SYNERGISTIC EFFECT OF CHITOSAN ON PHOTOSENSITIZATION OF *STAPHYLOCOCCUS AUREUS* AND *ESCHERICHIA COLI* O157:H7 BY SODIUM COPPER CHLOROPHYLLIN

by

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ABSTRACT OF THE THESIS

The Synergistic Effect of Chitosan on Photosensitization of *Staphylococcus aureus* and *Escherichia coli* O157:H7 by Sodium Copper Chlorophyllin

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Microbial photoinactivation is a sensitizing process where a photosensitizer inactivates microorganisms by generating reactive oxygen species in the presence of light. Sodium copper chlorophyllin (Na-Chl) is a green dye approved by the Food and Drug Administration as generally recognized as safe (GRAS) and commonly used in dry beverage mixes. Na-Chl is a hydrophilic anionic photosensitizer, which is known to be less effective than cationic photosensitizer due to repulsive electrostatic force with the negatively charged membrane of the bacterial cell. Chitosan is a positively-charged antimicrobial polysaccharide. In this study, we investigated the synergistic effect of chitosan on photosensitization of *Staphylococcus aureus* and *Escherichia coli* O157:H7 by sodium copper chlorophyllin.

Bacterial suspension in sterile water was incubated with different concentrations of Na-Chl prior to illumination with LED light at 400 nm to identify optimal concentration of Na-Chl for photosensitization. To examine the synergistic effect of chitosan, three different concentrations of chitosan were tested with the identified optimal concentration of Na-Chl. Three sample groups were tested: concurrent incubation of chitosan and Na-Chl prior to illumination, sequential incubation of chitosan first followed by addition of Na-Chl prior to illumination, and sequential incubation of Na-Chl first followed by addition of chitosan prior to illumination. We found that, in the experiment with *S. aureus*, the concurrent incubation group had a slight increase in log reduction compared to photosensitizer alone, whereas the sequential incubation with chitosan first reduced the effectiveness of photosensitizer. Interestingly, the sequential incubation with Na-Chl first followed by chitosan resulted in a synergistic effect, with an additional reduction of two log cycles compared to the photosensitizer alone. However, photosensitization had negligible killing effect on *E. coli* O157:H7. The results indicate that the intracellular localization of photosensitizer is important for the effectiveness of photosensitization, and that the ratio between chitosan and Na-Chl along with the sequence of treatment is important for the effectiveness of the hurdle system. Further studies are necessary to improve the effectiveness of photosensitization on gram-negative bacteria.

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DEDICATION

To my parents,

Phu Cong Dao and Bich-Thuy Thi Nguyen

and my sister,

Dan Minh Dao Nguyen

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1. INTRODUCTION

Effective reduction of initial microbial loads in food is critical to ensure food safety. As life quality is improved with the advancement in technology, quality of food is appreciated on a par with its safety. In the last few decades, there has been growing gravitation of consumers towards health-conscious diets. Such interest results in a lucrative market for convenient foods with nutritional and sensory attributes closest to fresh foods but still with an extended storage life. Non-thermal sanitizing methods have emerged as superior options due to the absence of heat destruction on organoleptic properties. Many techniques have been discovered and utilized to improve the wholesomeness of food supply.

In 1997, the American Food and Drug Administration accepted the application of gamma radiation as a method to decontaminate packed poultry (Food and Drug Administration, 2014a). Gamma rays provide the energy needed to split water for the generation of hydroxyl radicals, which are harmful to microorganisms. However, the reluctance in consumer acceptance on the irradiated food labels has hampered its potential usage (Loaharanu and Ahmed, 1991). High intensity pulsed light was introduced as a safe effective alternative to sanitize food. The major disadvantage of high intensity pulsed light is its negative effects on sensory quality and bioavailability due to the production of heat within foods (Dunn et al., 1995). Although high pressure processing has proven great potential on inactivation of bacteria and retention of nutrients, its operation costs pose a major drawback on practicality (Sampedro et al.,

2014). Therefore, a new effective sanitization technique that provides solutions to the disadvantages of the aforementioned methods is of considerable benefit.

Microbial photoinactivation is a method where a light-activated photosensitizer (PS) produces singlet oxygen to inactivate microorganisms. Photodynamic therapy (PDT) has a long history in medical field dated back to the 1950s. Many cancer treatments rely on photosensitization to destroy tumor cells (Capella and Capella, 2003; Dolmans et al., 2003; Wilson, 2002). A number of photosensitizers have been identified and well-studied for their effectiveness: rose bengal, methylene blue, toluidine blue, acridine orange, hypericin, etc. However, none has been identified with the generally recognized as safe (GRAS) status for applications in food. For that reason, the application of photosensitization has been strictly restricted to medical field. If scientists can identify a food additive as photosensitizer with effectiveness as comparable as that of those used in the medical field, photosensitization could offer an alternative for non-thermal sanitization methods.

The use of photosensitizers (PS) is promising for many reasons: (i) the PS is inactive in the absence of light; (ii) the light source required is at a safe wavelength within the range of visible light; and (iii) no heat is generated to alter sensory attributes. However, there are several restrictions on the selection of photosensitizers for use in the food industry. An ideal photosensitizer for food must assume the following properties: generally recognized as safe (GRAS) status, high quantum yield of singlet oxygen during photosensitization, free of toxic byproducts.

Sodium chlorophyllin (Na-Chl), a natural green food dye granted as safe by the United States Food and Drug Administration to use as food additive in beverage mixes, has recently drawn much attention of scientists as a promising photosensitizer for applications in food (Food and Drug Administration, 2014b). One major drawback of Na-Chl is its limited effectiveness in bacterial inactivation. The bacterial cell membrane barrier carries negative charge due to its structural components. Na-Chl is negatively charged when dissolved in aqueous solution. It is known that cationic photosensitizers are more effective than anionic photosensitizers because of the repulsive electrostatic force between the negative charge of the cell membrane and the negative charge of the photosensitizers (Akilov et al., 2006).

Chitosan is polycationic polysaccharide derived from chitin, which is a major component of crustacean shells. Chitosan have been approved and widely used in food in Japan and Korea to improve shelf life and enhance food safety (KFDA, 1995; Weiner, 1992). The multiple positive charged chitosan binds to the negatively charged cell membrane and subsequently disrupts the integrity of the cells, causing lethal damage.

This present study seeks to enhance the effectiveness of photosensitization by coupling chitosan as a secondary antimicrobial agent with the photosensitizer Na-Chl.

2. HYPOTHESIS and OBJECTIVES

The hypothesis of my study is that chitosan improves the photosensitization by sodium chlorophyllin.

