between samples; a new plate must be created for each sample (Tenover, 2009).

An effective reagent dye that can be used in combination with the broth dilution method is alamarBlue[TM], a cell viability indicator that works when living cells convert resazurin to resorufin, a fluorescent molecule. Resorufin produces a bright red/pink fluorescence that can be quantitatively measured by using a spectrophotometer. The amount of fluorescence is proportional to the number of living cells (ThermoFisher Scientific, 2018). One disadvantage of alamarBlue[TM] is that its mechanism works through metabolic pathways, which can be affected by each cell's reducing ability. However, alamarBlue[TM], unlike other reducing dyes, is not toxic to cells, is very sensitive, and is cost effective. AlamarBlue[TM] also works on both suspended and attached cells, making it additionally useful for biofilms (Zachari et. al, 2014).

Based on the above studies, the objective of this project is to evaluate the use of alamarBlue[TM] in the microbroth dilution assay as a bacterial viability indicator when testing antimicrobial emulsions. Carvacrol nanoemulsions and their effectiveness against *E.coli* O157:H7 and *S.* enteritidis in the microbroth dilution assay will be used as the model system for this study.

CHAPTER 2

MATERIALS AND METHODS

2.1. Bacterial Strains

Bacterial cultures *Escherichia coli* (*E. coli)* O157:H7 ATCC 43888 and

Salmonella enterica subspecies enterica serovar Enteritidis (*S. enteritidis*) ATCC BAA-

1045 were obtained from the American Type Culture Collection (Manassas, VA). Frozen

stock cultures were prepared by adding 1ml of tryptic soy broth (TSB) overnight growth

and 1ml of sterile 50% (w/v) glycerol to a cryotube vial and storing at -80 °C. Each

month, the frozen stock cultures were thawed and plated on tryptic soy agar (TSA) for *E.*

coli or xylose lysine deoxycholate agar (XLD) for *S*. enteritidis and incubated at 37 °C

for 24 h. This plate was used as the working stock. For each experiment, single colonies

of each were transferred to TSB and incubated for 24 h at 37 °C. The overnight growth

was the inoculum for microtiter dilution assays. Cell numbers were determined using the

serial dilution method (Landry et al. 2015).

2.2. Creation of Carvacrol and MCT Control Nanoemulsions

The 4% carvacrol nanoemulsion (40,000 ppm) was prepared by adding 1g of

carvacrol (Sigma-Aldrich, Cat# W224502-100G0K) to 1.5g of medium chain triglyceride

oil (Miglyol 812, Witten, Germany) and stirred at 125 RPM for 5 minutes. After mixing,

2.5g of Tween 80 (Sigma-Aldrich, Cat# P1754-500ml) was added and the mixture was

stirred for an additional 5 minutes at 125 RPM. The oil and Tween 80 mixture was then

titrated into 20g of 5mM sodium citrate buffer (pH 3.5) at a rate of 2mL/min. During

titration, the mixture was stirred continuously for 15 minutes at 600 RPM using a

magnetic stir bar. The emulsion was then filter sterilized by expelling through a sterile

0.45 µm syringe filter and stored in a sterile centrifuge tube at 4 °C for 3 weeks.

The MCT control emulsion was made using the same procedure, replacing the 1g of carvacrol with 1g of medium chain triglyceride oil (Chang et al. 2013).

2.3. Microtiter Plate Assay

The carvacrol nanoemulsion stock (40,000 ppm) was diluted 1:10 in TSB, then further diluted with a 1:2 dilution series in TSB to obtain 2000, 1000, 500, 250, 125, and 62.5 ppm, respectively. This dilution series was repeated with the MCT control emulsion, to create nanoemulsions without carvacrol. Aliquots (100 µl) of each dilution were then added to a 96-well microtiter plate (ThermoFisher, Cat# 14-245-197B) so that there were six wells containing each of six different concentrations of carvacrol or MCT control emulsion, in triplicate (Figure 1A). Into four wells, 100 µl of a negative control containing a mixture of 1ml of 40,000ppm carvacrol nanoemulsion, 1ml of TSB, and 1ml of saline was added. This was repeated using 1ml of MCT emulsion instead of carvacrol. Into four wells, 100 µl of TSB alone was added. Into eight wells, 100 µl of the inoculum was added. Four of these eight wells will have alamarBlue^{TM} dye reagent added, while the other four will not contain dye. Three of the six rows of nanoemulsion-containing wells were inoculated with 100 µl of microorganism. The microtiter plate was incubated for 24 h at 37 °C.

