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Influence of Inoculum Preparation upon Sensitivity of Common Food Borne Pathogens to Emulsion Based Antimicrobials

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INFLUENCE OF INOCULUM PREPARATION UPON SENSITIVITY OF COMMON

FOOD BORNE PATHOGENS TO EMULSION BASED ANTIMICROBIALS

A Thesis Presented

By

DILLON S. D. MURRAY

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Department of Food Science

DEDICATION

To my fiancée Arline, whose encouragement and patience did not go unnoticed.

ACKNOWLEDGMENTS

ABSTRACT

INFLUENCE OF INOCULUM PREPARATION UPON SENSITIVITY OF COMMON

FOOD BORNE PATHOGENS TO EMULSION BASED ANTIMICROBIALS

FEBUARY, 2016

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Antimicrobial optimization procedures use the most resistant bacterial culture that could be present in the food to determine the levels of treatment needed to ensure safety. These procedures usually only focus on one method of preparing these inoculums for testing despite prior research showing that the preparation of the culture can influence how the culture reacts to a treatment. In this work, planktonic cells grown in a liquid media and sessile cells grown on a similar solid media were subjected to identical emulsion based antimicrobial systems. The cultures were monitored over time and their numbers periodically enumerated. Weibullian destruction models were used to characterize bacterial death and the different inoculum preparations were separated using ANOVA statistical tests. Using these models highly significant differences between the different sessile and planktonic methods of growth were found. This difference was also found to not be related to the production of curli used in biofilm formation. These results suggest that the methods of inoculum preparation can be a significant factor in bacterial survival, a factor that should be included in food antimicrobial optimization procedures.

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ABBREVIATIONS

Media:

TSB= Tryptic Soy Broth

TSA = Tryptic Soy Agar

MCT = Medium Chain Triglycerides

PBS = Phosphate Buffered Saline

Other:

RPM = revolution per minuet

INTRODUCTION

All common antimicrobial concentration optimization procedures in foods use a single common inoculum preparation. Overnight liquid broth cultures are used due to the ease of producing reproducible cell numbers and the general simplicity of their preparation and application to foods.

Some research has been conducted looking at how bacteria grown on solid surfaces then subsequently tested in antimicrobial systems are more resistant to the stresses of desiccation when compared to an equivalent liquid culture (Uesugi et al., 2006). These tests only looked at desiccation, just one form of many antimicrobial treatments. If there is a form of bacterial inoculum more resistant to other antimicrobial treatments that current testing methods do not account for, than it would be easy for a company or agency to miscalculate and underestimate the amount of antimicrobial required for any particular application.

The purpose of this research is to evaluate how the growth of bacteria on surfaces alters the sensitivity or resistance to a chemical antimicrobial system. The objectives are as follows:

1) Evaluate the ability of growth conditions to alter antimicrobial systems

2) Determine the most resistant bacterial inoculum preparation for antimicrobial testing

3) Isolate some of the factors influencing the difference between inoculum preparations.

4) Streamline the inoculum preparation method to make its use in testing situations

   easy, and therefore more likely to be used

CHAPTER I

LITERATURE REVIEW

Food Borne Illness

Current

Although many diseases can be contracted through a food borne vector, there are sets of bacterial infections that are predominantly viewed as an issue in foods. The most recent reports by the CDC show that there are over nine million illnesses in the United States each year caused by food borne pathogens. Of this it is suspected that 56,000 are hospitalized and 1,300 die. Within the bacterial pathogens, nontyphoidal *Salmonella* spp. are the primary causative agent of foodborne illnesses with STEC *Escherichia coli* O157 and *Listeria monocytogenes* ranking at 9th and 16th respectively (Elaine et al., 2011).

These numbers do not completely illustrate the severity of the diseases caused by these bacteria. The high mortality rate of *L. monocytogenes* and *E. coli* O157 cause them to be ranked 2nd and 7th in deaths caused by foodborne bacterial infections. Because it is impossible to know exactly when and how an infection was transmitted these numbers are only estimates, but do give the best idea of how large of a problem foodborne illness is for this country (Jay et al., 2005).

Escherichia coli

Escherichia coli is a Gram negative rod shaped intestinal bacterium associated with the lower intestine of many warm blooded organisms. Although most *E. coli* spp. are harmless or opportunistically pathogenic some strains can be extremely dangerous to humans. The German sprout outbreak in 2011 involving Shiga toxin producing *E. coli* was responsible for 3950 illnesses and over 50 deaths (Buchholz et al., 2011).

Salmonella enterica

Salmonella enterica is a Gram negative rod shaped bacterium. Although the disease can travel through any fecal oral route contamination, it is estimated that 95% of cases are foodborne caused by contamination of the raw product or post processing contamination within a food facility (Hohmann, 2001).

S. enterica's ability to habituate and grow at water activities as low as 0.91 or survive in low water activity foods such as peanut butter (Aw=0.70) causes problems in the food industry with foods usually considered safe from microbial activity (Mattick et al., 2000). The reduced water activity environment has been shown to increase the organism's ability to withstand higher levels of heat treatments when compared with *S. enterica* grown under more regular conditions (Mattick et al., 2001)

Listeria monocytogenes

Listeria monocytogenes is the causative agent of listeriosis. It is a Gram positive facultatively anaerobic rod. It is most notable as a food borne pathogen for its unusual growth conditions and high mortality rate. Growth at refrigerated temperatures as low

as 1 °C allows the normally easily outcompeted *L. monocytogenes* to grow uninhibited by other bacterial populations (Farber and Peterkin, 1991). Due to its intercellular mechanism of travel within the body, *L. monocytogenes* can evade the host immune system as well as cross over the blood brain barrier and the infant placental barrier. Although healthy individuals can often fight the infection, immunocompromised individuals such as the elderly and pregnant can be at risk; contributing to *L. monocytogenes* high 23% mortality rate (Wilson and Salyers, 2011).

Antimicrobial Treatments

Food additives can be used for a wide variety of purposes in food systems, from food preservation to increased sensory attributes. Their use as bacteriostatic and bactericidal treatments inhibit the growth of or reduce the bacterial load of a food increasing safety and shelf life (Davidson et al., 2005).

Essential Oils

Essential oils have been used in food for a multitude of purposes for centuries. One of the most prevalent uses is to protect against foodborne illness using their bactericidal and bacteriostatic abilities. (Kalemba and Kunicka, 2003). In the late 1970's there was a resurgence in interest in use of essential oils as an alternative to nitrite use as an antimicrobial. Its continued use in modern food culture can be attributed to its use as a label friendly alternative to more traditional antimicrobials (Jay et al., 2005). One such example of an antimicrobial essential oil is carvacrol, a monoterpenoid phenol. Carvacrol (Figure 1.1) is an essential component of the aroma profile of thyme and oregano and has

promising antibacterial effects on a range of both gram positive and gram negative bacteria.
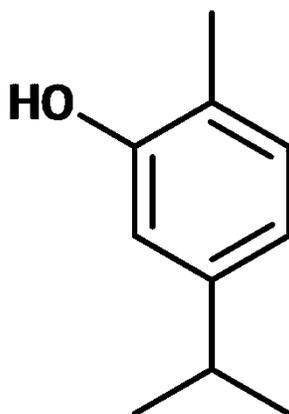
Figure 1.1: Carvacrol Molecule

Previous literature has found that carvacrol caused an increase in the ionic permeability of the cell membrane leading to the degradation of the ion potential required to sustain cell functions (Ultee et al., 1999). The hydrophobic nature of carvacrol and other essential oils is essential to its antibacterial effect; similar compounds with reduced lipophilicity had reduced antimicrobial efficacy. It has been speculated that their hydrophobic nature allows the compound to associate with the target membrane a necessary action to disrupt said membrane (Ben Arfa et al., 2006). Although the hydroxyl side group reduces the ability of carvacrol to associate with the cells membrane, its presence is essential to the antimicrobial efficacy of the chemical (Ultee et al., 2002).

Nanoemulsion

The antimicrobial activity of many essential oils has been amply demonstrated, however there are problems that must be overcome if they are to be used in a wide variety

of foods. The oils are only marginally soluble in an aqueous solution (Haynes, 2013); a problem that would prevent their effectiveness in a majority of food matrices. To combat this a protocol was developed to incorporate carvacrol into a nanoemulsion to increase its solubility in water while maintaining antimicrobial efficacy (Chang et al., 2013).

In a general sense, an emulsion are small droplets of one liquid dispersed into another immiscible liquid through the use of an emulsifier. A nanoemulsion refers to any emulsion that has a dispersed drop size between 100 and 600 nanometers (Bouchemal et al., 2004). By creating an oil in water (O/W) emulsion it is possible to deliver the essential oil into a food system that would otherwise be impossible. Nanoemulsions have been used as delivery systems for a wide variety of lipophilic bioactive compounds, from vitamins (Relkin et al., 2009) to other antimicrobial oils (Chang et al., 2012).

The nanoemulsion concurrently counteracts one of the other major problems with using essential oils in a food system. The oil's high volatility and very low sensory detection threshold as well as the low acceptable sensory threshold makes using an essential oil hard to incorporate into many foods where the levels needed to be an effective antimicrobial would be detrimental to the flavor profile. The encapsulation of the oil in the nanoemulsion helps to contain the oil, lowering its sensory effect onto the food system it is placed into (Hyldgaard et al., 2012).

The formation of a O/W emulsion requires the breaking of the oil phase into small droplets that can be surrounded by an emulsifier to stabilize the large interfacial tension between the two phases (McClements, 2005). This can be done using large high pressure homogenizers or sonicators to disrupt the phases, but these methods require a large

investment in machinery by a company wishing to produce the emulsion (Chang et al., 2013). The second option would be the low energy spontaneous formation of the emulsion and would require less of an investment by a company before production. The method relies on the interfacial budding of small droplets at the oil and water boundary. The advantage of these low energy methods is the low cost of the machines required to create the nanoemulsion and the low level of training required for the staff before making the emulsion stock solution.

