



**CPS 2012 RFP
FINAL PROJECT REPORT**

Project Title

Rapid assessment of oxidative stress induced in microbes to evaluate efficacy of sanitizers in wash water

Project Period

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Objectives

- 1.) *Develop magnetic resonance spectroscopy based approaches for rapid assessment of oxidative stress generated in microbes exposed to diverse sanitizers*
- 2.) *Evaluate correlation between oxidative stress levels in both pathogenic and non-pathogenic microbes and reduction in microbial count*
- 3.) *Evaluate influence of organic load on the correlation between threshold oxidative stress induced in microbes and reduction in number of microbes.*

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FINAL REPORT

Abstract

To assess and monitor the efficacy of sanitation processes, there is a need to rapidly measure microbial resistance to sanitizers and determine the critical concentration of sanitizers above which significant reduction in microbial load can be achieved in a complex environment. In this study, we have developed two complementary approaches (a) a rapid label free magnetic resonance (MR) imaging and relaxometry method was developed to measure resistance of *Escherichia coli* and *Listeria monocytogenes* to hydrogen peroxide; (b) a rapid label based optical approach to assess efficacy of both peroxide and chlorine based sanitizers. The results demonstrate that the MR and optical methods developed in this study can determine the critical concentration of sanitizers above which a significant reduction in bacterial culturability (at least 2 logs) was achieved. The results of this study also demonstrate that stationary phase microbes are more resistant to sanitizers compared to exponential phase microbes. The results that the methods developed in this study can predict the efficacy of sanitizers in high organic load environment and for both gram positive and gram negative bacteria.

Background

The primary goal of sanitation is to reduce microbial load in diverse products and process operations (Beck-Sague et al., 1990; Chu, McAlister, & Antonoplos, 1998; Doan et al., 2012; Nakazato et al., 2007; Sapers & Sites, 2003). For sanitation in food, environmental, and medical industry, various oxidizing agents such as hydrogen peroxide (H_2O_2), ozone, and chlorine are used. Among these diverse oxidizers, H_2O_2 and chlorine based sanitizers are the most widely used sanitizers due to its broad-spectrum microbiocidal activity (Linley, Denyer, McDonnell, Simons, & Maillard, 2012; Swartling & Lindgren, 1968).

To assess efficacy of sanitizers in diverse applications, a range of measurement approaches have been developed. In many process industries, such as water treatment and fresh produce washing, efficacy of sanitizers is assessed based on chemical methods to measure sanitizer concentration, total carbon content, oxidation reduction potential (ORP), turbidity, and/or pH of the aqueous phase (Newman, 2004; Suslow, 2000, 2004). Although chemical measurements such as ORP can assess the oxidation potential of water, these measurements are not effective in measuring reduction in bacterial count induced by sanitizers in complex environments with organic load. Furthermore, the ORP method is not a sensitive approach to measure the antimicrobial potential of H_2O_2 (Newman, 2004; Suslow, 2004).

In addition to chemical measurements, prior studies have investigated response of bacteria to sanitizers based on changes in membrane potential, membrane permeability, DNA double strand breaks, and lipid peroxidation (Baatout, Boever, & Mergeay, 2006; Farr & Kogoma, 1991; Imlay & Linn, 1986; Linley et al., 2012). The key advantage of these approaches is to directly assess the damage induced by sanitizers to various functional elements such as membrane and DNA of the bacteria. These measurements can aid in developing mechanistic understanding of the action of sanitizers. The major limitations of this approach are: (a) multiple steps for preparing microbial samples including labeling of microbes with specific fluorophores and isolation of DNA; (b) limited sensitivity of fluorescence measurements in

turbid environments; and (c) lack of correlation between the optical signal and the reduction in microbial count (Baatout, Boever, & Mergeay, 2006; Kramer & Muranyi, 2014; Wang, Claeys, van der Ha, Verstraete, & Boon, 2010).

Complementary to these approaches, prior studies have also used gene expression analysis to assess response of bacteria to sanitizers (Farr & Kogoma, 1991; Ming Zheng, 1999). Most of the gene expression studies are conducted using a sub-lethal level of sanitizer concentration, thus enabling bacteria to adjust their gene expression in response to the oxidative stress induced by sanitizers. However, process industry uses lethal concentration levels of sanitizers and short period of time, which may not provide sufficient time (less than 5-15 minutes) for microbes to adjust their gene expression. Thus, these studies have limited application in assessing efficacy of sanitation operations.

Traditional plate counting has also been used in laboratory settings to study efficacy of sanitizers (Asaumi, Watanabe, Taguchi, Tashiro, & Otsuki, 2007). Traditional plate counting is an effective approach for measuring biocidal activity of various sanitizers; however, plate counting methods require at least 12-24 hours to assess the reduction in microbial count.

The overall goals of this study were to develop novel spectrometry methods to measure bacterial resistance to H_2O_2 and $NaOCl$, and correlate MR measurements with reduction in bacterial culturability. For measuring resistance of bacteria to hydrogen peroxide, this study developed a magnetic resonance imaging (MR) and relaxometry approach. For rapid assessment of oxidative stress induced in microbes to evaluate efficacy of $NaOCl$, an optical spectroscopy approach was developed. Thus, the study evaluated both the optical and MR spectroscopy approaches to assess the oxidative stress induced in bacteria.

In MR imaging and relaxometry, signal intensity is based on the magnetic properties of the nuclei (protons) through their interaction with an applied radio frequency and a constant magnetic field (Zhang & McCarthy, 2012). The unique advantage of MR is its ability to measure reactions and interactions in turbid and opaque environments. In environments with high solid content, chemical measurements are also challenging as the electrodes for the oxidation-reduction potential measurements can be easily fouled. MR imaging and relaxometry are non-destructive methods that have been used in the food, chemical, oil and pharmaceutical industry as quality assessment tools for many years (Metz & Mäder, 2008). Therefore, the method developed in this study can be easily integrated with the current MR systems used in industry. The experimental setup is illustrated in Figure 1. Figure 1(e) illustrates an example of a portable MR relaxometry device that could be used in industrial settings

Using optical spectroscopy methods, radical sensitive fluorescence tracers were evaluated for measuring the oxidative stress induced in *E. Coli* O157:H7 upon treatment with $NaOCl$, measurements of changes in bacterial physiology (glucose uptake and membrane permeability) induced by sanitizers and comparison of these changes with the reduction in bacterial culturability.

In summary, the results from the MR and optical spectroscopy methods will aid in development of rapid assays for measuring efficacy of sanitizers in environments such as wash water used for fresh produce.

Research Methods and Results

A. Methods for measuring oxidative stress induced in microbes by hydrogen peroxide based sanitizers

a. H₂O₂ (30% v/v), phosphate buffered saline (PBS 10x solution), and microbial culture media (LB –Lysogeny broth Miller) were obtained from Fisher Scientific (Pittsburgh, PA). Catalase enzyme isolated from bovine liver and Tris(2,2'-bipyridyl)dichlororuthenium (II) hexahydrate (Ru(BPY)₃) were purchased from Sigma Aldrich (St. Louis, MO).

b. Bacterial strains and culture condition

E. coli BL21 #BAA-1025 was purchased from ATCC (Manassas, VA). A Rifampicin-resistant *E. coli* O157:H7 ATCC#700728 was provided by Dr. Linda Harris (University of California, Davis, USA). *L. monocytogenes* (DP-L1044(hly::Tn917-LTV3) was provided by Dr. Gary Smith (University of California, Davis, USA). All bacteria were stored in liquid nitrogen with glycerol. The stock liquid nitrogen bacteria was streaked onto a LB Agar plate and grown overnight at 37°C. A colony was picked from the agar plate, and cultured in LB broth for 16 hours (*E. coli*) or 24 hours (*L. monocytogenes*) to make an initial liquid culture. The initial liquid culture was then sub-cultured in LB broth. In the subculture step, *E. coli* BL21 was either grown for 7 hours to reach the stationary growth phase ($\approx 10^{10}$ CFU/mL or OD ≈ 1.3 at 600nm) or grown for 2 hours to reach the mid exponential growth phase ($\approx 10^9$ CFU/mL or OD ≈ 0.530); *E. coli* O157:H7 was grown for 16 hours to reach the stationary growth phase ($\approx 10^9$ CFU/mL or OD ≈ 1.4); *L. monocytogenes* was grown for 24 hours to reach the stationary growth phase ($\approx 10^9$ CFU/mL or OD ≈ 0.4). The exponential growth phase and the stationary of the selected bacterial strains were validated by spectroscopic evaluation of the growth curves (data not shown). All the culture media and agar for *E. coli* O157:H7 was supplemented with 50µg/mL of rifampicin (TCI America, Portland, OR). Before use in each experiment, the bacteria was washed twice with 1x PBS by centrifugation at 3,100xg (using Eppendorf Microcentrifuge 5414R) for 10 minutes at room temperature and resuspended in 1xPBS or LB broth for further analysis.

c. Nuclear magnetic resonance imaging

Signal intensity in MR imaging is a function of proton density, spin-lattice relaxation time (T₁), and spin-spin relaxation time (T₂). This study focuses on the T₁ and T₂ relaxation time measurements. These two relaxation time values are affected by the paramagnetic properties of a sample (As, 1992). The T₁ relaxation time describes energy exchange between nuclear spins and their surrounding environment (lattice). The T₂ relaxation time describes energy exchange among nuclear spins.