2.4. alamarBlue^{TM} Cell Viability Dye
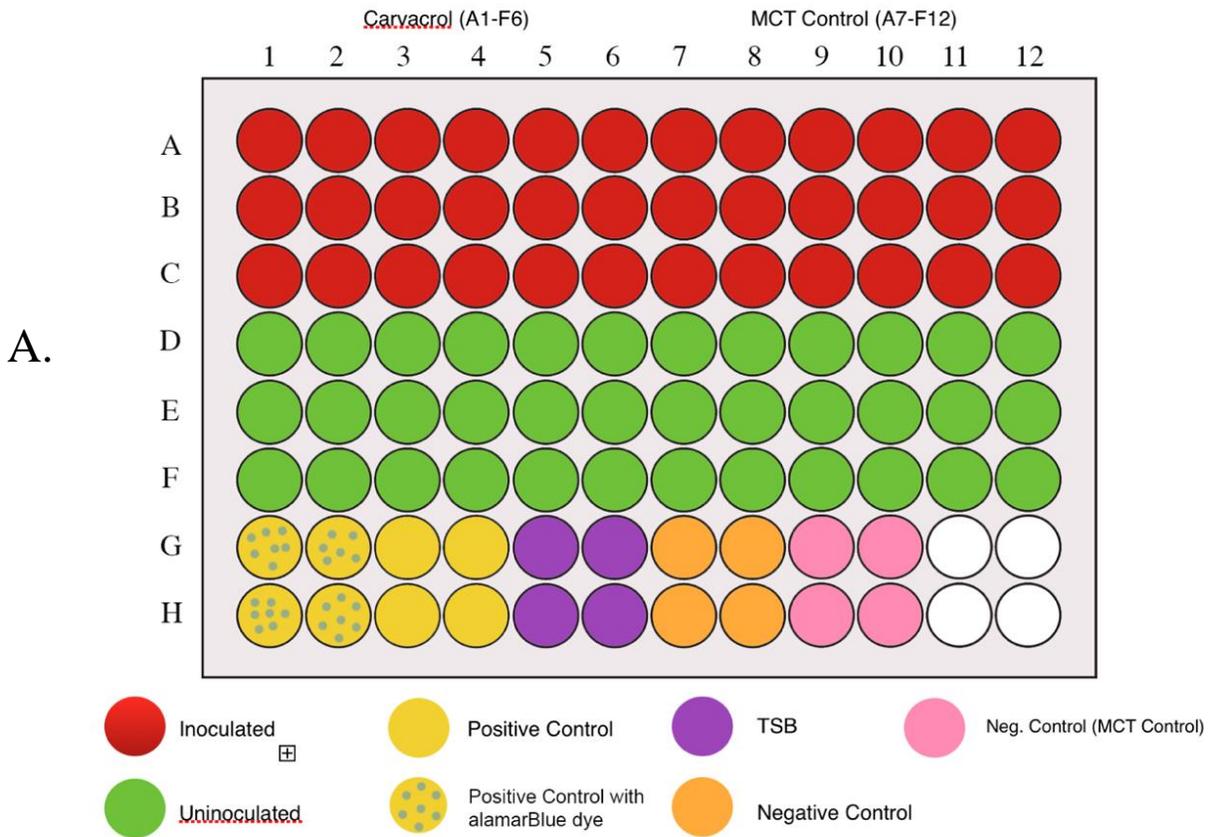
After 24h incubation, alamarBlue^{TM} (ThermoFisher) dye reagent was added to each of the inoculated and uninoculated wells after incubation, including four positive control wells (Figure 1B). 10 µl of the dye was added to wells. The microtiter plate was then incubated for one hour at 37 °C. After one hour, the color changes in the wells were

detected both visually and via fluorescence and absorbance. A blue color indicates wells with no cell growth, and a pink color indicates wells had cellular growth (Figure 1C).