Antimicrobial Testing and Optimization

Optimization of the parameters of an antimicrobial treatment is essential to the development of a new food product or antimicrobial system. There are multiple issues that must be overcome in the optimization of an antimicrobial. Cost vs. effectiveness is the most relevant to the implementation of an antibacterial treatment in a food processing plant. No matter how effective a compound is at mitigating bacterial load of a food, it will never be implemented if the company deems the cost too high. Part of the hazard analysis of any food product is the determination of what hazards can reasonably be present in a food and what needs to be done to control them. This includes what a reasonable level of bacterial contamination on a food can be expected, and what level of antimicrobial treatment is necessary to mitigate the risk while maintaining fiscal sense.

One problem in the optimization of antimicrobial protocols is determining the bacterial inoculums that will be used in testing. This includes both the species and strains of bacteria used in addition to the growth conditions of the bacteria prior to use in the

experiment. Many protocols (Concha-Meyer et al., 2014; Lathrop et al., 2014; Santillana Farakos et al., 2014) use overnight bacterial cultures in the stationary phase of growth as the bacterial cells to be tested. During the stationary phase the bacteria are not dividing at the high rate of exponential cells, leaving them more resistant to many antimicrobial treatments (Matsuo et al., 2011). While the stationary phase bacteria are the most resistant to death compared to exponential cells in a single liquid media, it does not mean they are the most resistant form of bacteria, nor does it mean that the broth grown cells resemble the bacterial load of a food prior to treatment. Surface contamination and growth is possible in a variety of situations (Glass and Doyle, 1989) and will have different characteristics than that of a liquid grown culture. Previous research has found that the survivability of S. *enterica* to certain stresses varied between different inoculum preparations (Uesugi et al., 2006).

Variation in Inoculum Preparation

Biofilm Antimicrobial Resistance

It has long been known that the antimicrobial resistance of a biofilm is greater than that of their planktonic twins and current literature points towards a multifactorial mechanism of resistance. The most obvious of these is the presence of an extracellular matrix that physically inhibits the rate of diffusion of any antimicrobial into the biofilm. The high level of cellular debris and other organics also can contribute by deactivating the bioactive compounds before they reach the core of the cells (Donlan, 2000).

Nutrient and oxygen depletion of the core of the biofilm forces many of the cells to enter a mode of growth similar to the stationary cells in a planktonically growing bacterial culture (Donlan, 2000). The rapid replication of cells in the exponential phase of bacterial growth is partially the case for increased effectiveness of certain antibiotics. The unraveled DNA in the nucleus is an easier target and the constant need to produce a wide variety of cellular proteins gives certain antimicrobials more of an opportunity to function (Matsuo et al., 2011).

Colony Model

Bacterial colonies share many characteristics with biofilms that make them an attractive choice for studying biofilms. One of the best similarities is that of the physical mass of the colony that would slow or inhibit the access of any antimicrobial into the system (Corcuera et al., 2013). They are not however a perfect representation of a biofilm as the protein expression, as viewed in a *Pseudomonas aeruginosa*, is more similar to that of planktonic cells than that of a true biofilm. (Mikkelsen et al., 2007)

One research group looked at the resistance of colony grown cells to desiccation and heat treatments outside of the solid mass of cells in order to remove the physical barrier the colony provides to the cells on the inside of the mass. By suspending a lawn of cells in the same liquid media and adjusting the concentration to match an overnight culture, the two cultures can be compared on equal footing (Uesugi et al., 2006). Although this study did not find a significant difference between the cells ability to resist long term heat treatments, they did see a very significant difference in the initial desiccation resistance of

the cells. The cultures were inoculated onto seed hulls at equal concentrations, but after drying the cells onto the hulls they found that the cells grown on a solid medium were more resistant to the stresses of imposed on cells while drying.

This finding shows that there is a difference in cell resistance to some stresses and is the basis for much of the research conducted in this thesis.

Bacterial Death Modeling

Bacterial growth and death modeling allow the researcher to predict how an organism will react in varying circumstances not explicitly tested and to give more options in comparing different treatments.

Modified Weibull Model

The Weibull kinetic death model used is based off of the probable distribution curve outlined by Waloddi Weibull (Weibull, 1961) which used the model to describe fatigue testing and failure rates of various materials. When the Weibull distribution is used to create a population survival curve based on the percentage of a population surviving gives the following equation.

$$S(t) = 10^{-\left(\frac{t}{\delta}\right)^{\beta}}$$

Equation 1-1: Weibull Survival Function

Substituting N_t/N_0 to calculate percentage of the bacterial culture surviving and converting all numbers to a log scale gives the equation (Mafart et al., 2002)

$$\log_{10} N_t = \log_{10} N_0 - \left(\frac{t}{\delta}\right)^{\beta}$$

Equation 1-2: Weibull Bacterial Survival Curve

Given that N is the number of surviving bacterial cells, N_0 is the original population,

δ and β determine the overall shape and location that the survival curve takes, and t is

time.

The parameters in these equations assumes an asymptotic approach to zero. Many

studies before have illustrated the existence of a certain subsection of a bacterial

population are more resistant to an antimicrobial treatment than others (Dawson et al.,

2011). To account for this in the model an additional perimeter of N_{res} is included. N_{res}

was introduced as a new minimum population in the model that the equation

asymptotically approaches (Albert and Mafart, 2005). In a bacterial model it is the number

of cells that are capable of resisting and surviving the antimicrobial treatment. The new

equation states

$$N_t = (N_0 - Nres) * 10^{-\left(\frac{t^{\beta}}{\delta}\right)} + Nres$$

Equation 1-3: Weibull Model plus Tail

Or

$$\log_{10} N_t = \log_{10}\left(\left(10^{\log_{10}(N_0)} - 10^{\log_{10}(N_{Res})}\right) * 10^{-\left(\frac{t}{\delta}\right)^{\beta}} + 10^{\log_{10} N_{Res}}\right)$$

Equation 1-4: log_{10} Weibull Model plus Tail

Perameter	Defines
t	Time
N_0	Original Bacterial Population
N_{Res}	Residual Risistant Bacterial Population
δ	Shape of Curve
β	Location of Curve

Table 1.1: Modified Weibull Parameters

<u>Curve Fitting</u>

Once a curve equation has been established and a dataset has been obtained, there must be a method of calculating a curves parameters based off of the data and calculating the accuracy of the predictive mode. Although hand calculation of these parameters is possible for simple linear or exponential curves, as the model increases in complexity it becomes exponentially more difficult to calculate these values. Statistical software such as SAS allow these complex models to be calculated and used.

These model parameters often utilize a least squares method to calculate the best fit. The user must begin the process with a model to be tested and a rough approximation of what the parameters of said models will be (SAS, 2013). This process must be done through knowledge of how each perimeter is related to the data to establish a general vicinity of the value followed by testing the least mean square error program to minimize the final error of the model.

The software then compares the model generated by the parameters provided and calculates the mean square error between the points of the model with the points of data from an experiment. Then a single perimeter is adjusted by a small amount and the mean square error is recalculated. After countless permutations of this process are performed,

a final fitted equation is generated and all of the parameters and information pertaining

to the error of the model compared with the data (Bertsekas, 1997).

CHAPTER II

OBJECTIVES

1) To study the influence of inoculum preparation for assessing effectiveness of carvacrol nanoemulsions using mathematical modeling.

2) Use mathematical modeling to characterize and compare antimicrobial destruction curves

3) Explore the mechanisms involved in relative inoculum sensitivity to carvacrol nanoemulsions

CHAPTER III

MATERIALS AND METHODS

Bacterial cultures and cultivation

E. coli (ATCC 43889), *L. monocytogenes* (ATCC BAA-19112), *S. enteritidis* (ATCC BAA-1045 and ATCC BAA-710) stock cultures were maintained at -80°C in tryptic soy broth (TSB) supplemented with 25% v/v glycerol. Working cultures were created by plating onto tryptic soy agar (TSA) and incubated at 37°C for 24h before storing at 4°C for one month.

Prior to each experiment, an isolated colony from the bacterial stock culture was transferred into TSB and incubated statically for 24h. Planktonic inoculum for antimicrobial destruction assays was prepared by transferring 100μL of overnight culture into 10mL of TSB and incubated at 37°C for 24h. Optical density was recording using a spectrophotometer at 600nm. Cell numbers were adjusted by dilution into 0.85% sterile saline and initial numbers were confirmed through plating inoculum onto TSA. Cultures were then diluted 3 log into 0.85% NaCl to approximately 1×10^6 CFU/mL with the final dilution containing the antimicrobial compound at specified concentrations if used.

Sessile bacterial inoculum for antimicrobial destruction assays was prepared by streaking two TSA plates with 500μL of an overnight culture followed by incubation at 37°C for 24 hours. Each bacterial lawn was removed using a sterile cell scraper then

transferred into a 5mL tube of TSB and vortexed at 8000rpm for 10 seconds to suspend. Suspended cells were pipetted into 10mL of fresh TSB to match the OD_{600} of the overnight planktonic inoculum. Cell numbers were adjusted by dilution into 0.85% sterile saline and initial numbers were confirmed through plating inoculum onto TSA. Cultures were then diluted into 0.85% NaCl to approximately 1×10^6 CFU/mL with the final dilution containing the antimicrobial compound at specified concentrations if used.