MR imaging experiments were carried out using a 1 Tesla (43.8 MHz) Aspect imaging unit (Shoham, Israel) with a 60mm radio frequency (RF) coil. A T₁ weighted Spin Echo 2D (SE 2D) sequence with a repetition time (TR) of 200 ms, an echo time (TE) of 12.8 ms, and a dwell time of 15µs were used. The number of phase encoding and frequency encoding steps were both 128; the field of view (FOV) was 50 mm. The number of slices was 5, each with a slice thickness of 8 mm. The voxel dimension of 1.22 mm³ was calculated using the equation below (Eq. (1)).

$$\text{Voxel volume} = \text{phase dimension} \times \text{frequency dimension} \times \text{slice thickness}$$

$$= \frac{\text{FOV}}{\text{phase encoding step}} \times \frac{\text{FOV}}{\text{frequency encoding step}} \times \text{slice thickness} \quad (1)$$

MR signal intensity (S) equation for the spin-echo sequence as provided by the Bernstein et al. (Eq. (2)) (Bernstein, King, & Zhou, 2004).

$$S = M_0 \left(1 - 2e^{-(TR-TE/2)/T_1} + e^{-TR/T_1} \right) e^{-TE/T_2} \quad (2)$$

where M_0 is the initial magnetization, TR is the repetition time and TE is the echo time.

Two milliliters of stationary growth phase *E. coli* BL21 at ($\approx 10^{10}$ CFU/mL) were pipetted into a cuvette. After treatment of bacteria with 0-1% v/v of H_2O_2 for 15 minutes at room temperature with stirring at a frequency of 250 rpm, the sample was placed in a cuvette holder for MR measurements. A reference sample containing 2 mL of PBS was placed next to the H_2O_2 treated sample in a same cuvette holder. The signal intensity of the treated sample was normalized with respect to the reference sample. All the experiments were performed in triplicate. The image files were analyzed using MATLAB™ v7.8.0 (Mathworks Inc., Natick, Mass., U.S.A.).

d Relaxometry

The T_1 and T_2 measurements were acquired in independent experiments with the same MR imaging unit. For the T_1 measurements, a 15 point saturation recovery sequence was used. For the T_2 measurements, a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence was used with an echo time (TE) of 1ms. Two milliliters of bacteria was transferred into a cuvette which was placed in a cuvette holder with an empty cuvette to maintain spatial orientation. Immediately following the addition of 0-1% H_2O_2 , changes in the T_1 or T_2 measurements were recorded. Measurements were acquired continuously during the incubation period after the initial 2 minutes of time required for power and frequency calibration of the instrument. Thus, the time $t=0$ minute as indicated in the result section is after the initial lag of 2 minutes. All the experiments were conducted in triplicate. The acquired data was analyzed using MATLAB™ v7.8.0.

e. Treatment of bacteria with hydrogen peroxide and reduction in culturability of bacteria

Stationary growth phase bacteria (*E. coli* BL21, *E. coli* O157:H7, and *L. monocytogenes*) was treated with 0%, 0.1%, 0.5%, and 1% v/v of H_2O_2 ; mid exponential growth phase *E. coli* BL21 was treated with 0%, 0.01%, 0.1%, 0.5%, and 1% v/v of H_2O_2 . Samples were then incubated for 15 minutes at room temperature with stirring at a frequency of 250 rpm; 50 μ g/mL final concentration of powder catalase was then added to 1 milliliter mixture of bacteria and H_2O_2 for 10 minutes to quench activity of the excess H_2O_2 in solution. The treated bacterial cells were washed twice with 1x PBS, plated onto a LB agar overnight, and the bacterial colonies were counted. Each experiment was performed in triplicate.

f. Statistical Analysis

Statistical analysis of the data was carried out using Microsoft Excel (Microsoft, Redmond, CA). The statistical significance was determined using the t-test, assuming unequal variances with an alpha of 0.05. Results are denoted with asterisks or noted in the text to indicate statistical significance (* $p < 0.05$, ** $p < 0.01$).

B. Methods for measuring oxidative stress induced in microbes by sodium hypochlorite based sanitizers

a. Bacterial strain

Rifampicin-resistant *Escherichia coli* O157:H7 ATCC#700728 (*E. coli* O157:H7) was kindly provided by Dr. Linda Harris (University of California, Davis, USA) and stored in liquid nitrogen with 50% v/v glycerol. For selective growth of *E. coli* O157:H7, rifampicin (TCI America, Portland, OR) was added to the medium at 50µg/mL. The liquid nitrogen *E. coli* stock was streaked onto a Luria Bertani (LB) Agar (Fisher Scientific, Pittsburg, CA) plate and grown overnight at 37°C. A colony was picked from the agar plate, cultured in LB broth (Fisher Scientific, Pittsburg, CA) for 16 hours and then sub-cultured in LB broth for 16 hours at 37°C before use in each experiment. In the subculture step, *E. coli* was grown to the stationary growth phase (OD \approx 1.4 at 600nm). The bacteria were washed twice with 1x PBS (phosphate buffered saline from Fisher Scientific, Pittsburg, CA) by centrifuging at 3,100xg (using Eppendorf Microcentrifuge 5414R, Hamburg, Germany) for 10 minutes at room temperature and resuspended in 1xPBS for further analysis.

b. Inactivation with sodium hypochlorite

The stationary growth phase microbes were diluted with 1xPBS to OD \approx 1 at 600nm before each experiment. Sodium hypochlorite (NaOCl from Sigma-Alrich, St. Louis, MO) or hydrogen peroxide (H₂O₂ from Fisher Scientific, Pittsburg, CA) was used as sanitizers for the inactivation of bacteria. The final concentrations of NaOCl used were 0, 2.2, 4.4, 8.8, 17.6, 35.2, and 70.4 mg/mL. After treatment with either NaOCl for 10 minutes, sodium thiosulfate with a final concentration of 0.1% w/v was added to the *E. coli* O157:H7 suspension to stop further activity of NaOCl. A previous study has shown that sodium thiosulfate in the concentration range of 0.01% to 5% does not affect *E. coli* survivability [12].

c. Direct measurements of intracellular reactive oxidative species

Direct measurements of intracellular reactive oxidative species (ROS) during NaOCl sanitation: To measure ROS generated during treatment of bacteria with NaOCl, *E. coli* O157:H7 were pre-incubated with 10 µM final concentration of Aminophenyl fluorescein (APF from Life Technologies, Carlsbad, CA) at room temperature for 30 minutes. Followed the incubation with APF, *E. coli* O157:H7 was washed twice with 1xPBS to remove excess APF, and the bacteria were resuspended in 1xPBS. The *E. coli* O157:H7 suspension was then treated with 0-70.4 mg/L NaOCl for 10 minutes. Sodium thiosulfate (Sigma-Alrich, St. Louis, MO) at the final concentration of 0.1% w/v was added to neutralize the excess NaOCl after the 10 minute treatment.

d. Metabolism activity and membrane damage measurements

Changes in glucose uptake in bacteria following treatment with sanitizers were measured using a fluorescent analog of glucose, 2-NBDG (2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose). Membrane damage was measured using the propidium iodide (PI) dye. After *E. coli* O157:H7 were treated with NaOCl, as described in the microbial inactivation section above, the bacteria were incubated with either 5µg/mL of 2-NBDG at 37°C or PI at room temperature for 20 minutes. The microbes were then washed once with 1xPBS and resuspended in 1xPBS. One hundred microliters of the *E. coli* O157:H7 suspension was transferred into each well of a 96-well plate for imaging. The fluorescence intensity was measured at excitation/emission wavelengths of 485/528nm and 530/590nm for 2-NBDG and PI respectively. The signal/background ratio was calculated as shown in the equation (1).

e. Statistical Analysis

Statistical analyses of the data were carried out using Microsoft Excel (Microsoft, Redmond, WA). The statistical significance was determined using the t-test, assuming unequal

variances with an alpha of 0.05. Results are noted in the text to indicate statistical significance ($p < 0.05$).