In order to investigate the synergistic effect of chitosan on photosensitization by sodium chlorophyllin, the objectives are:

1) To identify the optimal concentrations of photosensitizers and chitosan
2) To identify the optimal time length of irradiation for photosensitization
3) To identify the optimal sequence of treatment of chitosan and sodium chlorophyllin
4) To assess the difference in effectiveness of the treatment between Gram negative and Gram positive bacteria

3. LITERATURE REVIEW

3.1. Photosensitization in Pathogen Inactivation

3.1.1. Historical background

Photodynamic therapy (PDT) has a long history, dating back over 3000 years when the Indians employed psoralens to treat vitiligo (Ackroyd et al., 2001). However, not until the late twentieth century did the interest in PDT resurface with the discovery of a hematoporphyrin derivative by Lipson and Baldes (Lipson and Baldes, 1960). Having been utilized extensively in medical treatment to inactivate a wide range of microorganisms, PDT, however, remains relatively new for investigation in the area of food microbial inactivation.

3.1.2. Photophysics and Photochemistry

The photophysical and photochemical properties of photosensitization are illustrated in Figure 1. Photosensitizer (PS) is inactive in the absence of light. In this inactive ground state (also known as ground singlet state), the PS has a pair of electrons spinning at opposite directions in the low energy molecular orbital. Upon irradiation by light at a wavelength specific to the PS, one of the two electrons is excited into a high energy orbital while maintaining its spin direction (first excited singlet state). The electrons in this first excited singlet state have multiple ways to release the absorbed energy back to ground state. The electrons might return directly to ground state by emitting light (fluorescence), or by releasing heat (internal conversion). In these cases, the excited state lifetime is short (nanoseconds) (Castano et al., 2004).

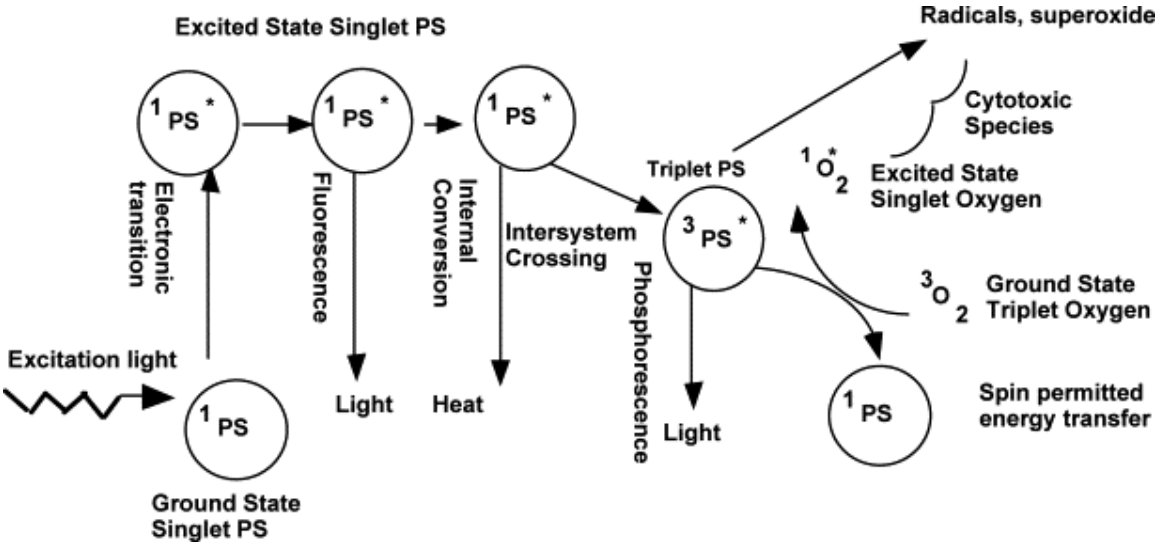

Figure 1: Illustration of photophysical and photochemical properties of photosensitization (Castano et al., 2004).

In less common occurrences, the substance undergoes intersystem crossing to enter a lower excited state (triplet state), where the excited electrons have spins parallel to one another after losing a part of the energy. The triplet state has lower energy than the first excited singlet state but higher than the ground state. To return to ground state from the triplet state, the PS has three ways, one of which is through the emission of light (phosphorescence). The other ways transfer the energy to other molecules in the surrounding, leading to photosensitization (Hamblin and Hasan, 2004). Photosensitization has two major pathways: Type I reaction and Type II reaction.

In Type I Pathway, the unstable energy of the PS triplet drives the PS to react with substrates in the surrounding, such as the cell membrane or a molecule. In this reaction, the PS transfers either a proton or an electron to the substrate to form anionic or cationic radical intermediates. These intermediates themselves have relatively low reactivity; however, they often further react with other substrates, especially oxygen, to form oxidized products, such as reactive oxygen species (ROS) and singlet oxygen ($^1O_2$) (**Table 1**) (Amor and Jori, 2000).

Table 1: Reactions in Type I and Type II Pathways in Photosensitization (Ormond and Freeman, 2013).

Excitation	$^1PS + h\nu \rightarrow {}^1PS^* \rightarrow {}^3PS^*$		
Photoprocess	**Reaction**		**Product**
Type I	$^3PS^* + {}^1PS$	$\rightarrow$	$PS^{-\bullet} + PS^{+\bullet}$
	$^3PS^* + D$	$\rightarrow$	$PS^{-\bullet} + D^+$
	$PS^{-\bullet} + O_2$	$\rightarrow$	$^1PS + O_2^{-\bullet}$
	$^3PS^* + O_2$	$\rightarrow$	$PS^{+\bullet} + O_2^{-\bullet}$
	$2O_2^{-\bullet} + 2H^+$	$\rightarrow$	$O_2 + H_2O_2$
	$Fe^{3+} + O_2^{-\bullet}$	$\rightarrow$	$Fe^{2+} + O_2$
	$Fe^{2+} + H_2O_2$	$\rightarrow$	$O_2 + OH^- + OH^{\bullet}$
Type II	$^3PS^* + {}^3O_2$	$\rightarrow$	$^1PS + {}^1O_2$

PS is the photosensitizer, ^{1}PS is PS in ground state, ^{1}PS* and ^{3}PS* are PS in singlet excited and triplet excited states, respectively, and D is an electron donor molecule. $PS^{-\bullet}$ and $PS^{+\bullet}$ are PS anion and cation radicals, respectively. D^+ is oxidized donor. $O_2^{-\bullet}$ is the superoxide anion.