Curli Morphology Determination

To identify curli production by *Salmonella sp*, overnight bacterial culture (TSB at 37°C) was streaked onto Congo Red Agar Plates (TSA supplemented with 40μg/mL Congo Red and 20μg/mL Coomassie Blue) and incubated at 32°C until the rdar colony morphotype could be observed (48-72h).

In experiments that take into account cellular curli production, the original inoculum and inoculum post experimentation were plated onto Congo Red Agar Plates to confirm initial morphotype and to check for morphological revertants (Monteiro et al., 2011). If ether test came back inconsistent then the data points from the test were excluded from statistical analysis.

Antimicrobial Destruction curves

Original bacterial sample (approx. 1×10^6 CFU/ml) was diluted in a series of 1:10 dilutions in sterile 0.1% PBS (pH 7.4). The dilutions were then plated using the drop plate method (Herigstad et al., 2001) plating five 10μL drops per dilution onto TSA plates.

Plates were incubated for 12h at 37°C before enumeration. During specified intervals the samples were collected, diluted and plated until the conclusion of the experiment

Nanoemulsion preparation

The carvacrol nanoemulsion was created using the protocol previously described in the literature (Chang et al., 2013). A solution containing 4g Carvacrol is placed onto beaker while magnetically stirring at 500rpm. 6g of MCT reported by the manufacturer to contain 50-65% caprylic acid (C8:0), and 30%-45% capric acid (C10:0) was added to the beaker and mixed until there was no visual separation between the reagents. This process was repeated after adding 10g of Tween 80 to the beaker. 80mL of a 5mM citrate buffer (pH=3.5) was placed into a beaker magnetically stirring at 500rpm. All 20g of the oil and emulsifier were then titrated into the citrate buffer at the rate of 2mL/min. The emulsion was left stirring for five minutes post addition of oil phase to stabilize. Once completed, the nanoemulsion was filter sterilized at 0.45μm and stored at 4°C for up to one month before use. A control emulsion was prepared using 10g of MCT and 10g Tween 80 titrated into 80mL Citrate buffer in an identical method as before.

Planktonic Cell Growth Curve

Overnight TSB bacterial culture was adjusted to an OD_{600} of 1.0. 100 μL of adjusted culture was used to inoculate 10mL of fresh TSB and grown with shaking at 100rpm. This culture was monitored every hour using spectrophotometer and every 4 hours plating cell numbers on TSA.

Sessile Cell Growth Curve

Black polycarbonate 0.22µm filters were placed on a sterile petri dish and exposed to UV light for 15 min. Filters were then flipped and exposed to 15 further min. of UV light. After treatment filters were moved to the surface of a TSB+A plate and stored at room temperature until experimentation. Culture adjusted to OD600 1.0 used for liquid inoculation was further adjusted with sterile TSSB down to an OD600 of 0.05. 20µL of this culture was pipetted onto the surface of the black polycarbonate filters on TSB+A to form a "colony".

Plates were incubated statically at 37°C. Periodically filters were placed into a sterile 1.5mL microcentrifuge tube, in triplicate, with 1.0 mL of sterile peptone. Tubes were the vortexed for 15 seconds creating the 10^0 sample. Samples were then further diluted in sterile peptone and enumerated using the drop plate method.

Colony Cellular Density

Once the cultures are grown on the .22um polycarbonate filters, they can be removed and weighed on an analytical balance. Once the colonies are weighed and subsequently enumerated, the g/CFU can be calculated to assess the cellular density.

Phase Dependent Bacterial Destruction Kinetics

Cells harvested in the exponential phase and stationary phase of growth, as determined by the previously describe methods and growth curves, will be used in the bacterial destruction kinetics experiments. Liquid culture cells were washed through centrifugation; centrifuged at 6000rpm for 5 min, then re-suspended in an equal volume

of sterile saline. Solidly grown cells were first suspended in sterile saline and washed

through identical centrifugation methods. Both methods were adjusted to create 5 mL of

approximately 10^7 CFU/mL

Volumes of the spontaneously emulsified carvacrol nanoemulsion were then added

to all samples in order to achieve a set of antimicrobial concentrations. The samples were

then stored statically at 20°C and sampled over time using identical methods to those

described above for the initial experiments.

Phase Dependent Antimicrobial Efficacy

Prepare sessile colonies identically to the Phase Dependent Bacterial Destruction

Kinetics experiments. Once inoculated, remove colonies in triplicate and weigh on an

analytical balance teared to uninoculated polycarbonate filter on TSA. Suspend cells into

a 5 mL solution and enumerate through plating on TSA.

Add stock solution of spontaneously emulsified carvacrol nanoemulsion to attain

optimal antimicrobial concentration (to be determined). Enumerate after four hours and

compare to cell concentration prior to antimicrobial treatment.

Compare level of cell destruction with the g/colony and g/CFU calculations.

CHAPTER IV

RESULTS AND DISCUSSION

Control study. Carvacrol nanoemulsions are prepared by mixing carvacrol with a carrier oil (MCT) and surfactant (Tween80) which is then added to a buffer system (5 mM citric acid pH 3.5). In order the assure that the antimicrobial activity was due to the carvacrol and not the surfactant or MCT oil, identical emulsions were prepared with and without the addition of carvacrol (Fig 4.1).   The control emulsions showed no antimicrobial activity.

Influence of inoculum preparation upon sensitivity to carvacrol nanoemulsions. Previous research has found that the survivability of S*. enterica* to certain stresses varied between different inoculum preparations (Uesugi et al., 2006). Therefore, we wished to determine if there were differences in antimicrobial susceptibility between inoculums prepared from broth or plate grown cells (planktonic vs sessile). The results can be seen in Figure 4.2. The destruction graphs show that when grown under identical temperature and nutrient conditions, *S. enterica* cultures grown under sessile and planktonic conditions were significantly different to a p=0.05 using a repeated measure ANOVA tests.   This definitively shows that cells grown on solid media are differentially effected by antimicrobials than their broth counterparts.

One hypothesis for the differences in antimicrobial susceptibility between sessile and planktonic grown inoculums, is that it is possible that plate grown cells may be producing curli fimbriae. Curli, or "thin aggregative fimbriae" are associated with biofilm formation
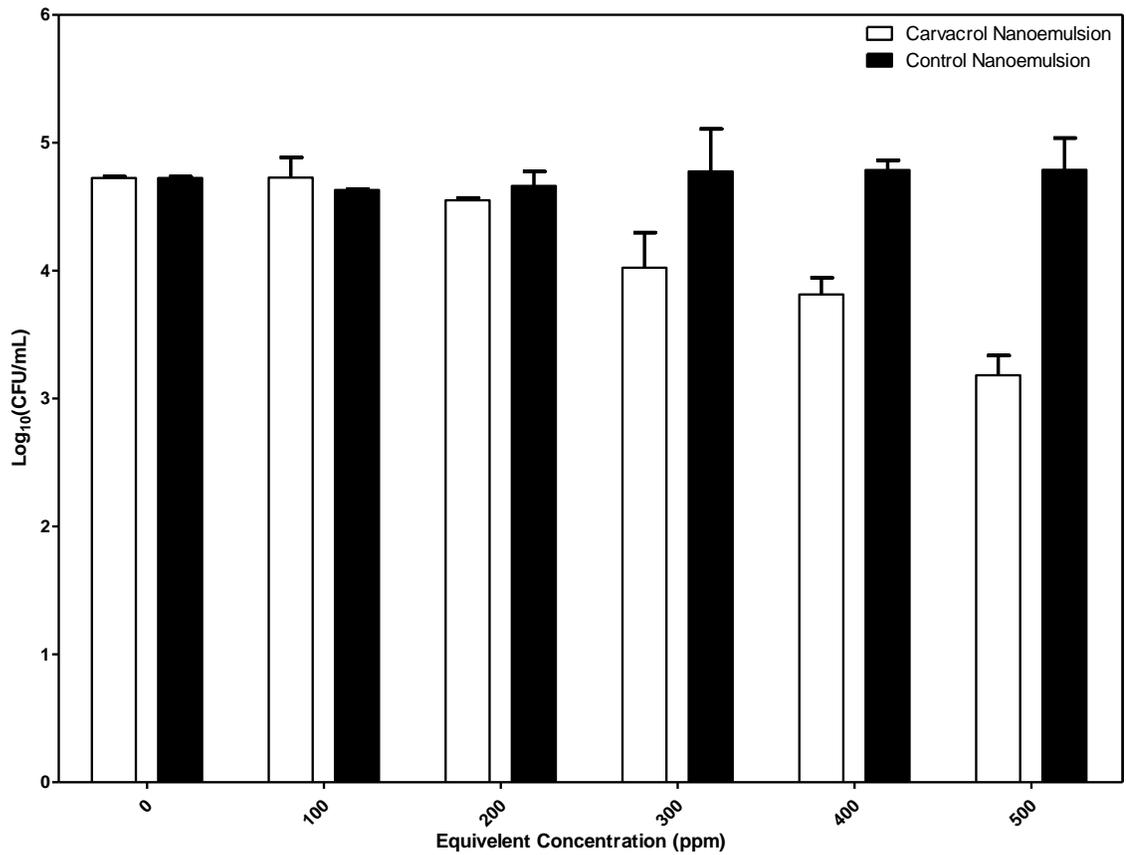
Figure 4.1: Comparison of antimicrobial activity of carvacrol MCT nanoemulsion to MCT alone (control) against S. enterica 1045 in 0.1% saline. Both emulsions were prepared in citrate buffer (pH 7.4) CFU calculated for each concentration and treatment after 8 hours