Results:

A: A MR approach to measure oxidative stress induced in bacteria by hydrogen peroxide

MR imaging contrast induced by interactions of microbes with hydrogen peroxide

Interactions of stationary phase *E. coli* BL21 with H₂O₂ (0- 1% v/v) were measured using T₁-weighted MR imaging as shown in Figure 2. Figure 2 (a) shows the MR images of stationary growth phase *E. coli* BL21 suspension upon treatment with the selected concentration range of H₂O₂ (0-1%). Quantification of the imaging data (Figure 2(b)) shows that the relative MR signal intensity in these microbial samples increase as a function of H₂O₂ concentration. The results show significant differences ($p < 0.01$) in the signal intensity between H₂O₂ treated and non-treated *E. coli* BL21 suspensions. An eightfold increase in signal intensity was observed for the *E. coli* BL21 sample treated with 1% H₂O₂ as compared to the control sample (not treated with H₂O₂). Furthermore, significant differences in MR contrast ($p < 0.01$) between the H₂O₂ treated and control sample (non-treated *E. coli* BL21 suspensions) can be detected at and above the concentration levels of 0.1% H₂O₂. Addition of H₂O₂ to PBS did not show any significant changes in the T₁ weighted MR signal intensity as shown in the supplementary Figure S1. To the best of our knowledge this is the first observation that indicates microbial interactions with H₂O₂ can induce significant changes in MR contrast properties, compared to the control, and these changes can be detected at concentration levels corresponding to both sub-lethal (0.1%) and lethal (above 0.1%) concentration levels of H₂O₂.

MR relaxometry to characterize interactions of microbes with hydrogen peroxide

To further characterize changes in the MR contrast induced by biochemical interactions of H₂O₂ with *E. coli* BL21, the T₁ and T₂ relaxation times were measured. MR signal intensity is a function of the T₁ and T₂ relaxation times, and the proton density. Thus, characterizing changes in the T₁ and T₂ relaxation times induced by interactions of *E. coli* BL21 with H₂O₂ could elucidate the potential mechanisms for the observed changes (Figure 2) in MR contrast properties. Results in Figure 3 show changes in the T₁ relaxation time as a function of H₂O₂ concentration and incubation time. The results show at least a ten-fold difference in the initial T₁ relaxation time (at 0 minute) of *E. coli* BL21 suspension upon treatment with 0.1% v/v H₂O₂ as compared to the control (*E. coli* BL21 suspension in PBS without H₂O₂). Figure 3 shows that the decrease in the initial T₁ time is a function of H₂O₂ concentration in the range of 0.1-0.5% H₂O₂. With an increase in H₂O₂ concentration above 0.5%, no significant difference ($p > 0.05$) was observed in the initial T₁ measurement. The results in Figure 3 also indicate that the T₁ relaxation time changes during incubation of *E. coli* BL21 with H₂O₂.

Reduction in the T₁ value can be induced by generation of paramagnetic species, oxygen, upon interactions of microbes with H₂O₂. Biochemical interactions of H₂O₂ with intracellular catalases are known to generate oxygen (Kauczor & Kreitner, 2000; H. E. Schellhorn, 1995). In addition, oxygen is a well-known paramagnetic contrast agent. To demonstrate generation of oxygen upon biochemical interactions of microbes with H₂O₂, an oxygen sensitive dye Ru(BPY)₃ was added to the aqueous phase of *E. coli* BL21 suspension. The fluorescence

intensity of this dye is inversely proportional to oxygen concentration. Result in Figure S2 shows a significant decrease in fluorescence intensity of the oxygen sensitive dye, indicating that interaction of bacteria with H₂O₂ results in generation of molecular oxygen. The decrease in fluorescence intensity of the oxygen sensitive dye upon interaction of *E. coli* BL21 with H₂O₂ followed a similar pattern as observed for the T₁ relaxation time measurements (Figure 3), although the dynamic range of fluorescence measurements was less as compared to the T₁ measurement using MR. This result validates that molecular oxygen generated by endogenous enzymatic degradation of H₂O₂ is the key paramagnetic contrast source for the observed changes in MR properties

Similar to the trend observed for the T₁ relaxation measurements, the T₂ relaxation time for the stationary phase *E. coli* BL21 suspension treated with H₂O₂ shows significant reduction ($p < 0.05$) as compared to the controls (supplementary Figure S3). Reduction in the T₂ relaxation time was also observed to be a function of both H₂O₂ concentration and incubation time. In contrast to changes in the T₁ relaxation time, addition of H₂O₂ to the control PBS solution (without microbes) did induce a relatively small but significant reduction in the T₂ relaxation time. Therefore, T₂ relaxation time may not be influenced only by the biochemical interactions between H₂O₂ and microbes and thus was not investigated further.

MR relaxometry measurements in the presence of organic load

To illustrate the potential of measuring biochemical interactions of bacteria with H₂O₂ in the presence of organic load using relaxometry, the MR measurements were conducted using the stationary growth phase *E. coli* BL21 in LB broth (a common growth media for microbes with approximately 15,000ppm of organic content). LB broth was chosen as the model organic load based on a prior study (Park, Hung, Doyle, Ezeike, & Kim, 2001). Figure 4 shows significant reduction in the initial T₁ relaxation time as a function of H₂O₂ concentration between 0 and 0.5%. The results also show that reduction in the initial T₁ relaxation time reaches a saturation value for H₂O₂ concentration greater than 0.5%, similar to changes in the initial T₁ relaxation time observed for the *E. coli* BL21 suspended in PBS (without organic content) (Figure 3). These results indicate that MR measurement approach can measure bacterial resistance to H₂O₂ in the presence of organic load.

Interactions of stationary and exponential growth phase bacteria with hydrogen peroxide

The next objective was to compare the differences in interactions of stationary and exponential growth phase *E. coli* BL21 with H₂O₂ using relaxometry. MR relaxometry measurements (Figure 5) show that reduction in the initial T₁ relaxation time upon incubation of *E. coli* BL21 with H₂O₂ was significantly smaller ($p < 0.01$) for the exponential phase microbes as compared to that for the stationary growth phase microbes (Figure 3). For example, at 0.1% H₂O₂, the initial T₁ value for the exponential phase *E. coli* BL21 suspension was 899.0 ± 49.9 ms, whereas the initial T₁ value for the stationary phase *E. coli* BL21 suspension was 248.3 ± 4.7 ms. The results also show that reduction in the initial T₁ relaxation time for the exponential phase *E. coli* BL21 reaches a saturation value (~ 900 ms, Figure 5) for H₂O₂ concentration equal or greater than 0.1%. For the stationary phase *E. coli* BL21, reduction in the initial T₁ relaxation time reaches a saturation value (~120 ms, Figure 3) only for H₂O₂ concentration levels at and above 0.5%. Together, these results indicate that the stationary phase *E. coli* BL21 can generate

significantly higher concentration of molecular oxygen upon interaction with H_2O_2 as compared to the exponential phase *E. Coli* BL21. These results indicate that the stationary phase *E. coli* BL21 have significantly higher catalase activity as compared to the exponential phase *E. coli* BL21. This indication based on MR measurements is in agreement with the current literature (Beatriz González-Flecha, 1995; Loewen & Switala, 1986; Herb E. Schellhorn, 2006). Elevated expression of catalases is one of the key factors responsible for higher oxidative stress resistance of the stationary phase bacteria as compared to the exponential phase bacteria.