In Type II Pathway, the triplet PS transfers the energy to only a limited number of molecules, only those with triple state multiplicity. O_2 is often the major substrate in type II pathway as its ground state is already in its triplet state but with a lower energy than the PS triplet state. The transfer of energy from the PS triplet to O_2 results in the generation of $^1O_2$ (Figure 2) (Inbaraj et al., 2005; Ormond and Freeman, 2013). The presence of oxygen is required as a prerequisite for photosensitization to take place. In aqueous solution, O_2 usually dissolves at a concentration of ~ $5\cdot 10^{-4}$mol/L (Shimoda, 1998).

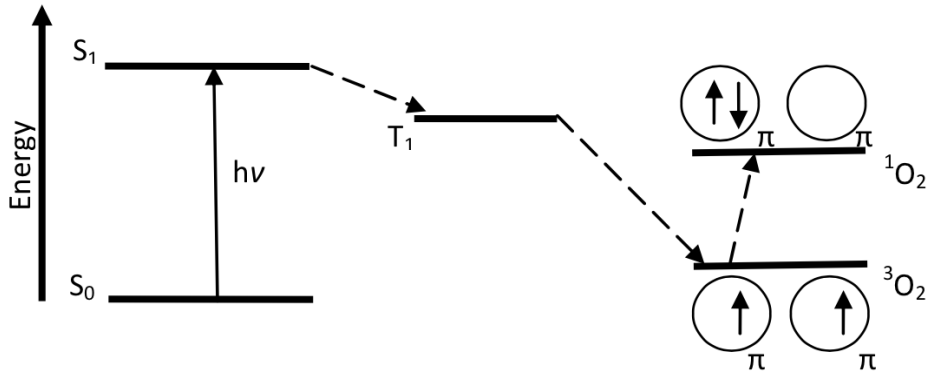

Figure 2: Type II pathway illustrated in simplified Jablonski diagram (Ormond and Freeman, 2013).

With this understanding of the photosensitization mechanism, a potential photosensitizer is expected to possess the following photophysical and photochemical properties (DeRosa and Crutchley, 2002):

a. A high absorption in the excitation spectrum (preferably in the visible spectral region)
b. A high quantum yield for triplet state generation (φ_T)
c. A high energy of the triplet state (E_T 95 kJ/mol)
d. A long lifetime of the triplet state (τ_T)
e. Low quantum yield of phosphorescence
f. High photostability

3.1.3. Mechanism of photosensitization

Although hydroxyl radicals, ROS, and singlet oxygen ($^1O_2$) can all cause lethal damage to bacterial DNA and cytoplasmic membranes, Martin and Logsdon (Martin and Logsdon, 1987) reported that $^1O_2$ is the primary sensitizing agent. The intrinsic hydroxyl radical scavengers in bacterial cells confer protection to the cells against the toxicity induced by radicals. $^1O_2$ alters cytoplasmic membrane proteins (Valduga et al., 1999) and disrupts cell wall synthesis (Nitzan et al., 1992), leading to inactivation of membrane transport systems and leakage of cellular contents. Both type I and type II pathways happen simultaneously at a ratio that depends on the type of photosensitizer, and on the relative concentrations of substrate and oxygen. $^1O_2$ has a short half-life and can only diffuse a distance of about 400 nm in water (Krasnovsky, 1998). The photosensitization must occur either within a proximal distance to the bacterial cells or inside the bacterial cells (Castano et al., 2004). For this reason, the interaction between bacterial cells and photosensitizers is critical to the efficiency of photosensitization.

3.1.4. Factors affecting the effectiveness of photosensitizers

Due to the short half-life of $^1O_2$, photosensitization is more effective when a photosensitizer is localized inside the cell. The uptake of the photosensitizer by bacteria is determined by two factors: the structure of the bacterial cell envelopes and the structure of the photosensitizer.

3.1.4.1. Gram positive bacteria vs. Gram negative bacteria

The membrane barriers of Gram positive (+) and Gram negative (-) bacteria are depicted in Figure 3. The cell membranes of Gram (+) bacteria consist of two parts: a relatively thicker porous cell wall surrounding a cytoplasmic membrane (Lambert, 2002) The cell wall is made up of interconnected peptidoglycans and lipoteichoic acids, residues of which render the negative charge to the cell wall of Gram (+) bacteria. Peptidoglycans and lipoteichoic acids allow passage to most photosensitizers with molecular weight lower than 1500-1800 Da (Lazzeri et al., 2004).

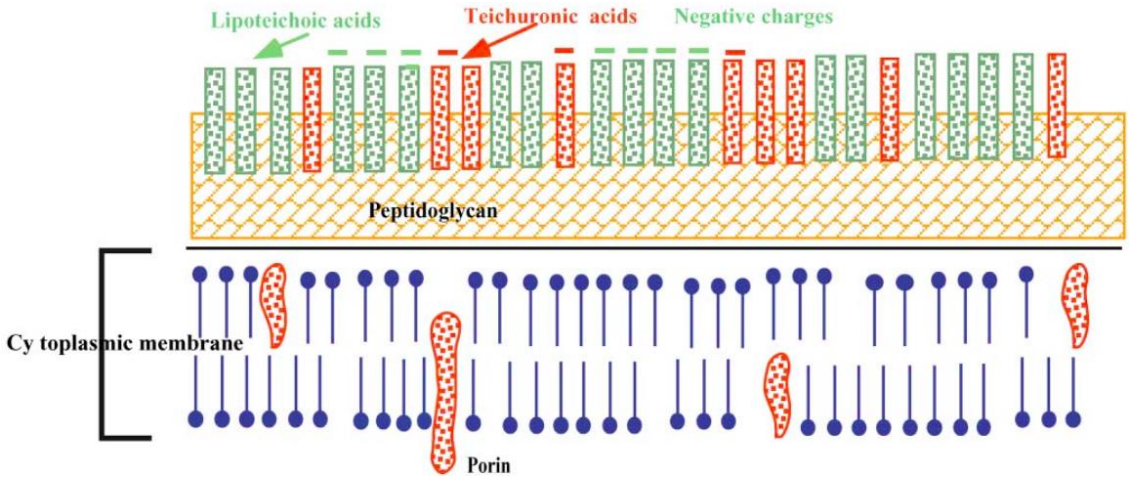

Figure 3: Structure of Gram positive (+) membrane barriers (Hamblin and Hasan, 2004).