.
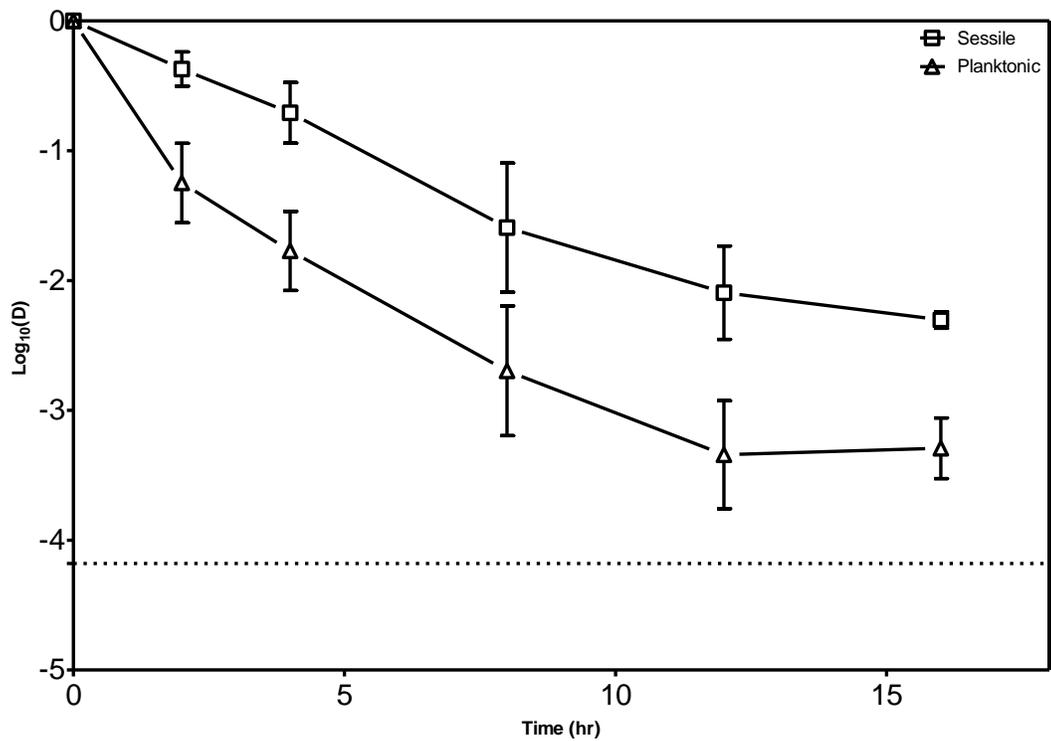
Figure 4.2: Comparison between varying growth conditions in S. enterica 1045 on cellular destruction. Cultures suspended in 0.85% saline and treated with 500ppm carvacrol nanoemulsion. Control data from culture treated with nanoemulsion made replacing all carvacrol with MCT's. Error bars represent SD of triplicate data. The ⎯ ⎯ line represents the detection threshold of the saline treatment

of *Salmonella,* and are often expressed along with the production extracellular cellulose –

an exopolymeric substance. In order to visualize the curli producing phenotype, *S. enterica*

1045 was streaked upon Congo Red agar (CRA) plates. Curli positive colonies on CRA

plates have a "red, dry and rough (rdr)" morphology, and it has been proposed that the

Congo red binding ability of curli is related to the hydrophobicity of these surface

structures (Kimizuka et al., 2009). This hydrophobic structure could disrupt the

stability of the nanoemulsion or directly interact with the more hydrophobic essential oils

to prevent the antimicrobial from reaching the membrane.

Salmonella expressing curli (red colonies) or not (white colonies) were grown either on

TSA plates (sessile) or in TSB in order to determine the contribution of red curli on this

difference (Figure 4.3). Using a repeated measure one way ANOVA test with a Tukey's

posttest, there was no significant difference (p=0.05) between curli and non-curli

expressing cultures, while the growth conditions effect was once again observed.

Plots were modeled using the log_{10} Weibull model plus tail and graphed side by side

with corresponding data (Figure 4.4, Table 4.1). With the Weibull plus tail model showed

significant differences between the level of remaining cells after death [$log_{10}(N_{res})$]. This

tailing suggests there is a percentage of the bacterial population that remains after

treatment than the planktonic cells while the increased δ values further support greater

overall resistance to the carvacrol by slowing the rate of destruction.

E. coli O157:H7 treated with identical concentrations of carvacrol nanoemulsion (Fig

4.5) showed greater resistance to the treatment than similarly grown *S. enterica*. However

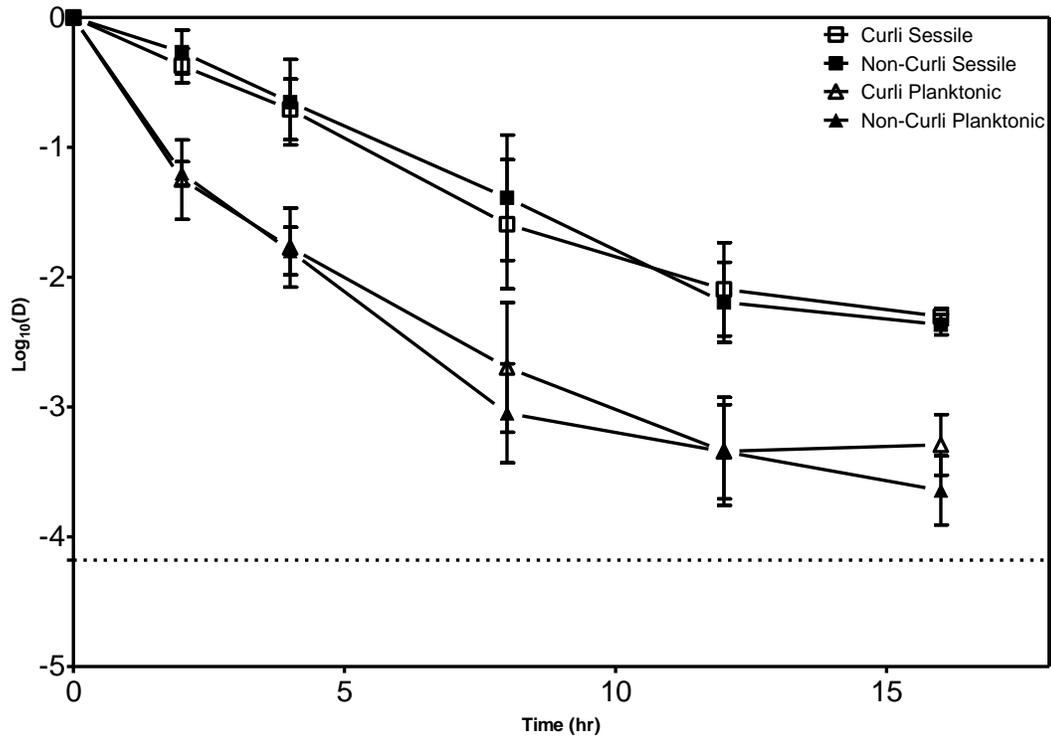**Figure 4.3: Comparison between varying growth conditions and expression of curli cell morphology in sensitivity of S. enterica 1045 to 500 ppm emulsion. Error bars represent SD of triplicate data**
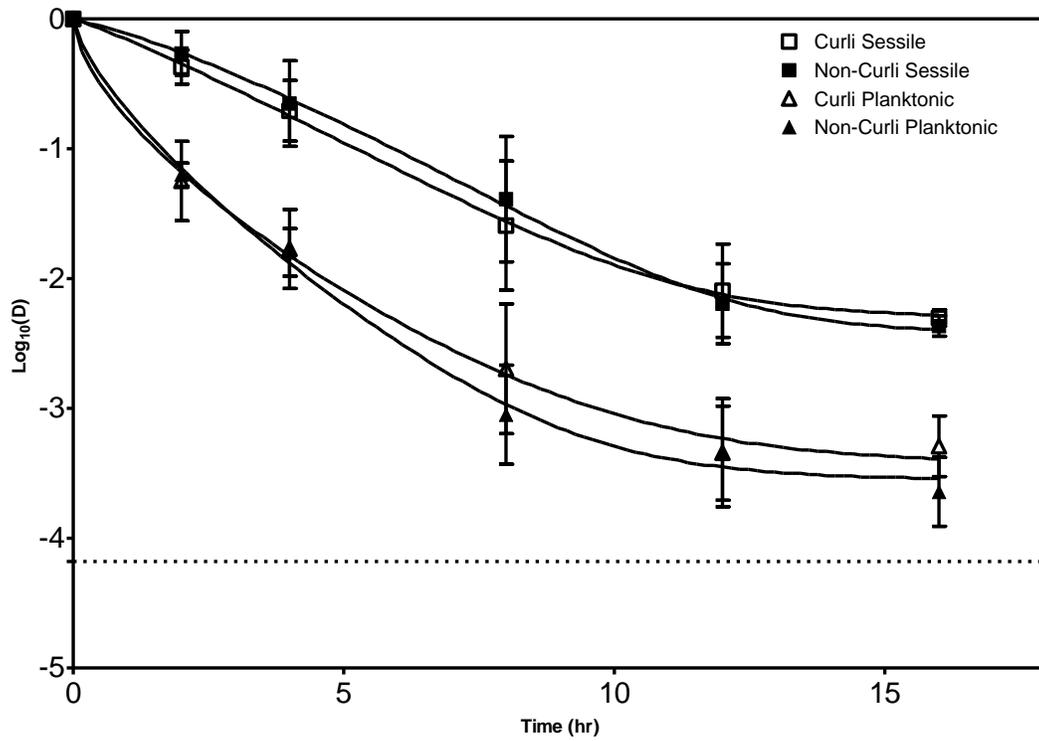
Figure 4.4: Weibull survival curves of varying growth conditions and expression of curli cell morphology in sensitivity of *S. enterica* 1045 to 500 ppm emulsion. Error bars represent SD of triplicate data