Correlation of MR measurements with reduction in culturability

Bacterial plate counting is the standard approach for measuring reduction in bacterial load following treatment with sanitizers. One of the goals of this study was to correlate the results of MR measurements with reduction in culturability of bacteria following treatment with H_2O_2 . Reduction in culturability of *E. coli* BL21 as a function of H_2O_2 concentration is shown in Figure 6. The results (Figure 6 (a)) show that in stationary phase *E. coli* BL21, a significant reduction (at or above 4 log) in culturability was observed at H_2O_2 concentration above 0.5%, while for the exponential phase *E. coli* BL21 (Figure 6 (b)) a significant reduction in culturability was observed at H_2O_2 concentration above 0.1%. These critical levels of H_2O_2 concentration for both the stationary and exponential phase *E. coli* BL21 correspond to the concentration levels of H_2O_2 above which no significant reduction in the initial T_1 relaxation times were observed (Figure 3 and Figure 5). These critical concentration levels of H_2O_2 for both the exponential and the stationary phase *E. coli* BL21 indicate concentration levels above which the H_2O_2 degrading enzymes (catalase) are saturated. Above these critical concentration levels, the excess concentration of H_2O_2 can induce lethal damage to *E. coli* BL21. Thus, the critical concentration of H_2O_2 that can be degraded by the *E. coli* BL21 is indicative of the resistance of the bacteria. Together, figures 3-6 demonstrate that resistance of *E. coli* BL21 to H_2O_2 is a function of physiological state of microbes and is not significantly influenced by organic load (LB broth, 15000 ppm).

MR measurements to characterize resistance of E. coli O157:H7 and L. monocytogenes to hydrogen peroxide treatments and its correlation with reduction in culturability

To demonstrate application of the MR approach to detect resistance of both gram-positive and gram-negative bacteria to H_2O_2 , stationary phase *E. coli* O157:H7 and *L. monocytogenes* were treated with H_2O_2 under the same set of experimental as described in Figure 3 (*E. coli* BL21). The results of MR measurements were correlated with reduction in culturability of the selected bacteria.

The results in Figure 7(a) and 7(b) shows changes in the T_1 relaxation time upon treatment of stationary phase *E. coli* O157:H7 and *L. monocytogenes* respectively with H_2O_2 . The results show that increase in H_2O_2 concentration results in decrease in the initial T_1 relaxation times, similar to the results obtained with the *E. coli* BL21 model system. Furthermore, these results also demonstrate that above a critical concentration of H_2O_2 no significant changes in the initial T_1 value were observed. For both the *E. coli* O157:H7 and *L. monocytogenes*, the critical concentration level was 0.5% H_2O_2 . This critical concentration level was same as measured for the stationary phase BL21 model bacteria, although the measured initial T_1 values for each species were different. For example, the initial T_1 relaxation time for

the stationary phase *E. coli* BL21 was 120 ms at 0.5% H₂O₂; whereas the initial T₁ relaxation time for the stationary phase *E. coli* O157:H7 was 215 ms at 0.5% H₂O₂. Surprisingly, the T₁ relaxation time for the stationary phase *L. monocytogenes* was similar to the measured T₁ value for the *E. coli* BL21 (124ms at 0.5% H₂O₂), under the same set of experimental conditions.

The results in Figures 8(a) and 8(b) show the reduction in culturability of the selected strains of bacteria as a function of H₂O₂ treatment. The results show that above 0.5% H₂O₂, a significant reduction in microbial culturability was observed in both *E. coli* O157:H7 and *L. monocytogenes*. This critical level of H₂O₂ concentration for both the stationary *E. coli* O157:H7 and *L. monocytogenes* corresponds to the concentration level of H₂O₂ above which no significant reduction in the initial T₁ relaxation times was observed (Figure 7(a) and Figure 7(b)). Overall, these results demonstrate that MR based measurement can determine the critical concentration of H₂O₂ above which significant reduction in culturability can be achieved in gram-positive and gram-negative bacteria expressing catalases. Although the methodology developed in this study is limited to catalase positive organisms, it has significant potential in improving food safety as many of the key food borne pathogenic bacteria including *E. coli* O157:H7, *Salmonella spp.*, and *Listeria monocytogenes* are catalase positive and are important targets of sanitation processes.

B: Results- Optical approach to measure oxidative stress induced in microbes by NaOCl and its correlation with reduction in microbial culturability

Assessment of culturability

Reduction in culturability of *E. coli* O157:H7 upon treatment with NaOCl was assessed based on the standard plating method (Figure 9(a)). The results in Fig. 1(a) shows no significant reduction in culturability of *E. coli* O157:H7 until the concentration of NaOCl reaches 8.8 mg/L. The microbial plate counting results show a linear reduction in bacterial counts (log scale) with increasing concentration of NaOCl in the range between 8.8 mg/L and 35.2 mg/L.

Loss in culturability and oxidative stress

To investigate intracellular generation of reactive oxygen species (ROS) in bacteria upon treatment with NaOCl, the bacteria cells were pre-labeled with the Aminophenyl fluorescein (APF) dye. APF is a fluorescein derivative, which is specific for measuring levels of ⁻OCl and *OH in cells upon exposure to HOCl or NaOCl[13]. This ROS reporter dye has been previously used in mammalian cells to measure ROS generated by endogenous hypochlorous acid[8]. Upon interacting with ⁻OCl ions and *OH radicals, the non-fluorescent APF dye is activated resulting in an increase in fluorescence signal intensity. Figure 1(b) shows that exposure of APF labeled bacteria to NaOCl resulted in a significant increase in fluorescence signal, indicating generation of intracellular ROS in bacteria upon exposure to NaOCl in the concentration range of 0-4.4 mg/L NaOCl. For the NaOCl concentration above 4.4 mg/L, there was no significant increase in the fluorescence intensity in bacterial cells with an increase in concentration of NaOCl.

The results in Figure 9(a) indicates that the concentration of NaOCl required for inducing statistically significant reduction in culturability of *E. coli* O157:H7 was higher than 8.8 mg/l of NaOCl. The APF dye labeled bacteria could only assess the increase in oxidative stress at sub-lethal concentration levels of NaOCl (0-4.4 mg/l). Therefore, the ROS measurement using the APF dye was not effective in predicting the critical concentration of NaOCl above which

significant reduction in culturability was achieved. The limited dynamic range of this measurement could be attributed to the limited permeation of APF dye in the bacteria. The outer membrane of Gram-negative bacteria, such as *E. coli* O157:H7 used in this study, has low permeability, hindering the amount of dye uptake [14]. These results also indicate that although sub-lethal concentrations of NaOCl can induce oxidative stress in bacteria, these stress levels may not be adequate to reduce the bacterial plate count.

Membrane damage and reduction in culturability:

Membrane damage induced by NaOCl was measured using the propidium iodide (PI) dye. PI dye is a membrane impermeable DNA interchelating dye. The PI dye can permeate into bacteria cells with compromised cell membrane. Upon permeation, the dye has a high affinity to bind DNA molecules, resulting in enhancement of its fluorescence intensity[4]. Figure 9(c) shows that exposure of bacteria to NaOCl results in an increase in normalized fluorescence intensity ratio of the PI dye at a NaOCl concentration level equal to or higher than 8.8 mg/L ($p < 0.05$). In the concentration range of 0-4.4 mg/L no significant increase ($p > 0.05$) in fluorescence intensity ratio of the PI dye was observed.

Comparing the results of Figures 9(a) and (c) suggest that the significant increase in membrane permeability of the PI dye is correlated with reduction in culturability. These results indicate that lethal concentration levels of NaOCl induce significant membrane damage and hence increase permeability of the PI dye. These results are in agreement with the results of previous studies that have indicated the key role of membrane damage for inactivation of bacteria upon treatment with lethal concentration of NaOCl [15, 16]. Membrane damage, assessed using the PI dye, was linearly correlated (the Pearson correlation was 0.96) with reduction in culturability for the stationary phase *E. Coli* O157:H7.