Gram (-) bacteria have an additional layer of membrane barrier compared to Gram (+); it is called outer membrane. This outer membrane is rich in lipopolysaccharides and functional proteins (Leive, 1974). Lipopolysaccharides have negative charge that attracts cations, such as calcium (Ca^{2+}) and magnesium (Mg^{2+}), the binding of which ensures the thermodynamic stability of the outer membrane. There are three types of functional proteins in the outer membrane: enzymatic proteins, structural proteins, and transporter proteins. All these proteins have different levels of interactions with PS during photosensitization to determine its effectiveness. The most notable in the protein transporters is porins, which facilitate the uptake of hydrophilic compounds with low molecular weight (about 600-700 Da). Porins were initially reported to be located on the Gram (-) outer membrane and later they were also found on the cell wall of some Gram (+) bacteria (George et al., 2009b). In addition to porins, lipopolysaccharides on the outer membrane of Gram (-) allow, to some extent, diffusion of hydrophilic low molecular weight molecules.

Whether or not and to what degree a PS is bound to or transported to the inside the cell depends on the structure and the electrostatic charge of the PS (Hancock, 1984). The fact that Gram (-) has an outer membrane in addition to the two layers of Gram (+) makes Gram (-) bacteria less susceptible to photosensitization. Addition of cationic agents such as polymixin, Tris-EDTA has been shown to increase permeability of the outer membrane in Gram (-) bacteria (Malik et al., 1992).

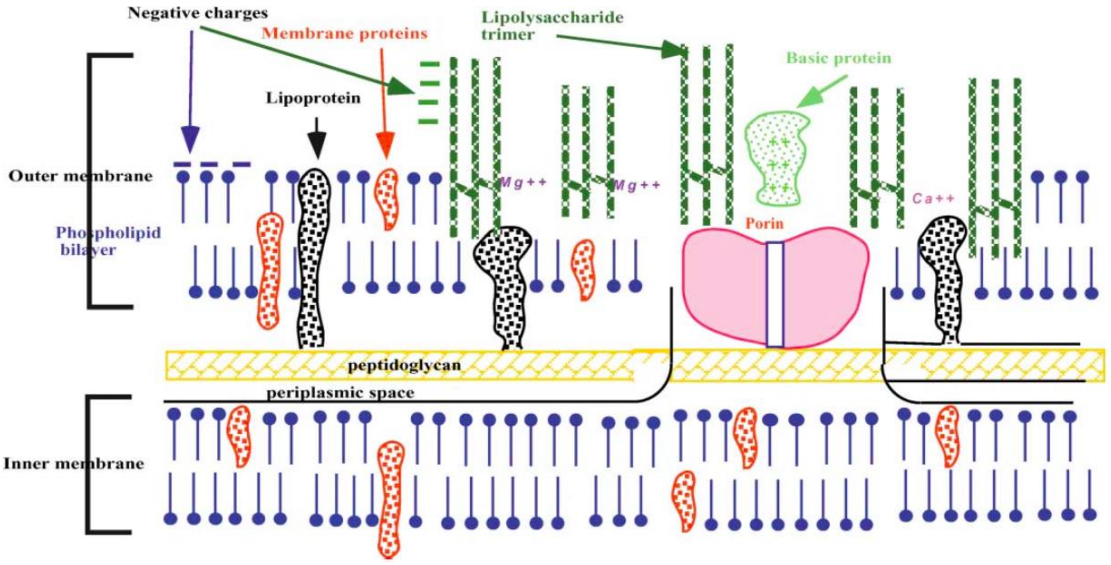

Figure 4: Structure of Gram negative (-) membrane barriers (Hamblin and Hasan, 2004).

3.1.4.2. Physical and Chemical Properties of Photosensitizers

The membrane barriers of Gram (+) and Gram (-) are negatively charged due to the presence of lipoteichoic acids and by lipopolysaccharides, respectively. These negatively charged entities provide binding sites to PS with positive charge, or cationic PS. If the PS is hydrophobic, positively-charged, and has low molecular weight, the membrane barriers of Gram (+) allow uptake of the PS into the cell through simple diffusion. Anionic and neutral PS's are not ready to diffuse through the cell membranes due to electrostatic repulsion between the negative charges of the PS and that of the cell membrane. A study by George et al. (2009) reported that bacterial cells did allow uptake of anionic photosensitizers to a lesser extent than cationic photosensitizers through the route of protein transport machinery embedded in the cell membrane. For this reason, it is known that cationic PS is more effective than anionic PS (Akilov et al., 2006).

The effectiveness of photosensitization by anionic PS can be further improved by coupling with a cationic entity, such as divalent agents (Ca^{2+} or Mg^{2+}) and cationic peptides (Hancock and Bell, 1988). The presence of a cationic entity destabilizes the outer membrane of Gram (-) bacteria by competing with Mg^{2+} and Ca^{2+}, which function as bridges between cell surface lipopolysaccharides. Cationic peptides increase membrane permeability by creating transient "cracks" on the cell surface. Their positive charge creates binding affinity towards the negatively charged cell membrane. Because peptides are bulky in size, the initial binding of multiple cationic peptides to the cell

surface consequently disrupts the structural arrangement of the cell membrane, creating a passage for molecules to pass through (Akilov et al., 2006).

3.2. Chlorophyllin

3.2.1. Background and Synthesis

Discovered in the 18th century, chlorophyll is a vital molecule in plants as it is responsible for photosynthesis, the process in which plants absorb sunlight energy to produce carbohydrates from CO_2 and water. The prefix *chloro*- in chlorophyll finds its root in the Greek word *chloros*, which means yellowish green. Such naming of the molecule commemorates the fact that chlorophyll imparts the green color to plants (Streitweiser and Heathcock, 1981). The basic structure of chlorophyll consists of a porphyrin ring centered by a magnesium atom and a long hydrocarbon (phytol) tail, which makes chlorophyll hydrophobic. Two major types of chlorophylls in higher plants, chlorophyll a and chlorophyll b, are determined by slight variations in the functional groups on their side chains. Chlorophyll a has a methylene group whereas chlorophyll b has an aldehyde group (Hendry, 2000).

Sodium copper chlrophyllins (Na-Chl) are semi-synthetic salt derivatives of chlorophyll a. In the industrial synthesis of Na-Chl, dehydrated alfalfa is the main source for the extraction of chlorophylls. Chlorophylls are structurally modified by saponification in an alkaline medium to form chlorophyllins. The process involves opening of the isocyclic ring and hydrolysis of the phytyl tail, the removal of which makes chlorophyllins hydrophilic (Humphrey, 1980). Usually, the magnesium is replaced by copper to give chlorophyllins its desirable chemical stability (Bobbio and Guedes, 1990).