Table 4.1: Weibull with tail curve parameters for stationary phase S. enterica inactivation kinetics at 500 ppm carvacrol

Variable	Non-Curli Planktonic		Curli Planktonic		Non-Curli Sessile		Curli Sessile	
	Value	SEM	Value	SEM	Value	SEM	Value	SEM
$Log10(Nres)$	-3.55	0.15	-3.43	0.26	-2.42	0.23	-2.30	0.20
δ	1.65	0.40	1.55	0.56	5.91	0.99	5.15	0.95
β	0.72	0.11	0.63	0.14	1.28	0.34	1.12	0.29
$Log10(N_0)$	0.00	0.14	-0.01	0.19	-0.01	0.15	0.00	0.15

The trend difference between sessile and planktonic cells remained significant at a p=0.05. The Weibull plus tail model on *E. coli* O157:H7 data (Figure 4.6 and Table 4.2) also showed that that the sessile cells completed their curve before the planktonic cells leveled off. This difference did not hold up on carvacrol treated *L. monocytogenes* (Figure 4.7 and Table 4.3). The planktonic cells remained significantly more resistant than their sessile counterparts.
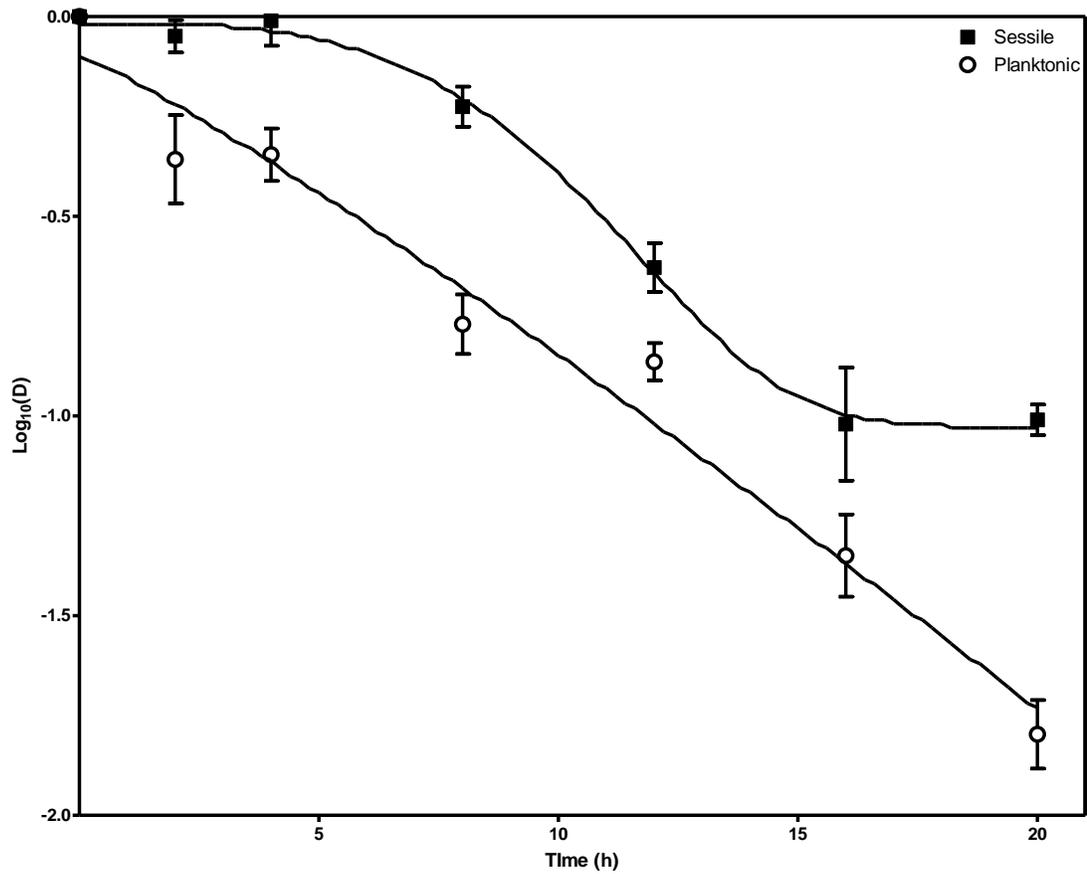
Figure 4.5: Comparison between varying growth conditions in E. coli O157:H7 on cellular destruction. Cultures suspended in 0.85% saline and treated with 500ppm carvacrol nanoemulsion. Control data from culture treated with nanoemulsion made replacing all carvacrol with MCT's. Destruction curves are Weibull curves with tailing where applicable. Error bars represent SD of triplicate data

Table 4.2: Weibull with tail curve parameters for stationary phase E. coli O157:H7 inactivation kinetics at 500 ppm carvacrol

| Variable	Planktonic		Sessile	
	Value	SEM	Value	SEM
Log10(Nres)	No Tailing		-1.03	0.04
δ	12.90	1.38	12.79	0.04
β	1.12	0.27	3.27	0.58
Log10(N_0)	-0.10	0.27	-0.02	0.03
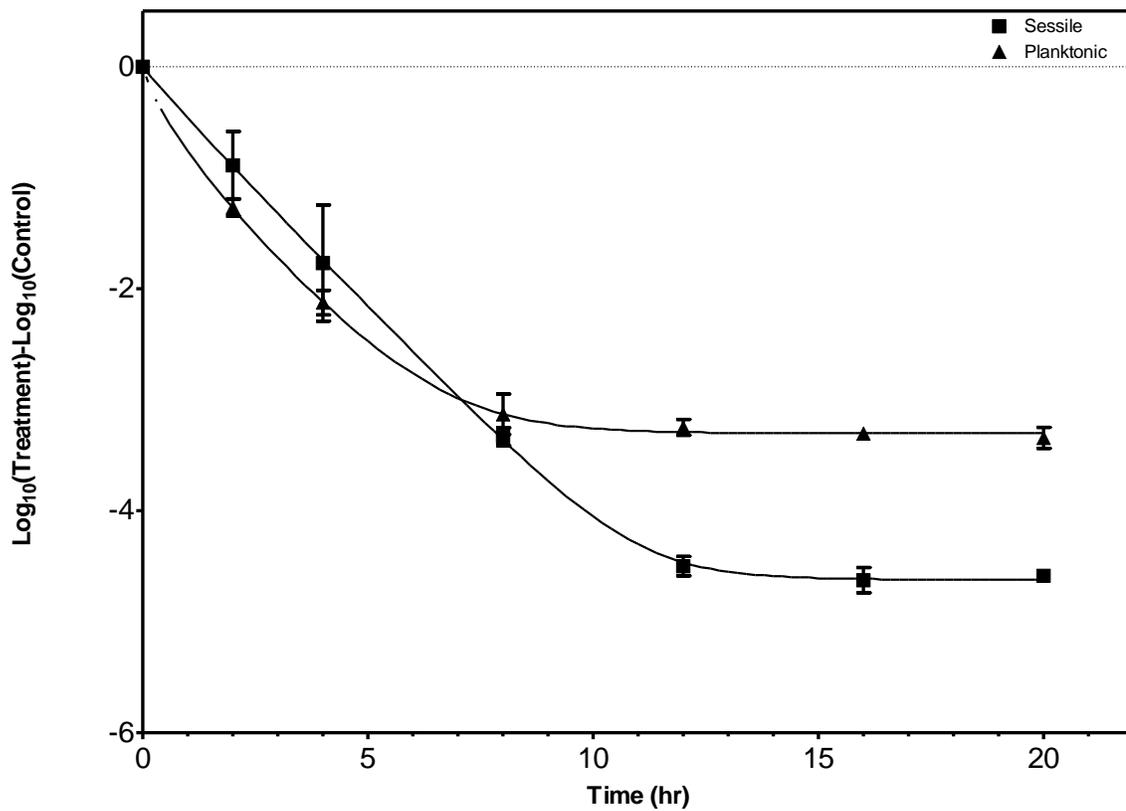
Figure 4.6: Comparison between varying growth conditions in *L. monocytogenes*
on cellular destruction. Cultures suspended in 0.85% saline and treated with
1500ppm carvacrol nanoemulsion. Control data from culture treated with
nanoemulsion made replacing all carvacrol with MCT's. Destruction curves are
Weibull curves with tailing. Error bars represent SD of triplicate data

Table 4.3: Weibull with tail curve parameters for stationary phase L. monocytogenes inactivation kinetics at 1500 ppm carvacrol

Variable	Planktonic		Sessile	
	Value	SEM	Value	SEM
$Log10(Nres)$	-4.62	0.08	-3.30	0.03
δ	2.24	0.30	1.44	0.12
β	0.96	0.08	0.75	0.04
$Log10(N_0)$	0.00	0.11	0.00	0.05

Mechanism of Carvacrol nanoemulsion effectiveness. Initial studies were performed to determine the appropriate diluent to use for carvacrol nanoemulsion treatment (pH 3.35) and recovery. It was observed that when cells were treated in PBS (10 mM HPO_4, 0.80% NaCl, pH 7.4), that the carvacrol nanoemulsion was less effective (Figure 4.8). In fact, at when treated in saline, 1000 ppm of carvacrol emulsion reduced cells to below detectable limits, while just under a 2 log reduction was observed in PBS. When treated in 0.85% NaCl, a relatively linear dose response to carvacrol was observed once the level of carvacrol was increased above 100 ppm (Figure 4.9). The buffering capacity of the PBS, may have changed the pH of the solution, and suggests ether a low pH of the carvacrol emulsion may enhance antimicrobial effectiveness.