Glucose uptake and reduction in culturability

The results in Figure 9(d) show a significant drop in glucose uptake in the bacterial cells at the NaOCl concentration level of 8.8 mg/L. At concentrations of NaOCl higher than 8.8 mg/L, no significant further decrease in the uptake of 2-NBDG was observed. The result in Figure 9(d) shows that reduction of the intracellular uptake of this glucose analog following treatment of bacteria with NaOCl is a step change process as compared to a linear change observed in the case of membrane permeability measurements. These results indicate that reduction in the intracellular uptake of glucose could be an indicator for the critical concentration of NaOCl at and above which significant reduction in culturability can be achieved. The reduction in the intracellular uptake of glucose could be attributed to changes in activity of intracellular enzymes and glucose transporters upon treatment of bacteria with NaOCl. The activity of intracellular enzymes including glycolytic and phosphotransferase enzymes could induce changes in the metabolic state of cells upon treatment with NaOCl. Furthermore, the function of glucose transporters could also be influenced by membrane damage induced by NaOCl treatment (Figure 9(c)).

Outcomes and Accomplishments

- Development and validation of a novel label-free MR imaging and relaxometry method to rapidly assess the resistance of *E. coli* (*non-pathogenic and pathogenic*) and *L. monocytogenes* to H₂O₂.
- Demonstrated application of the MR method to measure oxidative stress induced in microbes in the presence of organic content and turbid samples

- Developed an optical approach to rapidly assess the oxidative stress induced by NaOCl in bacteria in simulated wash water
- Characterized the role of microbial physiology in influencing the oxidative stress resistance of the microbes
- Compared the oxidative stress induced damage in microbes with membrane damage, uptake of glucose and reduction in culturability.

Summary of Findings and Recommendations

- MR methods can predict the oxidative stress resistance of microbes to hydrogen peroxide. This implies that this approach has significant potential to measure efficacy of peroxide based sanitizers.
- The results demonstrated good correlation between the oxidative stress resistances measured based on MR measurements and reduction in microbial load.
- The results also demonstrated no significant differences in microbial resistance of pathogenic and non-pathogenic bacteria but significant differences in microbial resistance between stationary and exponential phase microbes.
- Optical approach based on the uptake of glucose was effective in predicting the critical concentration of NaOCl above which significant reduction (greater than 2 log) in microbial load can be achieved.
- Chlorine based sanitizers' induced significant damage to cell membrane while no significant membrane damage was detected using hydrogen peroxide.

APPENDICES

Publications and Presentations (required)

Phuong Le and N. Nitin, Fluorescence Spectroscopy Approach for Rapid Assessment of Antimicrobial Efficacy of Wash Water for Post-Harvest Processing of Fresh Produce, IFT, 2014.

Phuong Le and N. Nitin , Measuring oxidative stress response of microbes using magnetic resonance (MRI) and spectroscopy (NMR), IFT, 2013.

P Le, L Zhang, V Lim, MJ McCarthy, N Nitin, A novel approach for measuring resistance of Escherichia coli and Listeria monocytogenes to hydrogen peroxide using label-free magnetic resonance imaging and relaxometry; - Food Control, 50, 560-567, 2015.

Budget Summary (required)

Tables and Figures (optional)

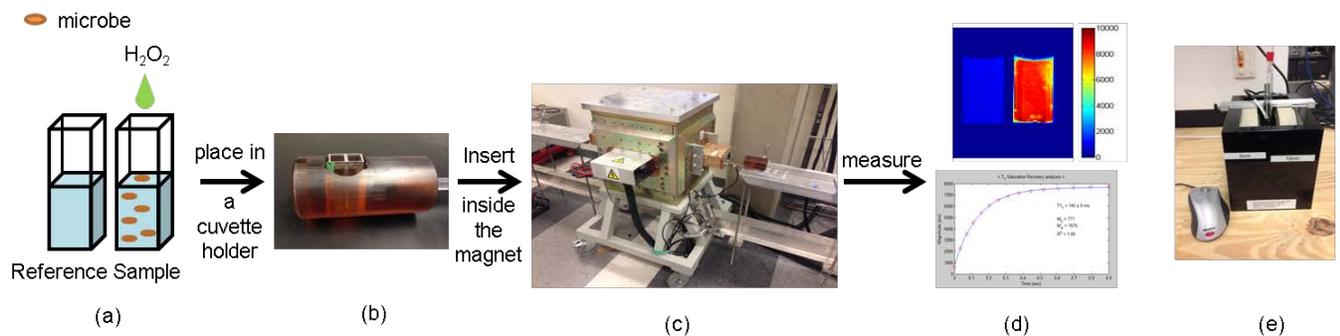


Figure 1. Illustrates the experimental approach to detect MR contrast based on interactions of microbes with H₂O₂. (a) The reference (PBS) and microbial suspension in PBS (sample) were treated with H₂O₂. (b-c) The samples were placed in a cuvette holder and placed inside a 1 Tesla (43.8 MHz) Aspect imaging unit with a 60mm radio frequency coil. (d) Illustrates the representative MR imaging and relaxometry measurements based on interactions of microbes with H₂O₂. (e) Shows an example of a bench top MR relaxometry set up. The size of this system is compared with a standard computer mouse to illustrate portability and compactness of the device.

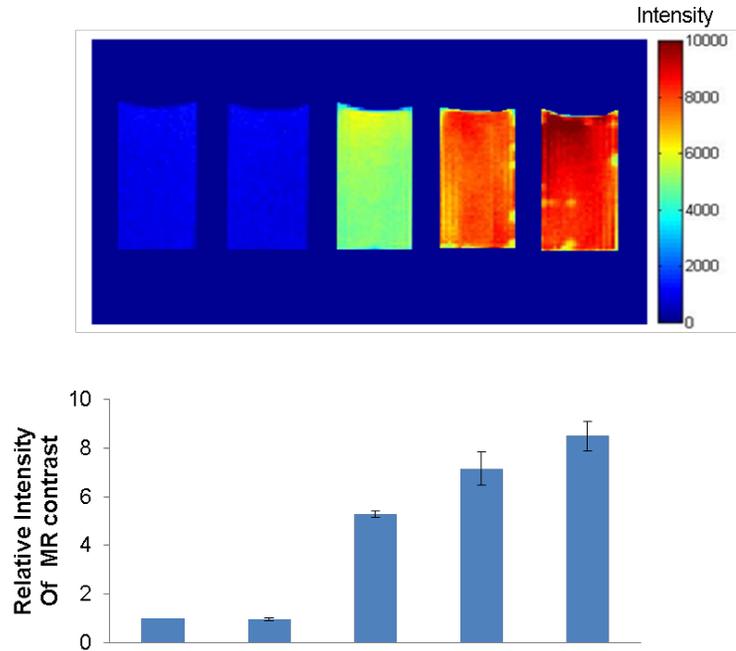


Figure 2: T₁ weighted MR imaging measurements to characterize interactions of stationary phase *E. coli* BL21 with H₂O₂. The control sample is 1x PBS. (a) Representative MR images to illustrate changes in MR contrast properties of BL21 suspension in PBS as a function of H₂O₂ concentration and (b) Relative quantification of MR signal intensity as a function of H₂O₂ concentration. The data was normalized with respect to the MR signal intensity in the reference sample. The results represent mean value \pm standard deviation based on three independent repeat measurements. (**p<0.01 compared to 0% H₂O₂).