2.5. MIC By Traditional Plating Assay

To confirm the minimum inhibitory concentration of carvacrol that inhibits bacterial growth, 0.1ml of the inoculum was added to tubes containing 9ml of serially diluted carvacrol containing 2000, 1000, 800, 500, 250, and 125 ppm, respectively. The carvacrol was diluted in TSB. Tubes were incubated for 24 h at 37 °C, after which the tube samples were plated on TSA, and incubated for 24 h at 37 °C. After incubation, the plates were observed for growth.
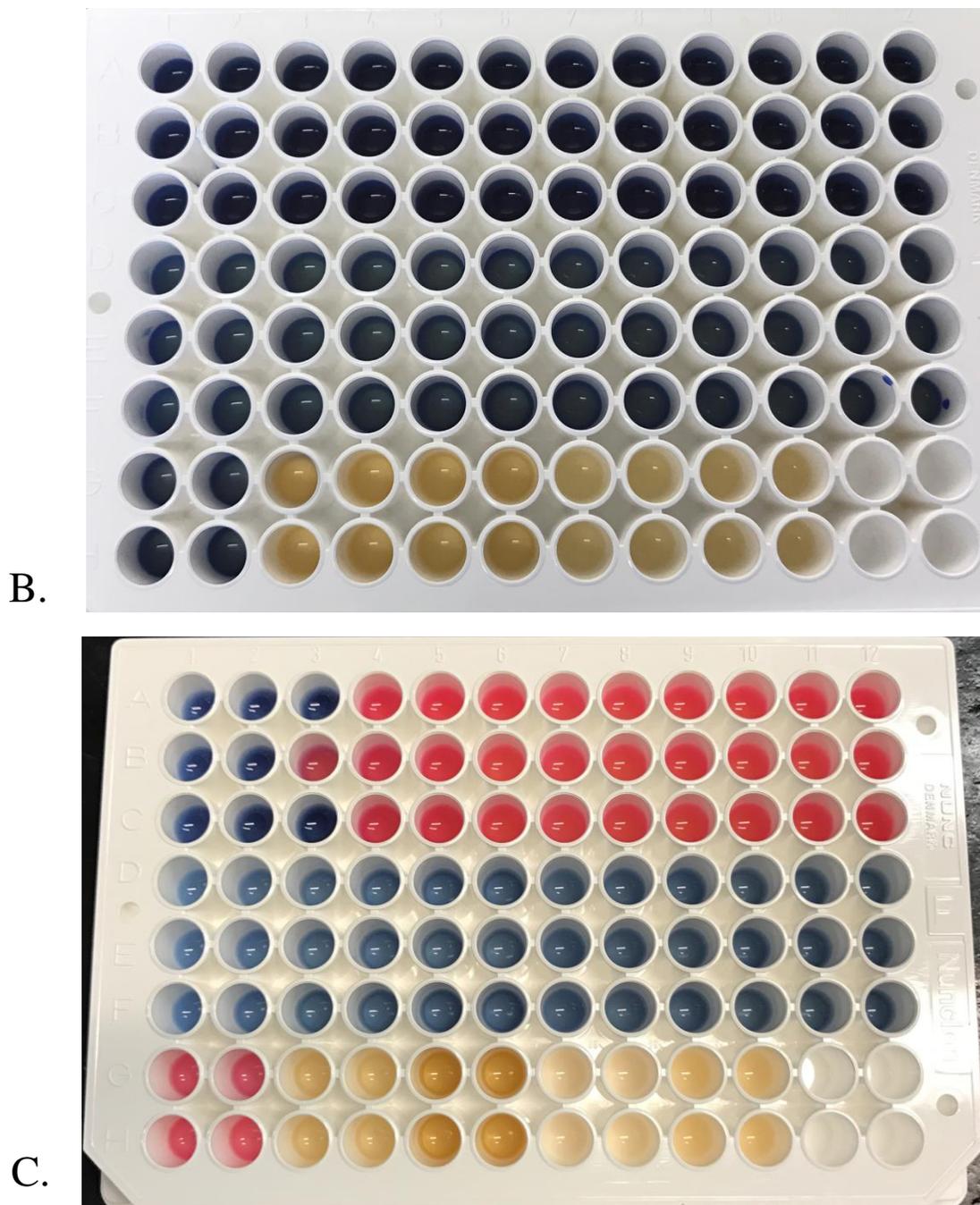
A.

Figure 2.1. Organization of the microtiter plate. A) 100 μl of each nanoemulsion dilution was added to a 96-well microtiter plate so that there were six wells containing each of six different concentrations of carvacrol or MCT control emulsion, in triplicate. Four wells contained 100 μl of a negative control containing carvacrol. Four wells contained 100 μl of a negative control containing MCT. Four wells contained 100 μl of TSB. Eight positive control wells contained 100 μl of the inoculum and TSB. Three of the six rows of nanoemulsion-containing wells were inoculated with 100 μl of microorganism. B) Microtiter plate with alamarBlue[TM] prior to 1h incubation. C) Microtiter plate with alamarBlue after 1h of incubation at 37°C. Pink wells indicate bacterial growth.