In order to test this hypothesis, acidified carvacrol emulsions were prepared and compared to the a control oil emulsion (without carvacrol) prepared in 5 mM citric acid, the original carvacrol emulsion prepared in 5 mM citric acid, and carvacrol emulsions prepared in 5 mM acetic, levulenic or hydrochloric acid (Figure 4.10).

Initial experiments showed complete destruction of all organisms at the 8 hour time point, therefore experiments were performed after a 30 minute exposure to all emulsions. The carvacrol nanoemulsion had a minor but significant effect compared with the control MCT emulsion. The addition of 5mM sodium hydroxide removed any antimicrobial efficacy of the nanoemulsion and allowed for significant bacterial growth. Three different acids were added due to their GRAS status in foods and increased the efficacy of the antimicrobial system over 3 log, beyond the detection threshold of the experiment.

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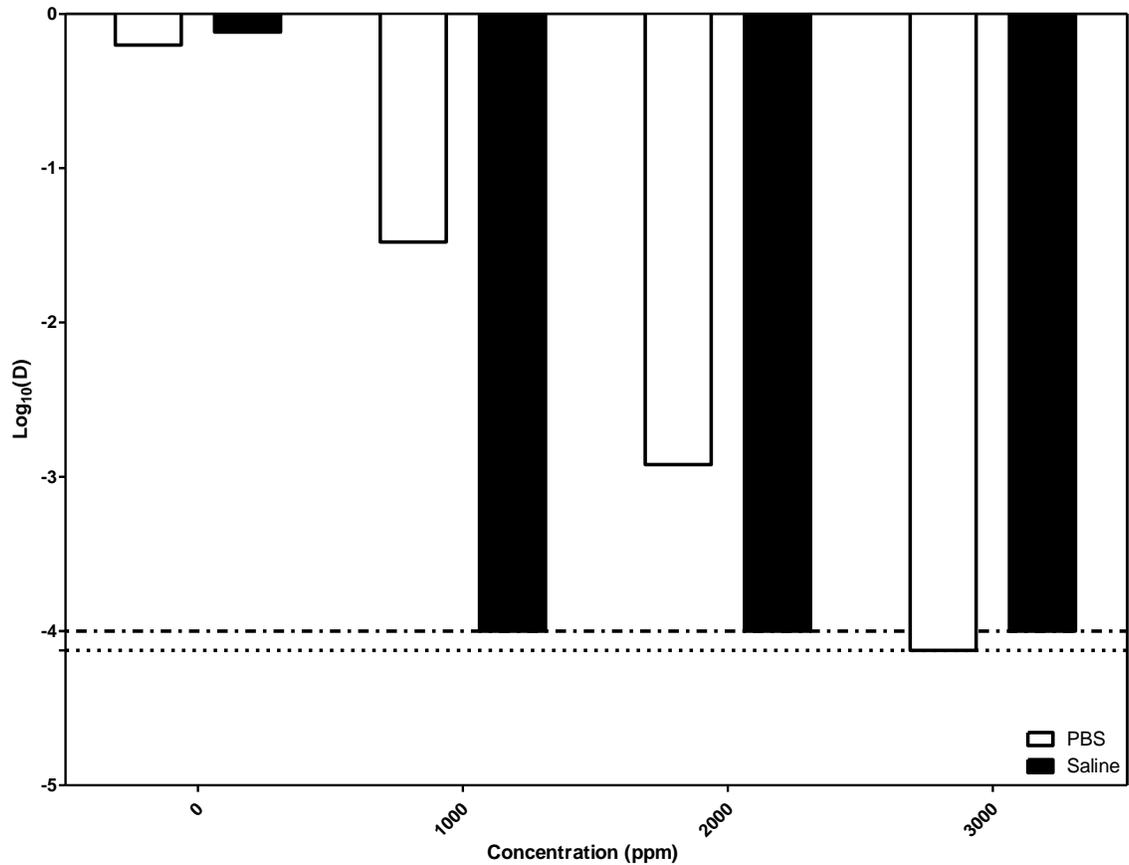
Figure 4.7: Comparison between varying diluents of *S. enterica* 1045 on cellular destruction. Cultures suspended in 0.85% saline or pH 7.4 phosphate buffered saline and treated with 500ppm carvacrol nanoemulsion for 8 hours prior to enumeration. Control data from culture treated with nanoemulsion made replacing all carvacrol with MCT's. The ― · ―line represents the detection threshold of the saline treatment while the ······· line represents the detection threshold for PBS.
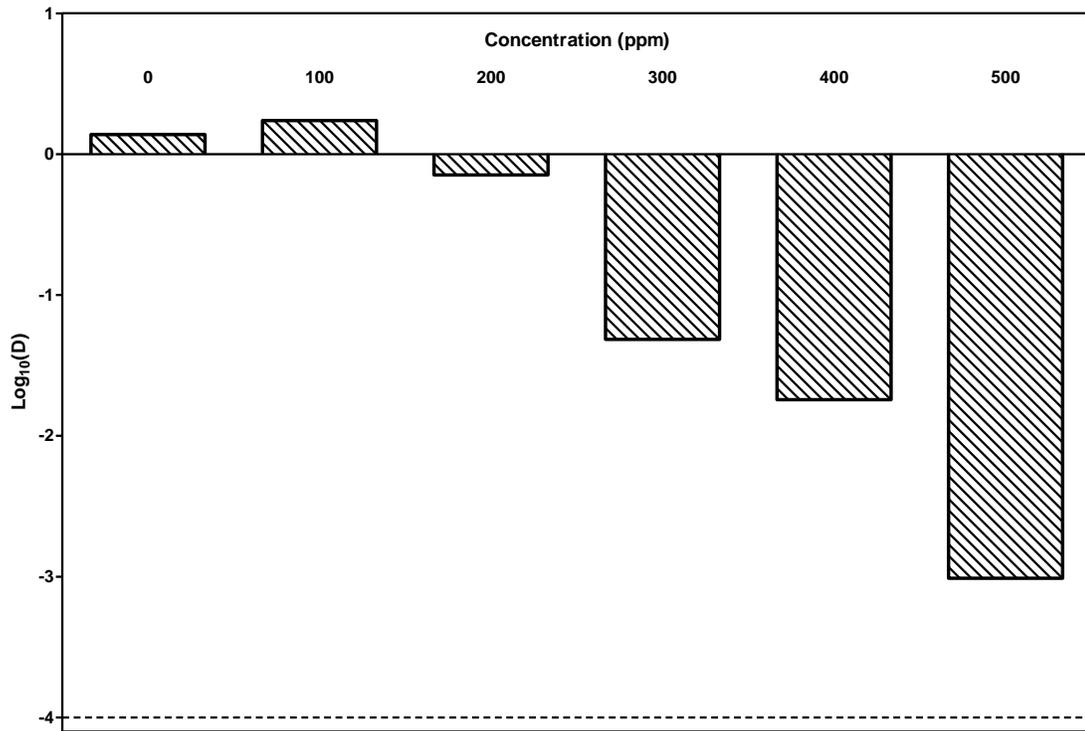
Figure 4.8: *S. enterica* 1045 cellular destruction in saline. Cultures suspended in 0.85% saline and treated with 500ppm carvacrol nanoemulsion for 8 hours prior to enumeration. Control data from culture treated with nanoemulsion made replacing all carvacrol with MCT's. The ······· line represents the detection threshold.
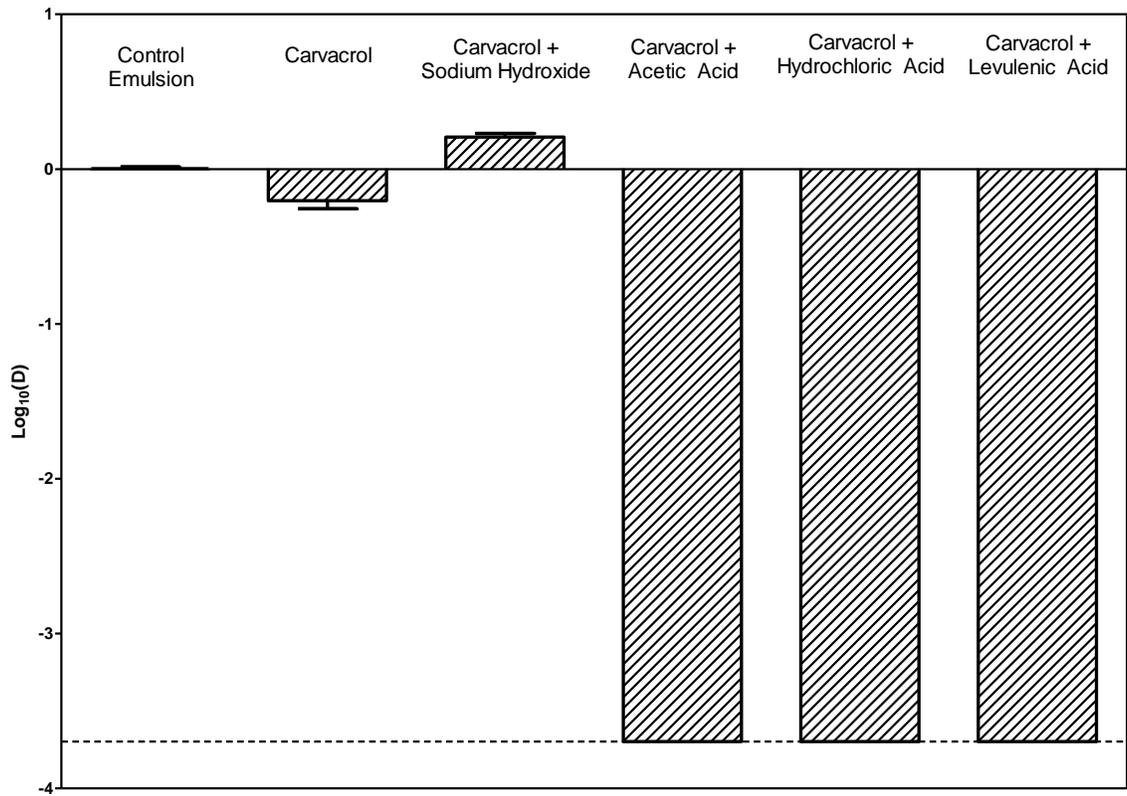
Figure 4.9: Influence of acid type upon *S. enterica* **1045 destruction by 500ppm carvacrol nanoemulsion in 0.85% saline. Reduction calculated for each point comparing original inoculum with CFU/mL after 30 min. Control Nanoemulsion made with only MCT and added to samples in an equivalent volume to that of the carvacrol nanoemulsion. Acidic components added at a level of 5mM final concentration Error bars represent SD of triplicate data**

This strongly suggests the use of the nanoemulsion is best suited for use in acidic foods for the greatest efficiency in use of carvacrol.