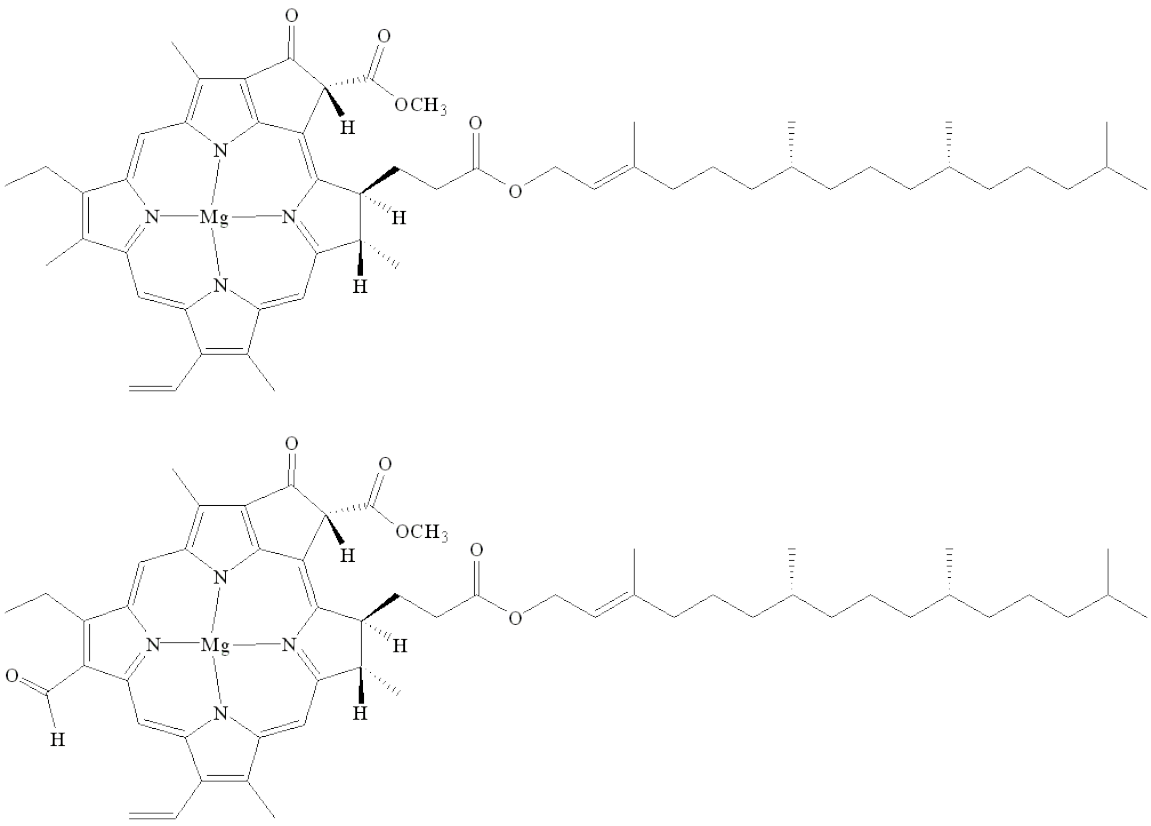

Figure 5: Molecular structure of Chlorophyll a (top) and Chlorophyll b (bottom)

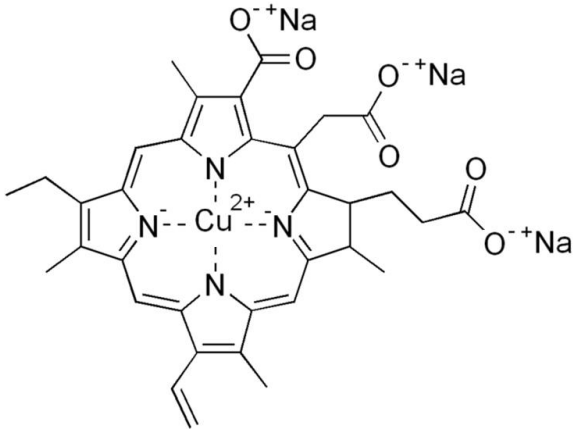

Figure 6: Molecular structure of Sodium Copper Chlorophyllin (Na-Chl). Na-Chl is derived from chlorophyll a with the opening of the isocyclic ring and removal of the phytyl tail.

Na-Chl is generally recognized as safe (GRAS) by the Food and Drug Administration to use as a coloring agent in citrus-based dry beverage mixes (Food and Drug Administration, 2014b)

Na-Chl has a long history of more than 50 years in medical treatment without causing any known serious side effects. It has been used both topically and internally to reduce odors related to wound, injuries, incontinence and colostomies, etc. In addition, Na-Chl demonstrates superior anti-carcinogenic ability by scavenging free radicals and by complexing with chemicals suspected to cause cancer (Pietrzak et al., 2003; Tachino et al., 1994). Recently, Na-Chl has risen as a potential photosensitizer with applications widely employed in the treatment of dental cavities. However, the potential of Na-Chl as a sensitizing agent in the food industry still remains a promising field for investigation.

3.2.2. Properties

Na-Chl has a blackish green color in powder form and a vibrant green color in solution. With a low molecular weight (Mw = 724.5 g/mol) and high hydrophilicity, Na-Chl easily dissolves in water. The replacement of Mg^{2+} by Cu^{2+} gives Na-Chl good thermal and photo stability (Hiroshi and Kunio, 2013). Na-Chl has strong affinity to biomolecules, and can be rapidly eliminated from the body with no negative effects.

3.2.3. Photochemistry

Na-Chl in water solution has strong absorption at 403 nm (Figure 7) (Wang et al., 2013a).

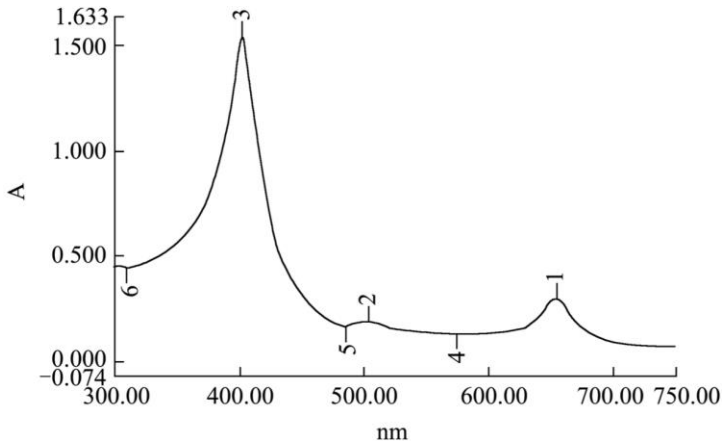

Figure 7: Absorption spectrum of Chlorophyllin in water (Wang et al., 2013a). Na-Chl has a strong absorption peak at 403 nm.