2.6. Standard Curve Assay

A standard curve assay was done to analyze the sensitivity of alamarBlue™. The inoculum was serially diluted tenfold in tubes. 100 µl of each dilution and 10 µl of alamarBlue were then added to microtiter wells. The plate was incubated for 1h at 37 °C. After incubation, the color changes in each dilution were analyzed both visually and via fluorescence and absorbance.

CHAPTER 3

RESULTS & DISCUSSION

3.1. Effectiveness Of alamarBlue™ as a Cell Viability Indicator

Due to the background turbidity of antimicrobial emulsions, screening the anitimicrobial effectiveness cannot be performed utilizing a microdilution assay in a microtiter plate. For this reason, we used alamarBlue™, a cell permeable resazurin dye (blue color, non-fluorescent, Figure 3.1A) that is reduced to resorufin (bright red color, and fluorescent, Figure 3.1B) in viable cells. Initial experiments were performed to show that with control emulsion (MCT alone) growth turbidity of *E.coli* and *S. enteritidis* could not be detected using a microtiter plate reader (results not shown), however after an hour incubation with alamarBlue™, the color change indicated that bacterial growth was present. This is similar to growth Figure 3.1B (Rows A-C, wells 7-12). Since the dye can be both qualitatively and quantitatively analyzed via fluorescence, we chose to use alamarBlue™ as a cell viability indicator and in determining MIC with antimicrobial emulsions.

3.2 Determination Of Level Of Viable Bacteria Needed To Cause A Color Change With alamarBlue™.

The initial bacterial number for the *E.coli* O157:H7 inoculum was 6.1×10^7 CFU/ml, and for *S. enteritidis* was 8.9×10^8 CFU/ml. Plates that contained less than 10^4 CFU/ml of *E. coli* did not display color change, while plates containing less than 10^5 CFU/ml of *S. enteritidis* did not display color change.

3.3. Effect Of Carvacrol on *E.coli* in Microtiter Plate Assay

The minimum inhibitory concentration of carvacrol was found to be 500ppm for

the inhibition of *E.coli* O157:H7. The color of the alamarBlue^TM dye changed from blue to pink when the concentration of carvacrol dropped below 500ppm (Figure 3.1). This is further confirmed in the fluorescence plate readings, which showed a sharp decrease in relative fluorescence units (RFU) in wells containing 500ppm of carvacrol or greater (Figure 3.2). When attempting to read the absorbance, color changes were far less clear due to the turbidity of the carvacrol nanoemulsion. Because of this, fluorescence readings were used for the remaining experiments.

3.4. Effect Of Carvacrol On *S.* enteritidis in Microtiter Plate Assay

The minimum inhibitory concentration of carvacrol was found to be 1000ppm for the inhibition of *S. enteritidis*. The color of the alamarBlue^TM due changed from blue to pink when the concentration of carvacrol dropped below 1000ppm (Figure 3.4). This is further confirmed in the fluorescence plate readings, which showed a sharp decrease in relative fluorescence units (RFU) in wells containing 1000ppm of carvacrol or greater. (Figures 3.5).

3.5. Effect Of Carvacrol On *E.coli* O157:H7 in MIC by Traditional Plating Assay

The minimum inhibitory concentration of carvacrol was found to be 500 ppm for the inhibition of *E.coli* O157:H7. Additionally, growth was reduced to undetectable levels at 800, 1000, and 2000 ppm.