Influence of growth phase upon sensitivity to nanoemulsions. Initial experiments used inoculum preparations were from stationary phase of growth for both the broth and the sessile cells. Growth curves were performed in broth (Figure 4.11) and on solid media using, polycarbonate membranes were inoculated with cells and at various time points, the number of cells were determined (Figure 4.12). Based the growth curves in Figure 4.11, time points were selected that represented exponential phases of growth. This was an OD of 0.5 for planktonic cells, or approximately 4 hours for *S. enterica* and *E. coli* and 12 hours for *L. monocytogenes.* A target inoculum of 7-8 log for all sessile cultures was achieved after 4 hours of growth planktonic *S. enterica* isolated in the exponential phase of growth (Figure 4.12) were more resistant than the exponential phase sessile cells, the reverse of the trend that was seen in the stationary phase cultures (Figure 4.4). This flip in inoculum sensitivities did not hold up in the exponential phase *E. coli* (Figure 4.13), as the sessile cells remained more resistant to the treatment then the planktonic in both cell growth phases. The exponential growth phase *L. monocytogenes* (Figure 4.14) also mirrored the stationary phase carvacrol sensitivity studies as the planktonic growth continued to remain more resistant to the treatment then the sessile.
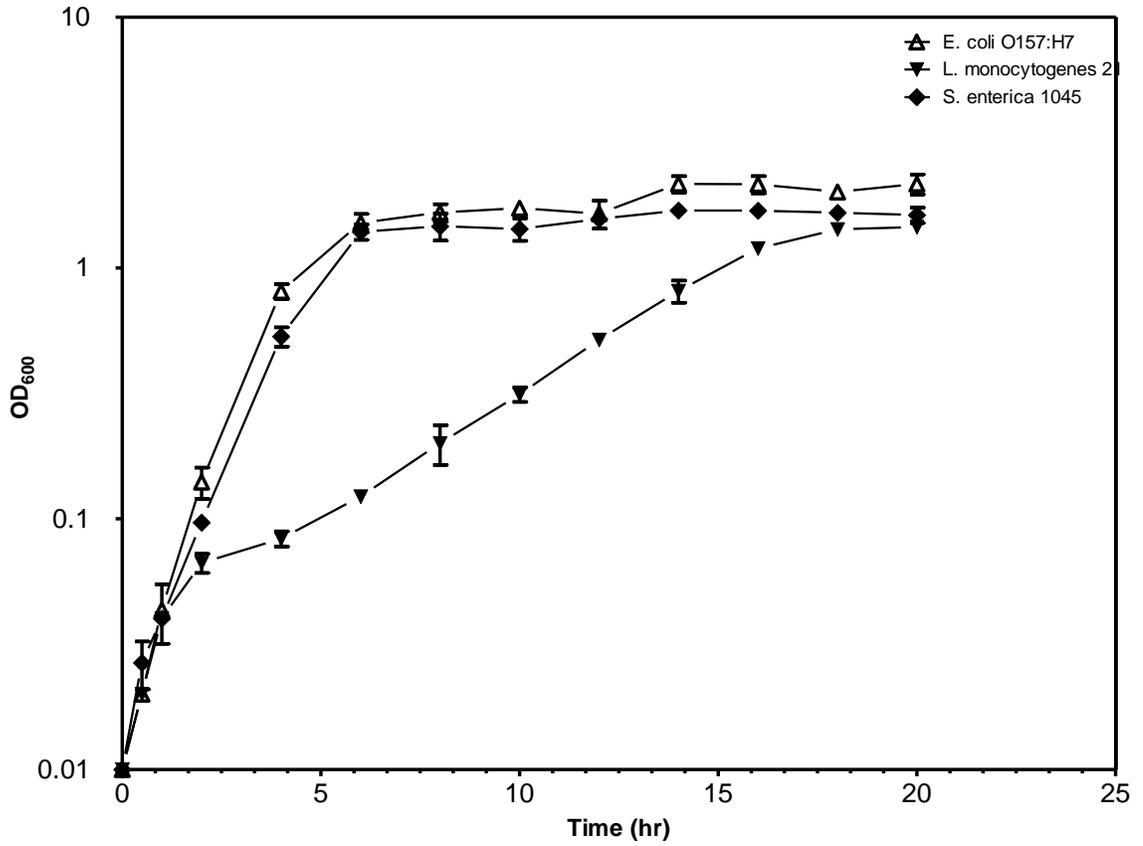
Figure 4.10: Growth of various bacterial cultures in liquid broth. TSB broth cultures grown at 37°C. Measurements collected in triplicate. Error bars represent SD.
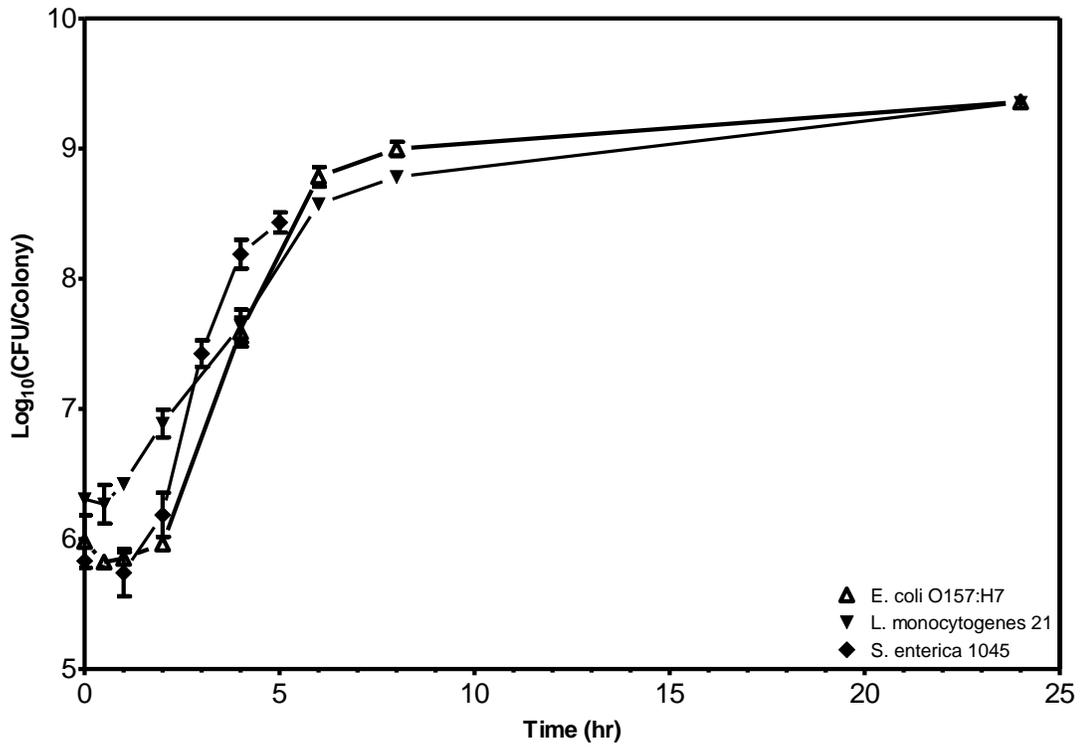
Figure 4.11: Growth curves on a solid agar surface. Colonies grown at 37°C and collected in triplicate. Colonies grown on polycarbonate filters on the surface of TSA plates. Error bars represent SD.
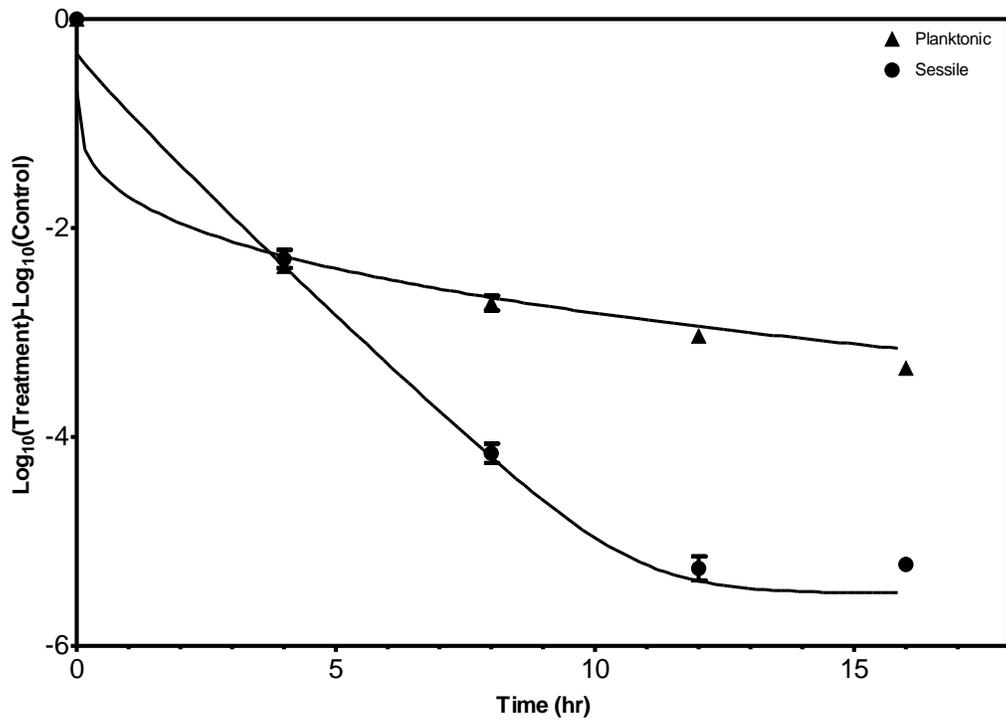
Figure 4.12: Comparison between varying growth conditions in *S. enterica* 1045 collected in the exponential growth phase on cellular destruction. Cultures suspended in 0.85% saline and treated with 600ppm carvacrol nanoemulsion. Control data from culture treated with nanoemulsion made replacing all carvacrol with MCT's. Destruction curves are Weibull curves with tailing. Error bars represent SD of triplicate data.