A study by (Salin et al., 1999) reported that Na-Chl underwent photobleaching at a rate dependent on illumination time and temperature. Photobleaching is a process where a fluorophore permanently loses the ability to fluoresce due to covalent modification by photo-induced chemical damage. Photobleaching of Na-Chl is negligible when illumination time is limited to 5 minutes or below (Figure 8). Temperature of 40°C or above hastens the photobleaching of Na-Chl with prolonged illumination time (Figure 9). Regarding my current study on photosensitization and its subsequent applications, which will be conducted at room temperature and with illumination time less than 5 minutes, photobleaching of Na-Chl in principle should be negligible under the conditions set in my study.

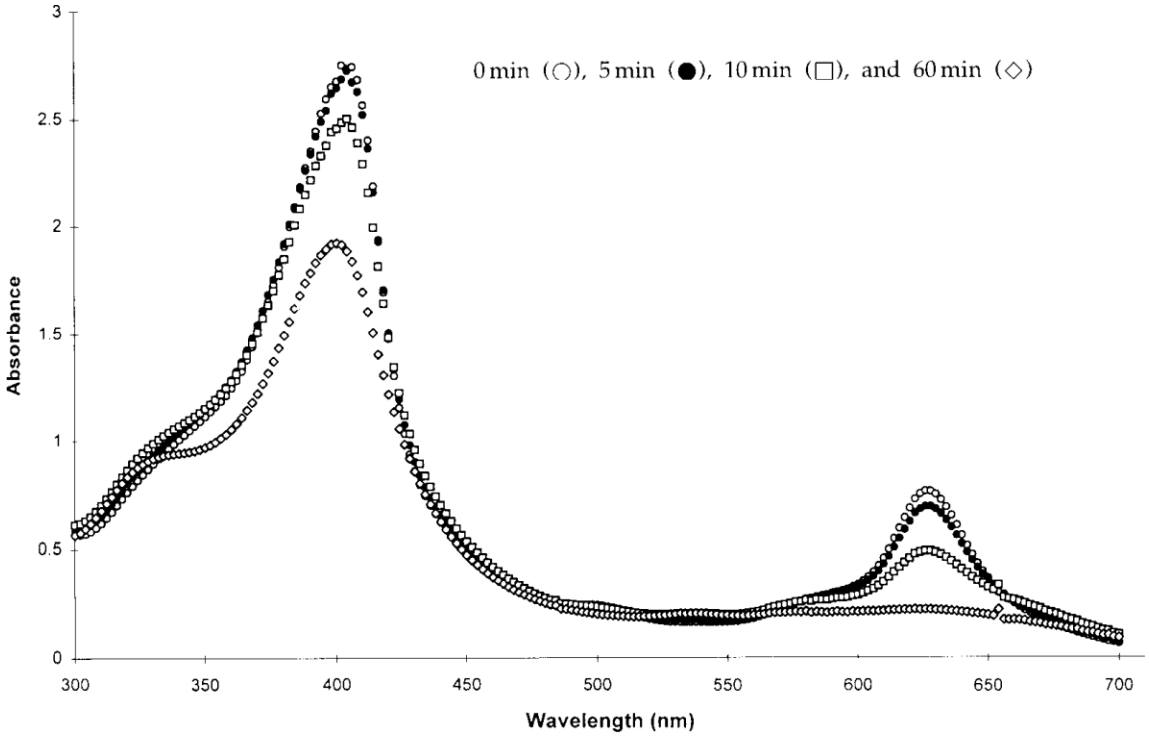

Figure 8: Absorption spectra of chlorophyllin as a function of time of photobleaching.

Readings were taken with UV-Vis photodiode array spectrophotometer at 20 s intervals for periods of up to 2h. Total radiation reaching the sample was approximately 14 Wm^{-2} at 630 nm (Salin et al., 1999).

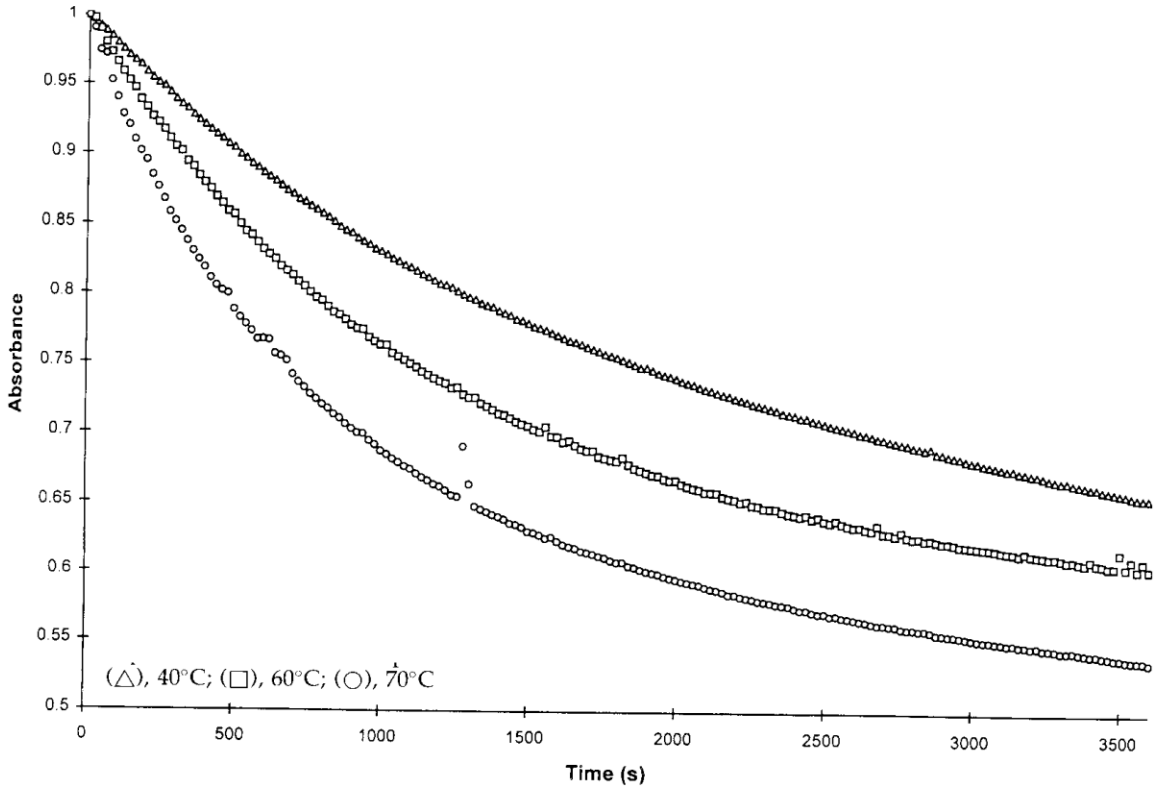

Figure 9: Time course of chlorophyllin photobleaching at three temperatures, 40°C, 60°C, and 70°C (Salin et al., 1999).