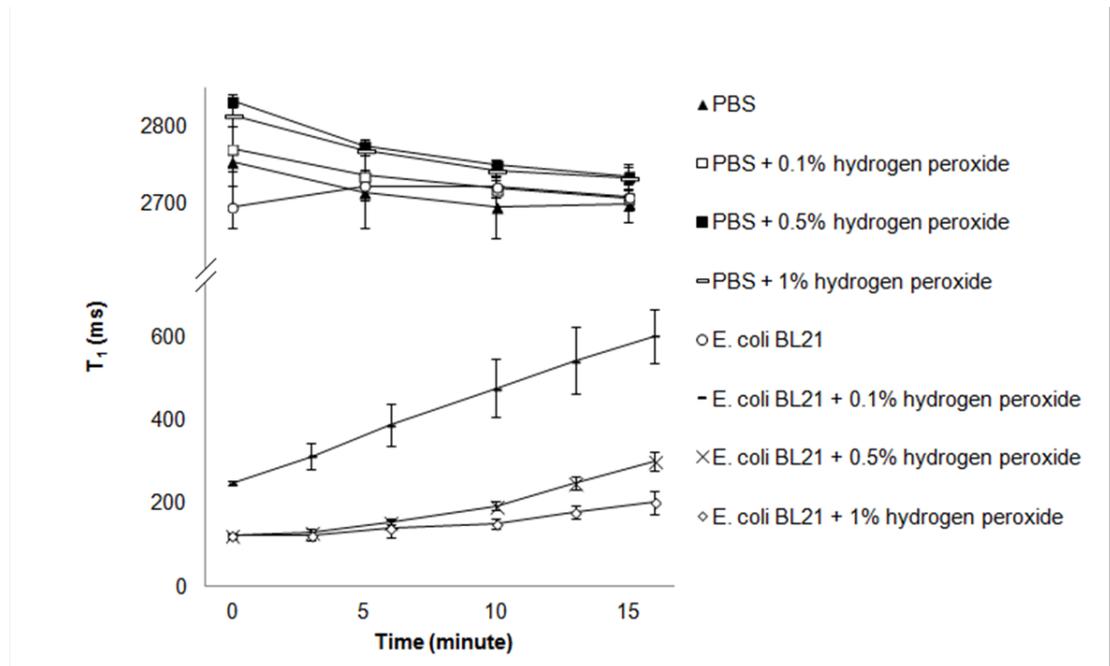


Figure 3. Changes in the T₁ relaxation time following treatment of microbial suspension (stationary growth phase *E. coli*) with H₂O₂ in PBS. The controls were PBS solutions incubated with different concentration (% v/v) of H₂O₂ and microbial suspension in PBS without H₂O₂. The results represent mean value ± standard deviation based on three independent repeat measurements.

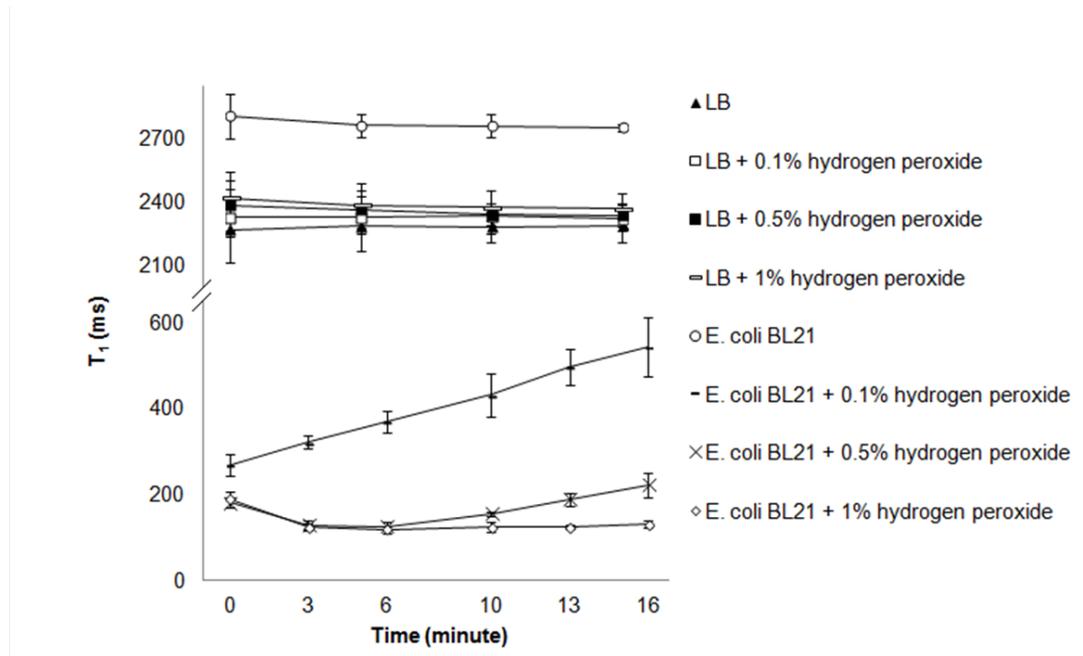


Figure 4. Demonstrates application of MR measurement approach to measure interactions of stationary phase *E. coli* BL21 with H₂O₂ in presence of organic content (15,000 ppm). Illustrates changes in T₁ relaxation time following treatment of BL21 suspension in LB media with H₂O₂. The controls were different concentrations of H₂O₂ in LB media and microbes in LB media without H₂O₂. The results represent mean value ± standard deviation based on three independent repeat measurements. The controls (without bacteria) were based on two independent repeat measurements

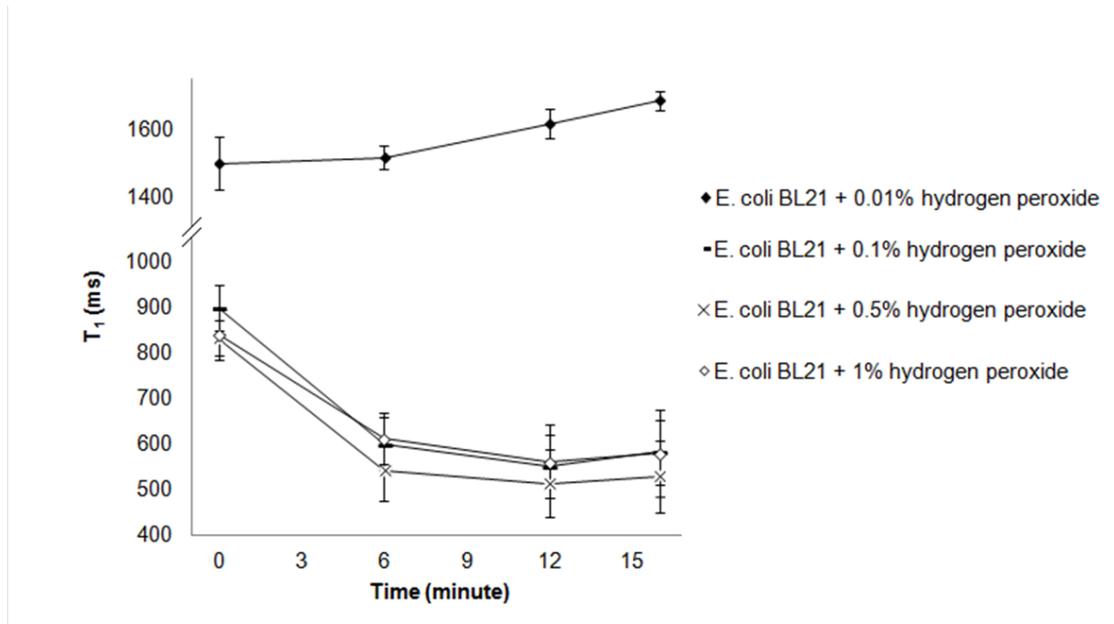


Figure 5. Changes in T₁ relaxation time following treatment of microbial suspension (exponential growth phase *E. coli* BL21) with H₂O₂. The results represent mean value ± standard deviation based on three independent repeat measurements.