Table 4.4: Weibull with tail curve parameters for exponential phase S. enterica inactivation kinetics at 600 ppm carvacrol

| Variable	Planktonic		Sessile	
	Value	SEM	Value	SEM
$Log10(N_{res})$	No Tailing		-1.03	0.12
δ	0.95	0.93	1.86	0.35
β	0.32	0.11	0.93	0.11
$Log10(N_0)$	-0.68	0.21	-0.33	0.14
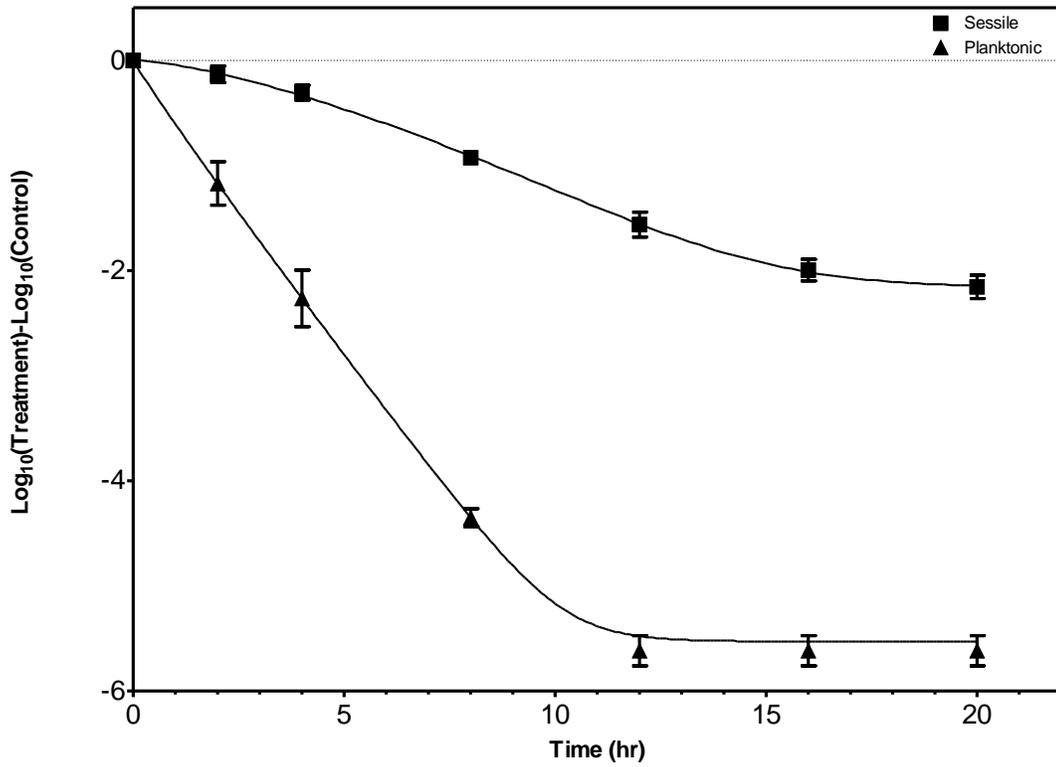
Figure 4.13: Comparison between varying growth conditions in *E. coli* **O157:H7 collected in the exponential growth phase on cellular destruction. Cultures suspended in 0.85% saline and treated with 500ppm carvacrol nanoemulsion. Control data from culture treated with nanoemulsion made replacing all carvacrol with MCT's. Destruction curves are Weibull curves with tailing. Error bars represent SD of triplicate data.**

Table 4.5: Weibull with tail curve parameters for exponential phase E. coli inactivation kinetics at 500 ppm carvacrol

| Variable	Planktonic		Sessile	
	Value	SEM	Value	SEM
Log10(Nres)	-2.17	0.04	-5.53	0.18
δ	8.37	0.31	1.69	0.04
β	1.44	0.11	0.95	0.58
Log10(N$_0$)	0.01	0.03	0.00	0.03
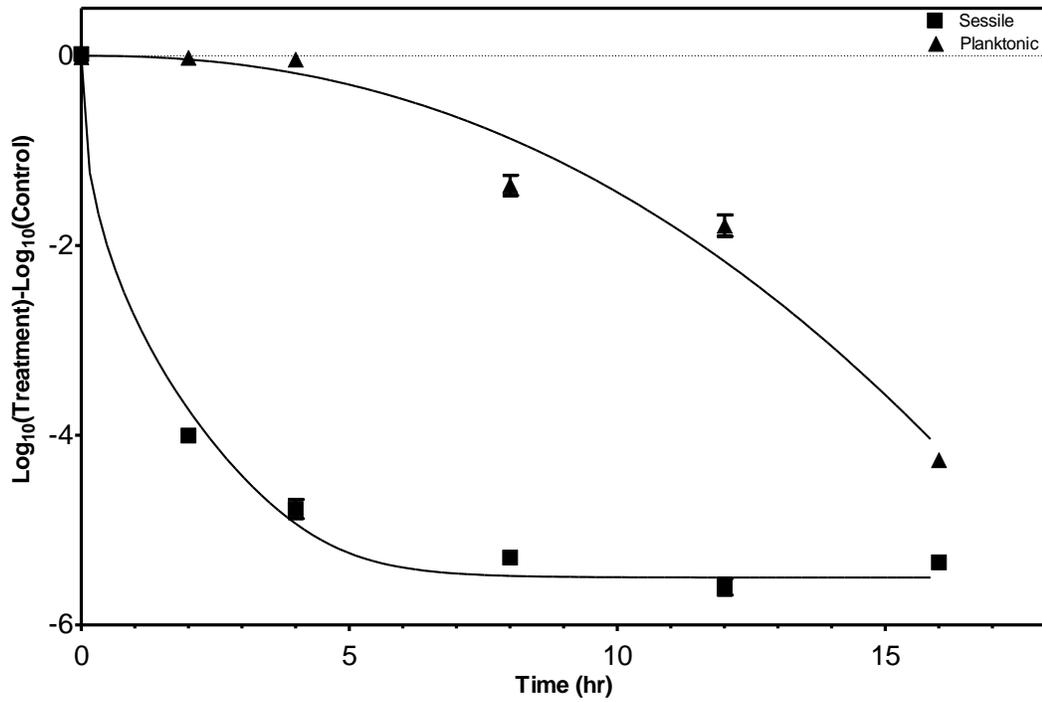
Figure 4.14: Comparison between varying growth conditions in L. monocytogenes collected in the exponential growth phase on cellular destruction. Cultures suspended in 0.85% saline and treated with 1500ppm carvacrol nanoemulsion. Control data from culture treated with nanoemulsion made replacing all carvacrol with MCT's. Destruction curves are Weibull curves with tailing where applicable. Error bars represent SEM of triplicate data.

Table 4.6: Weibull with tail curve parameters for exponential phase L. monocytogenes inactivation kinetics at 1500 ppm carvacrol

Variable	Planktonic		Sessile	
	Value	SEM	Value	SEM
Log10(Nres)	No Tailing		-5.50	0.07
δ	8.50	0.60	-0.10	0.12
β	2.24	0.24	-0.44	0.04
Log10(N_0)	0.00	0.10	0.00	0.15

CONCLUSION

1) Curli production are not related to the increased resistance to emulsion based antimicrobial systems of stationary phase *S. enterica*.

2) The growth phase of S. enterica was strongly correlated with increased resistance to emulsion based antimicrobials. With planktonic cells being more resistant in the exponential phase of growth and sessile being more resistant in the stationary phase.

3) Sessile cells of *E. coli* were more resistant than broth grown cells to antimicrobial nanoemulsions. This observation consistent with cells in both exponential and stationary phases of growth.

4) Sessile cells of *L. monocytogenes* were more sensitive to antimicrobial nanoemulsions than broth grown cells and this observation was consistent with both exponential and stationary phase cells

Although no overarching conclusion about antimicrobial sensitivities, and therefore testing, can be made there is evidence that the current methods are inadequate for taking account for the many variables of the tests. A recommendation that each organism be assessed for the most resistant phase of growth to a particular antimicrobial system be assessed by individuals before they continue to optimize the concentration for its use.

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