Photobleaching increases as the temperature increases. At 40°C, 2 minutes of illumination caused negligible photobleaching of chlorophyllins.

3.2.4. Literature review

A thorough understanding of sodium chlorophyllin (Na-Chl) is necessary to optimize the potential of Na-Chl as a photosensitizer. Unfortunately, the photochemical properties of Na-Chl have not yet been investigated as thoroughly as chlorophyll a. However, Na-Chl is expected to exhibit comparable photosensitizing properties with chlorophyll a because it is derived from chlorophyll a and has an absorption spectrum like that of chlorophyll a (Figure 10). The following section of this paper will discuss the photophysical properties of chlorophyll a to provide some understanding of the photophysical properties of chlorophyllin.

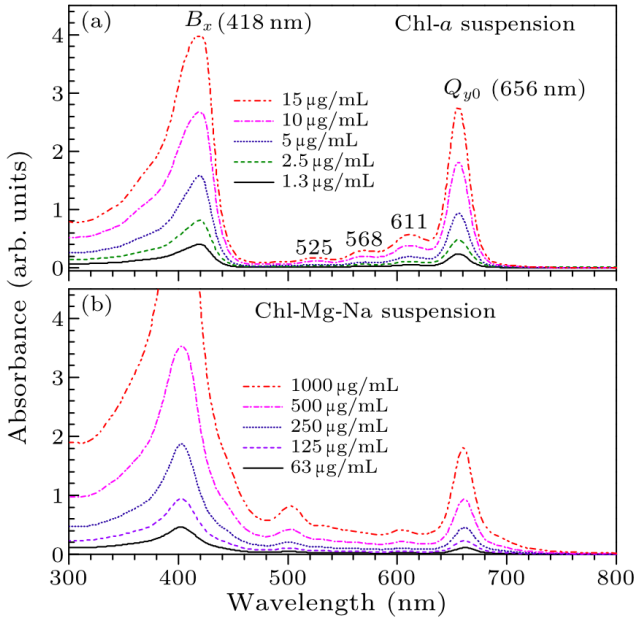

Figure 10: Absorption spectra of Chlorophyll a and Chlorophyllin.

Chlorophyllin salt and chlorophyll a have comparable absorption spectra with two peaks, one in the 400 region (405 nm for Sodium Magnesium Chlorophyllin and 418 nm for Chlorophyll a) and the other in the 650 nm region (Wang et al., 2013b)

3.2.4.1. Generation of singlet oxygen ($^1O_2$) by chlorophyll a:

Substantial studies on chlorophyll a show that the compound possesses photochemical properties of a powerful photosensitizer. Photoexcitation of chlorophyll a generates triplet states T_1 at a high quantum yield (0.55 at 77°K) (Egorov et al., 1990) with a long lifetime (2.0-2.7 ms), while the quantum yield of phosphorescence is significantly low (<1-3· 10^{-5}) (Semenova, 1973). These data demonstrate that phosphorescence does not compete with photosensitization reactions in the release of energy from the triplet state T_1 to the ground state S_0. Type I and Type II pathways in photosensitization are the preferred routes leading to the generation of singlet oxygen, whose quantum yield has a value close to that of the quantum yield of triplet state T_1 (S.Yu.Egorov, 1988).

One study investigated the photosensitization of different photosensitizers: Chlorophyll a (Mg-Chl), bacteriochlorophyll (Mg-BChl), protochlorophyll (Mg-PChl), pheophytin (Mg-PhC), and magnesium complex of phthalocyanine (Mg-PhC). The study quantified the quantum yield of $^1O_2$ and its oxidation products, anthracene endoperoxide ($AthO_2$) and hydrogen peroxide (H_2O_2), in different organic solvents (**Table 2**). Magnesium chlorophyllin had a high quantum yield for $^1O_2$ of 0.57 in CCl_4 solvent. Results demonstrated the powerful photosensitizing activity of Chlorophylls and its analogs (Lobanov et al., 2014).

Table 2: The quantum yield of singlet oxygen and its oxidation products by different photosensitizers in different solutions (Lobanov et al., 2014).

TPS	Solvent (support)	$\Phi(^1O_2)$	$\Phi(AthO_2)$	$k_{ef}(AthO_2)$, L/mol s	$\Phi(H_2O_2)$	$k_{ef}(H_2O_2)$, L/mol s
Mg–Chl	CCl_4	0.57 [2]	0.012	68	–	–
"	CCl_4/H_2O	~0.57*	0.012	66	0.00011	12
"	$CHCl_3$	~0.06*	0.0021	12	–	–
"	$EtOH/H_2O$	<0.02*	<0.001	<0.1	$<10^{-5}$	<0.1
"	H_2O/SG	–	–	–	0.00017	–
"	TEA/H_2O/SG	–	–	–	0.00026	–
"	$Morph/H_2O$/SG	–	–	–	0.00031	–
"	Hid/H_2O/SG	–	–	–	0.00043	–
"	TX-100/H_2O	~0.06*	0.0021	10	0.00002	4.1
"	TX-100/D_2O	0.35 [2]	0.0072	36	0.00007	8.1
Mg–BChl	CCl_4	0.60 [2]	0.013	73	–	–
Mg–PChl	TX-100/D_2O	0.84 [5]	0.019	86	0.00016	18
PhP	TX-100/D_2O	~0.35*	0.0024	94	–	–
"	H_2O/SG	–	–	–	0.00042	–
Mg–PhC	TX-100/D_2O	0.06–0.3**	0.0068	34	0.00008	10.2
"	$DMFA/H_2O$	–	<0.001	<0.1	$<10^{-5}$	<0.1

* The approximate values of $\Phi(^1O_2)$ were estimated on the basis of the known data on the lifetime of $^1O_2$ in different solvents or on the basis of the values of $\Phi(^1O_2)$, which are known for related compounds under similar conditions (e.g., PhP and bacteriopheophytin) [2, 5, 17–19].

** Presumably, the value of $\Phi(^1O_2)$ for Mg–PhC should be close to $\Phi(^1O_2)$ for the complexes of phthalocyanines with nontransition metals [20, 21].