3.6. Effect Of Carvacrol On *S. enteritidis* in MIC by Traditional Plating Assay

The minimum inhibitory concentration of carvacrol was found to be 800 ppm for the inhibition of *S. enteritidis*. Additionally, growth was reduced to undetectable levels at 1000 and 2000 ppm.
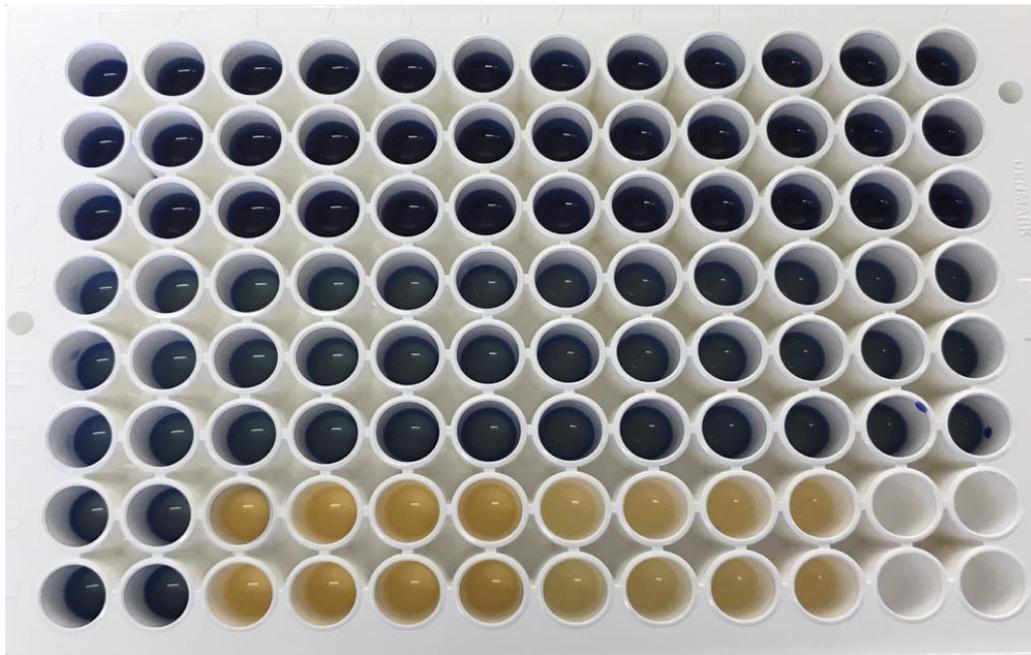
3.7. Effect Of Carvacrol On Other Bacteria

Carvacrol was additionally tested on *Listeria* monocytogenes, *Pseudomonas* aeruginosa, and *Lactobacillus* acidophilus, and it was found that it did not inhibit the growth of these bacteria.

3.8. Effectiveness Of Carvacrol As An Antimicrobial

Based on the above findings, carvacrol is shown to be effective as a bactericide for *E.coli, Salmonella*, and possibly other Gram-negative bacteria. It was able to reduce the bacterial growth of *E.coli* at 500ppm and *S.* enteritidis at 800ppm (Table 3.1). In its nanoemulsion form, it has the ability to be used in a wide range of food applications, including water-based food and drink (Chang et. al., 2013).

A.

B.

Figure 3.1. Microtiter plate assay utilizing alamarBlue™. A) Microtiter plate of *E. coli* O157:H7 (24h growth at 37°C) after addition alamarBlue™. B) Color change after 1 hour incubation at 37°C. Un-inoculated negative controls (rows D-F) all show blue color indicating no microbial growth. Inoculated positive controls either in TSB (G1 and 2, and H1 and 2), and MCT emulsion controls (Rows A-C, wells 7-12), all show red color, indicating bacterial growth. Antimicrobial emulsions were added at concentrations of 2000 (A1, B1, C1), 1000, (A2, B2. C2), 500 (A3, B3, C3), 250 (A4, B4, C4) 125 (A5, B5, C5), and 62.5 (A6, B6, C6)
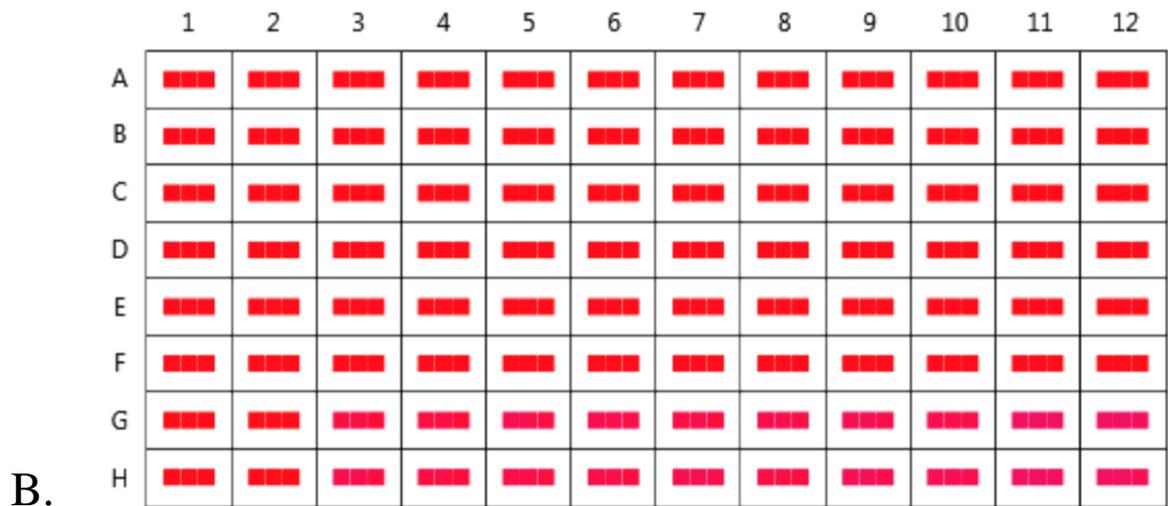