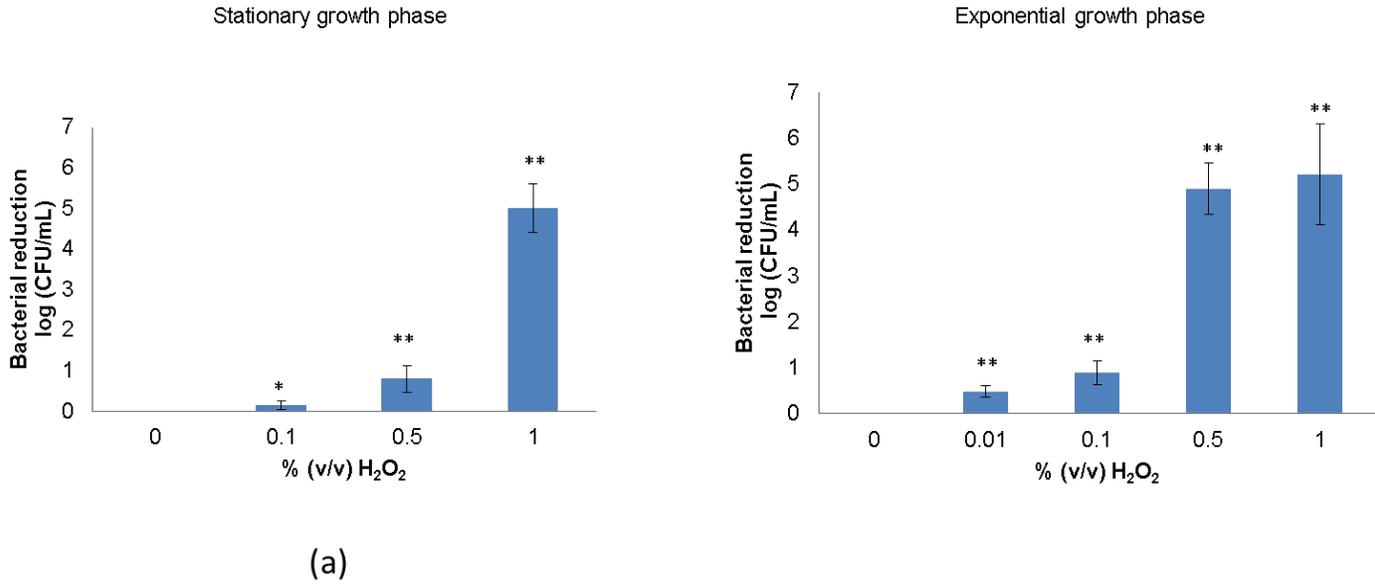


Figure 6. Measurement of reduction in microbial count for (a) Stationary growth phase, and (b) Exponential growth phase *E. coli* BL21 upon incubation with different concentration levels of H₂O₂. The results represent mean value \pm standard deviation based on three independent repeat measurements. (*p < 0.05, **p < 0.01 compared to 0% H₂O₂).

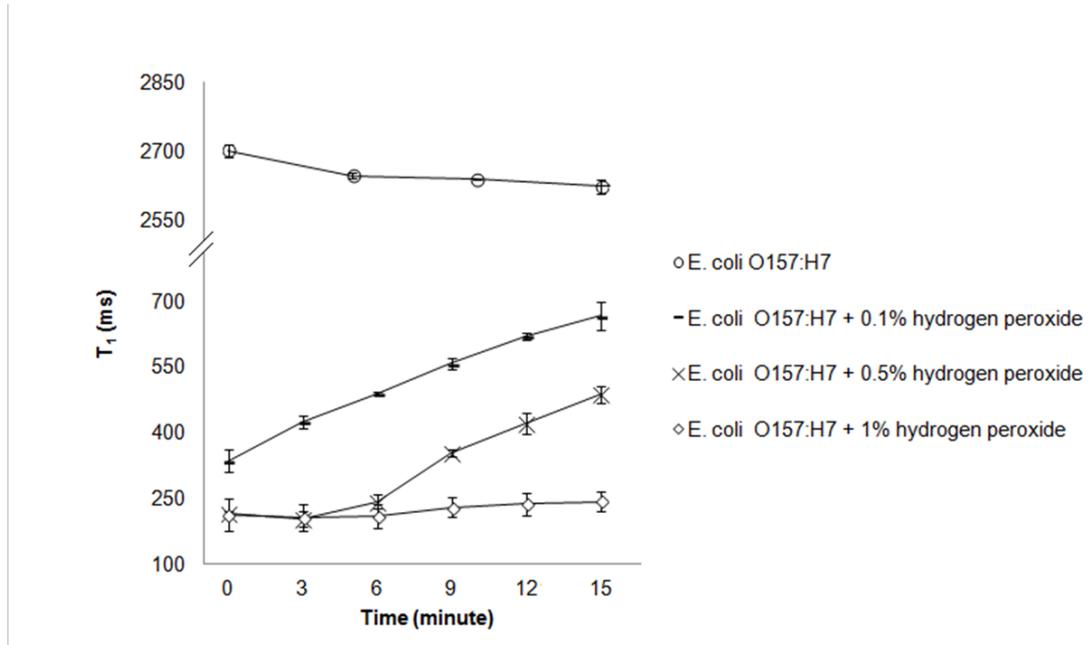


Figure 7. (a) Changes in the T₁ relaxation time following treatment of stationary phase *E. coli* O157:H7 suspension with H₂O₂ in PBS. The control was *E. coli* O157:H7 in PBS without H₂O₂. The results represent mean value ± standard deviation based on three independent repeat measurements.

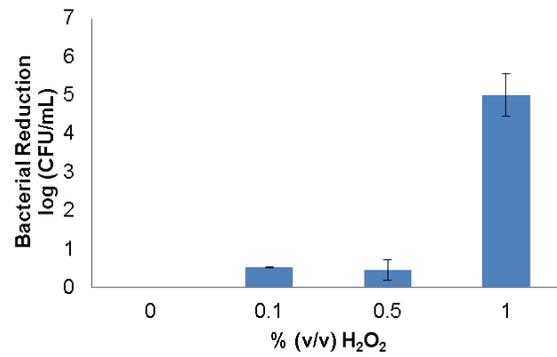


Figure 7. (b) Measurement of reduction in microbial count for stationary phase *E. coli* O157:H7 upon incubation with different concentration levels of H₂O₂. The results represent mean value \pm standard deviation based on at least two independent repeat measurements. (*p < 0.05, **p < 0.01 compared to 0% H₂O₂).

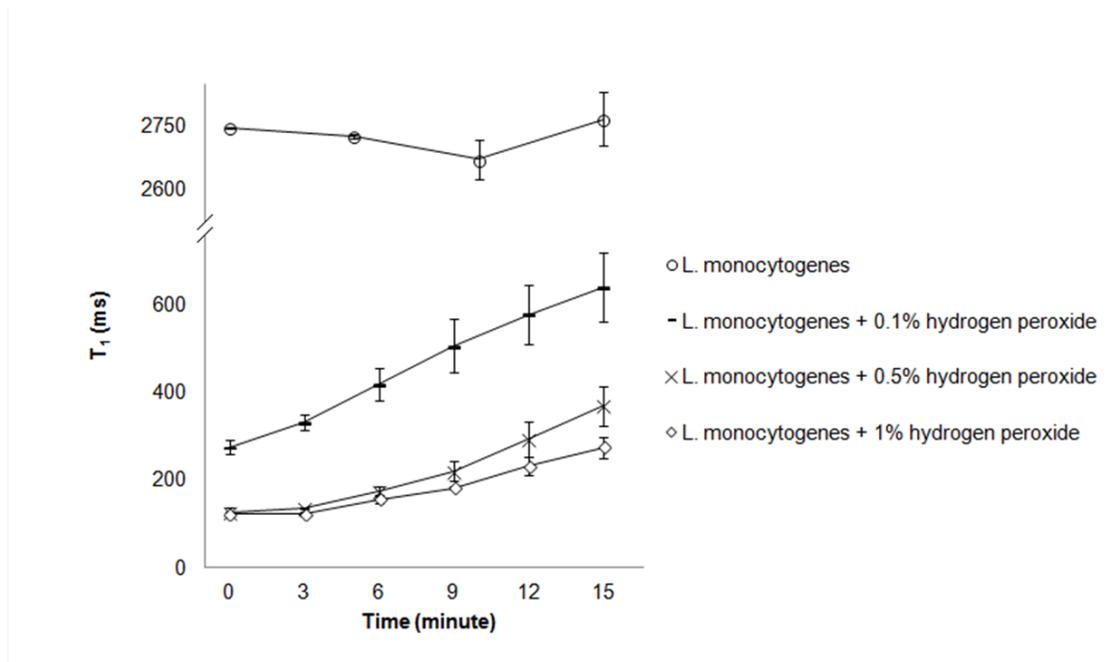


Figure 8. (a) Changes in the T₁ relaxation time following treatment of stationary phase *L. monocytogenes* suspension with H₂O₂ in PBS. The control was *L. monocytogenes* in PBS without H₂O₂. The results represent mean value ± standard deviation based on three independent repeat measurements. The non-treatment results (without H₂O₂) were based on two independent repeat measurements

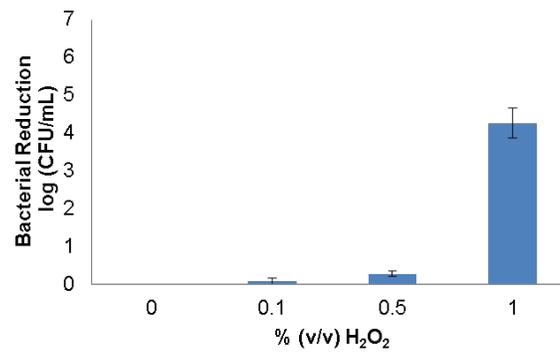


Figure 8. (b) Measurement of reduction in microbial count for stationary phase *L. monocytogenes* upon incubation with different concentration levels of H₂O₂. The results represent mean value \pm standard deviation based on three independent repeat measurements. (*p < 0.05, **p < 0.01 compared to 0% H₂O₂).

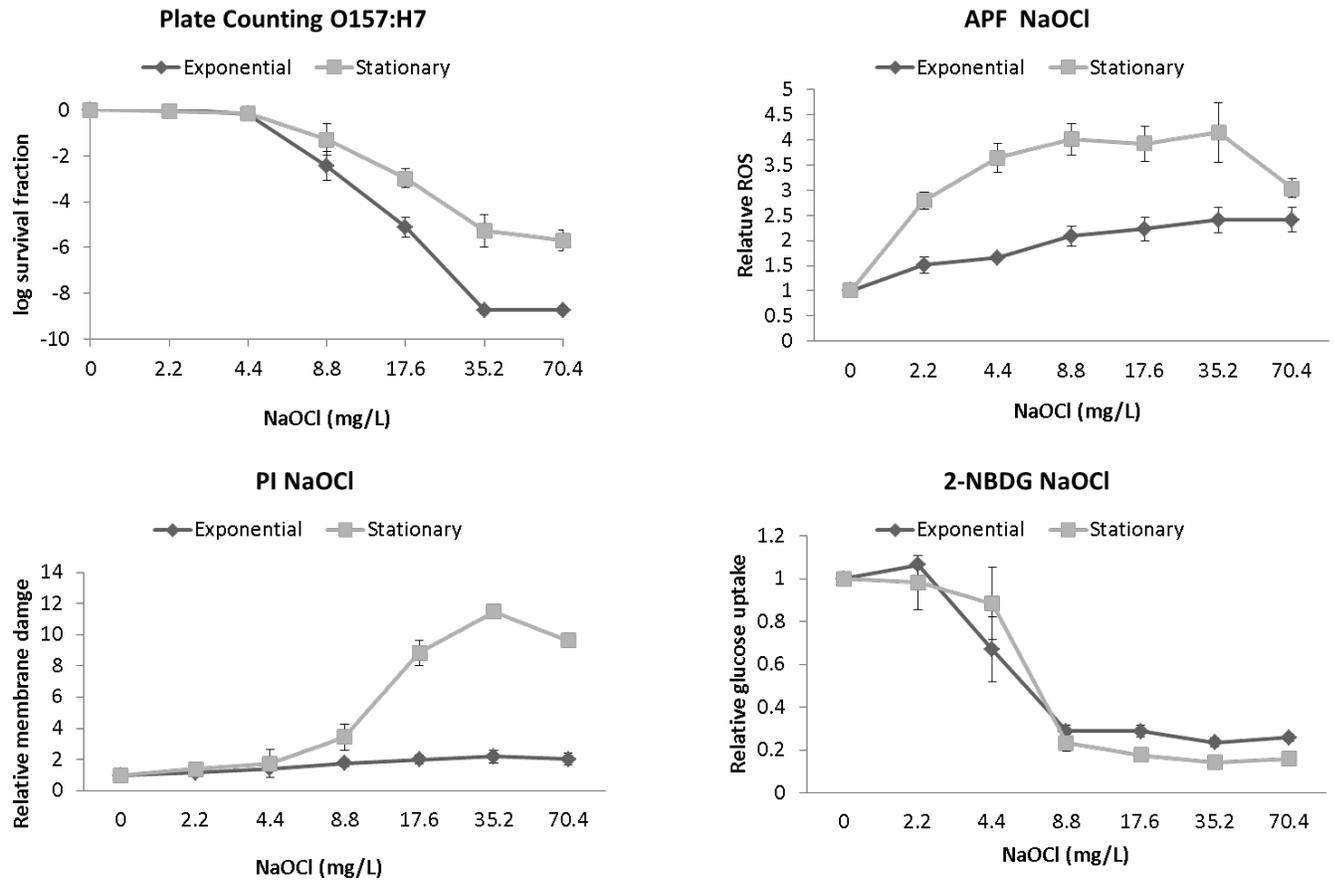


Figure 9 Treatment with NaOCl (a) Plate counting; (b) ROS measurements using APF; (c) Membrane damage measured with PI; (d). Glucose uptake measured with 2-NBDG

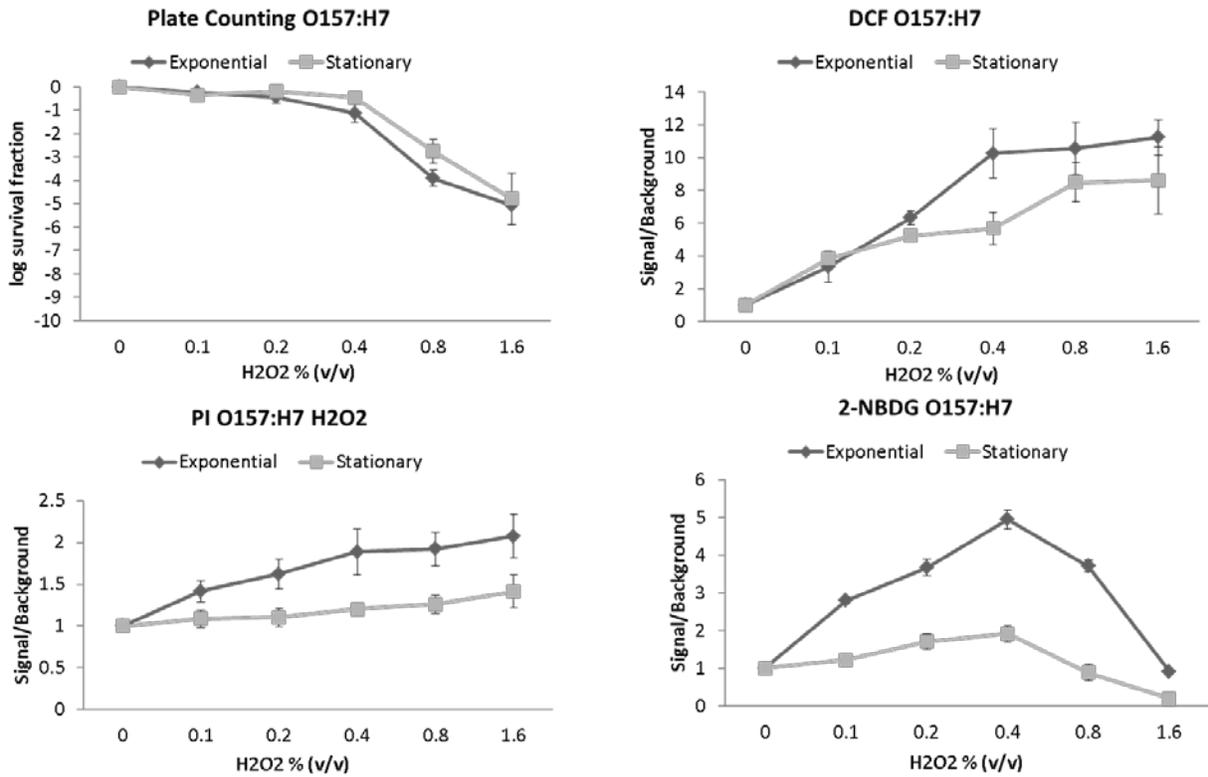


Figure 10 Treatment with H₂O₂ (a) Plate counting; (b) ROS measurements using H₂DCFDA; (c) Membrane damage measured with PI; (d). Glucose uptake measured with 2-NBDG

Suggestions to CPS (optional)