A.

Figure 3.2. Well scan fluorescence and absorbance readings of the microtiter plate for *E.coli* O157:H7. A) Fluorescence reading. Color change is evident after the third well (500ppm.)
B) Absorbance reading. Note the lack of color change, indicating that absorbance is not an ideal way of analyzing the color changes of alamarBlue^{TM} dye reagent.
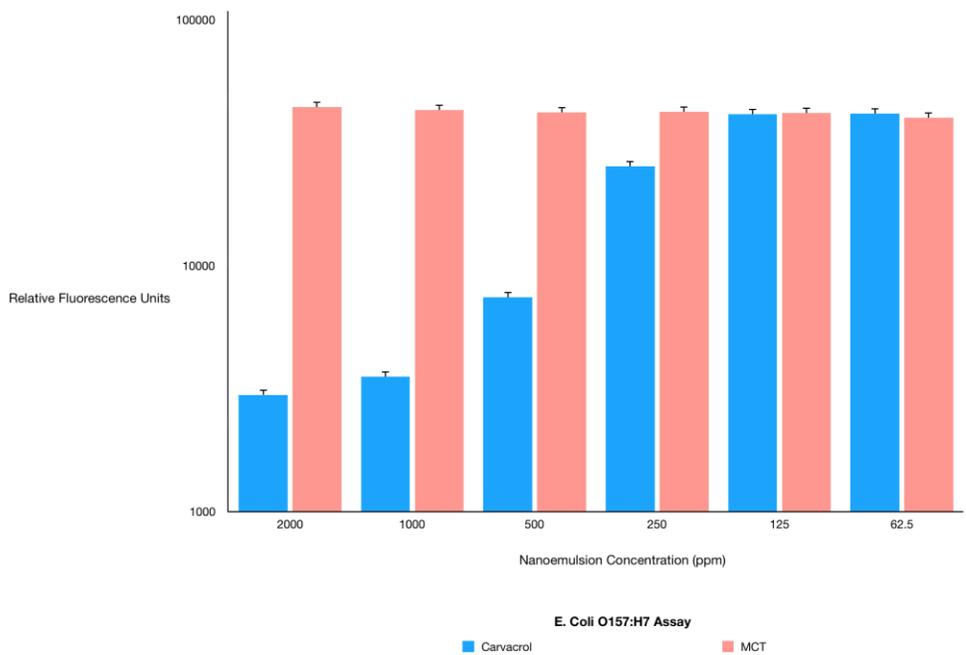

Figure 3.3. RFU versus concentration of nanoemulsion. *E.coli* O157:H7 is shown to be inhibited at 500ppm.

Figure 3.4. The effect of carvacrol on *S. enteritidis*. Color change is evident after the second well (1000ppm.)
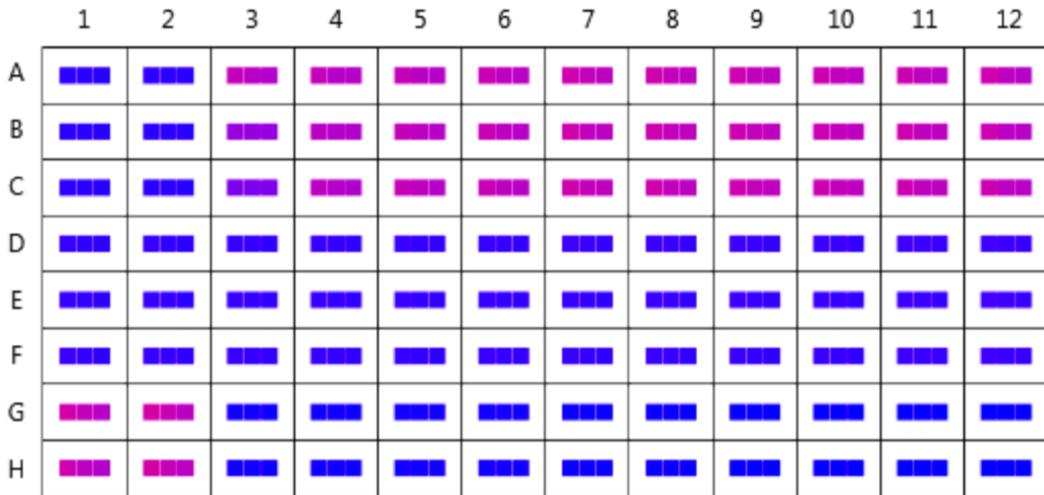

Figure 3.5. Well scan fluorescence reading of the microtiter plate for *S. enteritidis*. Color change is evident after the second well (1000ppm.)

Figure 3.6. RFU versus concentration of nanoemulsion. *S. enteritidis* is shown to be inhibited at 1000ppm.

MIC

	Color	Fluorescence	Traditional Plating
E. Coli	500 ppm	500 ppm	500 ppm
Salmonella	1000 ppm	1000 ppm	800 ppm

Table 3.1. MIC as determined visually (color), by fluorescence, and by traditional plating. Based on the traditional plating method, which is considered the most accurate, the MIC of carvacrol for E. coli is 500ppm and for *S.* enteritidis is 800ppm.

3.9. Effectiveness Of The Microtiter Plate Assay

The microtiter plate assay was shown to be effective in determining the MIC of both *E.coli* and *S. enteritidis.* By diluting the emulsion from 2000 to 62.5 ppm across the wells, it was easy to see where bacterial growth stopped as determined by the color change of alamarBlue^{TM}. Using white plates instead of clear plates was also effective for reading the fluorescence of alamarBlue^{TM}. One issue that was experienced in this assay was in-between color changes that were not clear to read. Because of this, the traditional plating method for determining MIC was necessary in order to confirm the concentrations at which bacterial growth was reduced. It was also necessary to develop a standard curve assay to determine the sensitivity of alamarBlue^{TM} and the lowest CFU/ml of growth detectable by the dye.
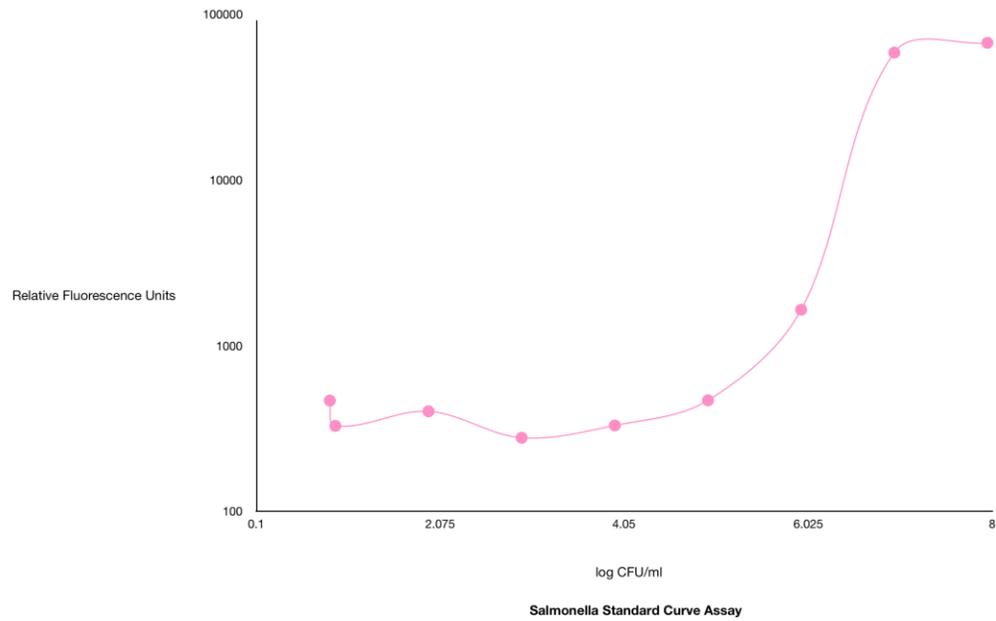
3.10. Effectiveness Of The Sensitivity Assay

The sensitivity assay was necessary to determine the lowest CFU/ml detectable by alamarBlue^{TM}. By diluting the inoculum in a 1:10 dilution series across the microtiter plate wells and adding dye to each well, it was clear to see which wells displayed color change. Because the initial cell numbers of the inoculum were known, it was simple to calculate the cell number in each well (Figure 3.7).

3.11. Discussion

Because carvacrol and other antimicrobial essential oils are derived from use and effectiveness of carvacrol as an antimicrobial has been observed in many studies. Carvacrol works by disrupting the cytoplasmic membrane of a bacterial cell, effectively causing cell death (Landry et. al., 2015). Because pure carvacrol has poor water solubility, it is necessary to emulsify it so that it can be used in aqueous solutions. One

18

A.

Salmonella Standard Curve Assay

B.

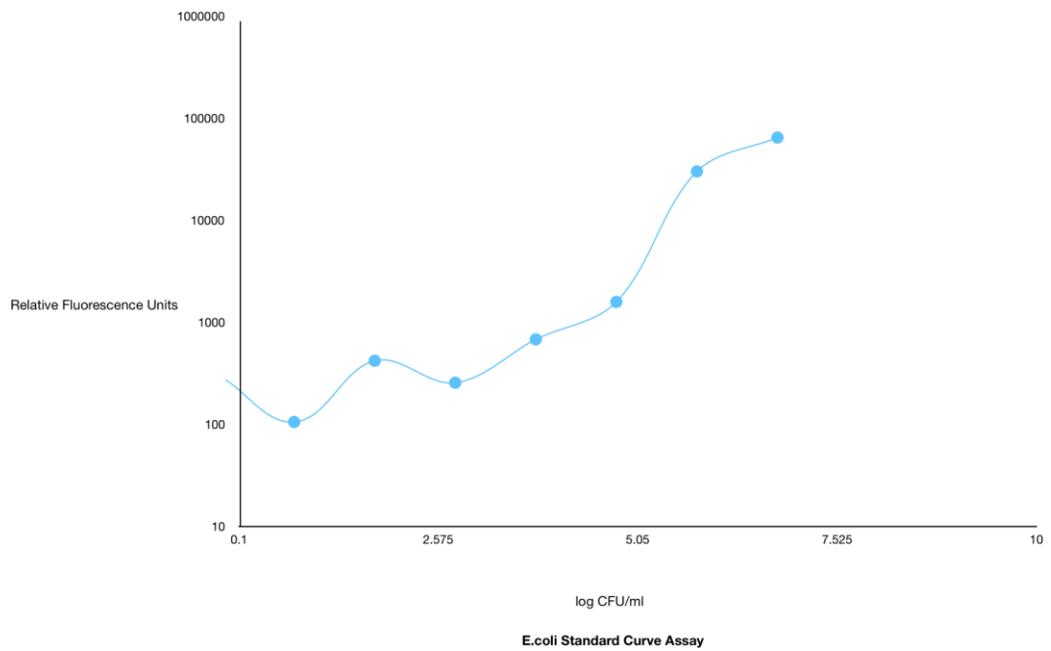E.coli Standard Curve Assay

Figure 3.7. Sensitivity of alamarBlue^{TM}. A) The lowest CFU/ml of *E. coli* detectable by alamarBlue^{TM}. is 10^4 CFU/ml.

B) The lowest CFU/ml of *S. enteritidis* detectable by alamarBlue^{TM}. is 10^5 CFU/ml.

issue that occurs with carvacrol nanoemulsions is that they are thermodynamically unstable, and the oil droplets can only remain dispersed for about three weeks at 4 °C before separation occurs. However, creating the nanoemulsion is a relatively fast and simple process.

Carvacrol was able to successfully inactivate *E. coli* at 500ppm and *S. enteritidis* at 800ppm. The use of essential oils as antimicrobial agents is of interest in the food processing industry, as consumers are increasingly becoming more interested in the use of natural compounds in the place of traditional agents. Developing an antimicrobial treatment that follows food grade and GRAS standards can provide an alternative to antibiotics, which cause bacterial resistance that poses a threat to human health. While the success of carvacrol as an antimicrobial treatment is apparent, a solution is still needed for reducing the growth of *Listeria monocytogenes* and other commonly encountered foodborne pathogens. In future studies, testing the efficiency of antimicrobial essential oils in reducing the growth of other pathogens is necessary before they can eventually be used as treatments in the food industry.

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