3.2.4.2. Photosensitization by Sodium Chlorophyllin:

The effectiveness of bacterial photoinactivation by Na-Chl has been investigated by various studies on different microorganisms. **Table 3** compiles the data from different authors. The effectiveness of photosensitization by Na-Chl varies depending on multiple parameters: PS concentration, type of PS solvents, testing methods, characteristics of the microorganisms being tested, light dosage, and length of irradiation. Generally, sterile water as the PS solvent resulted in fewer log reductions than sodium chloride solution or methanolic potassium hydroxide (KOH). Bacteria were more susceptible in suspended culture than on solid surface. Vegetative cells were more susceptible to photosensitization than spores. Gram (-) and thermal resistant bacteria were more resistant than Gram (+) bacteria. Higher light dosage and longer irradiation resulted in more log reductions. Luksiene and colleagues (2010) (Luksiene et al., 2010) obtained 7 log reductions of Bacillus *cereus* after irradiating the suspended bacterial culture mixed with 0.75 µM Na-Chl for 5 minutes. However, 60 minutes of irradiation on the same bacteria, B. *cereus*, fixated on agar surface yielded only 3.1 log reduction (Kreitner et al., 2001).

The effectiveness of photosensitization varies immensely with the level of bacteria-photosensitizer interaction. When incorporated in the food matrix, photosensitizer was unable to inactivate microorganisms as effectively as in aqueous solution. A 7 log reduction of *Listeria monocytogenes* was obtained with 0.75 µM Na-Chl and 5 minute illumination (Luksiene and Paskeviciute, 2011a), while 1000 µM Na-Chl with 20 minute illumination resulted in less than 1 log reduction of the same strain

adhered to strawberry surface (Luksiene and Paskeviciute, 2011b). Another study investigating photosensitization in food liquids did not provide noticeable reductions. No log reduction was observed in milk and lychee juice with pulp as opposed to 4 log reductions in clear cranberry juice and salt water covered with packaging materials (Wang et al., 2013a). These findings indicate that the more suspended particulates are present in a food matrix, the more light scattering and quenching of the singlet oxygen by organic matter could happen, leading to reduced effectiveness of photosensitization.

This current study is dedicated to developing a hurdle system combining Na-Chl as a photosensitizer and chitosan as a secondary sensitizing agent to improve applications in food.

Table 3: Summary of literature review on photoinactivation of different microorganisms by Sodium Chlorophyllin

Authors	Bacterial Strains	PS Solvent	Testing methods	Light λ (nm)	Intensity (mW/cm^2)	Log reduction	PS concentration [μM]	Exposure to Light (mins)
(Kreitner et al., 2001)	*S. aureus* ATCC8096	H_2O	Culture (10^6) on agar	N/A	N/A	3.1	10	60
	B. cereus ATCC9634	H_2O	Culture (10^6) on agar	N/A	N/A	3.1	10	60
	B. subtilis ATCC1904	H_2O	Culture (10^6) on agar	N/A	N/A	4.2	10	60
	R. mucilaginosa H10007	H_2O	Culture (10^6) on wort agar	N/A	N/A	0.3	10	60
	S. cerevisiae H70449	H_2O	Culture (10^6) on wort agar	N/A	N/A	2.5	10	60
	K. javanica DSMZ	H_2O	Culture (10^6) on wort agar	N/A	N/A	3	10	60
(Luksiene et al., 2010)	*B. cereus* ATCC 12826	0.9 NaCl	Culture in flat bottom wells	405	20	7	0.75	5
	B. cereus ATCC 12826	0.9 NaCl	spore in vitro (10^8)	405	20	4	7.5	5
	B. cereus ATCC 12826	0.9 NaCl	Surface-attached cells	405	20	4	0.75	5

Table 4 (continued): Summary of literature review on photoinactivation of different microorganisms by Sodium Chlorophyllin

Authors	Bacterial Strains	PS Solvent	Testing methods	Light λ (nm)	Intensity (mW/cm^2)	Log reduction	PS concentration [µM]	Exposure to Light (mins)
(Erzinger et al., 2011)	Mosquito larvae	Methanolic KOH	10 larvae added to aq. mixture	N/A	3	100%	22.25 mg/L	120
(Luksiene and Paskeviciute, 2011a)	*L. monocytogenes* 56Ly	H_2O	Culture in flat bottom wells	405	12	7	0.75	30
	L. monocytogenes ATCL3 C 7644	H_2O	Surface-attached cells	405	12	4.5	150	15
(Luksiene and Paskeviciute, 2011b)	*L. monocytogenes* ATCL3 C 7644	0.9 NaCl	Strawberry immersed in TSB (10^7)	400	12	1.8	1000	20
	L. monocytogenes ATCL3 C 7644	0.9 NaCl	Strawberry immersed in TSB, Plated TSYEA	400	12	1.7	1000	20
	L. monocytogenes ATCL3 C 7644	0.9 NaCl	Strawberry immersed in TSB, Plated dichloran glycerol agar	400	12	0.86	1000	20

Table 5 (continued): Summary of literature review on photoinactivation of different microorganisms by Sodium Chlorophyllin

Authors	Bacterial Strains	PS Solvent	Testing methods	Light λ (nm)	Intensity (mW/cm^2)	Log reduction	PS concentration [μM]	Exposure to Light (mins)
(Wang et al., 2013a)	*S. aureus*	H_2O	Milk	400	1	0	10	10
	S. aureus	H_2O	Lychee juice without pulp	400	1	4	10	10
	S. aureus	H_2O	Lychee juice with pulp	400	1	0	10	10
	S. aureus	H_2O	Salt water	400	1	4	10	10
	S. aureus	H_2O	Covered with different packaging materials	400	1	4	10	5

3.3. Chitosan

3.3.1. Background and Synthesis

Chitosan is derived from chitin, which is a major component of the exoskeleton of *Crustacea* and insects, as well as the cell wall of many fungi. Chitin is a heteropolysaccharide composed of two monosaccharides, N-acetylglucosamine (GlnNAc) and D-glucosamine, joined at β-1,4-glycosidic bonds (Tharanathan and Kittur, 2003).

The synthesis of chitosan results from deacetylation of chitin in strong NaOH solution or in the presence of enzyme chitinase (Figure 9) (Friedman and Juneja, 2010). The main source of chitin for the commercial production of chitosan comes from the seafood industries, such as shrimp canning, where the removed shells are subjected to further chemical processing.

Due to its great antimicrobial activity and the ability to form polymer film, it has gained significant interest in the food industry with a wide range of applications, especially in extending shelf life. The usage of chitosan as safe food additive has been accepted in Japan and Korea since 1983 and 1995, respectively (KFDA, 1995; Weiner, 1992). Chitosan has a great potential to make its way into the generally recognized as safe (GRAS) list in the United States.

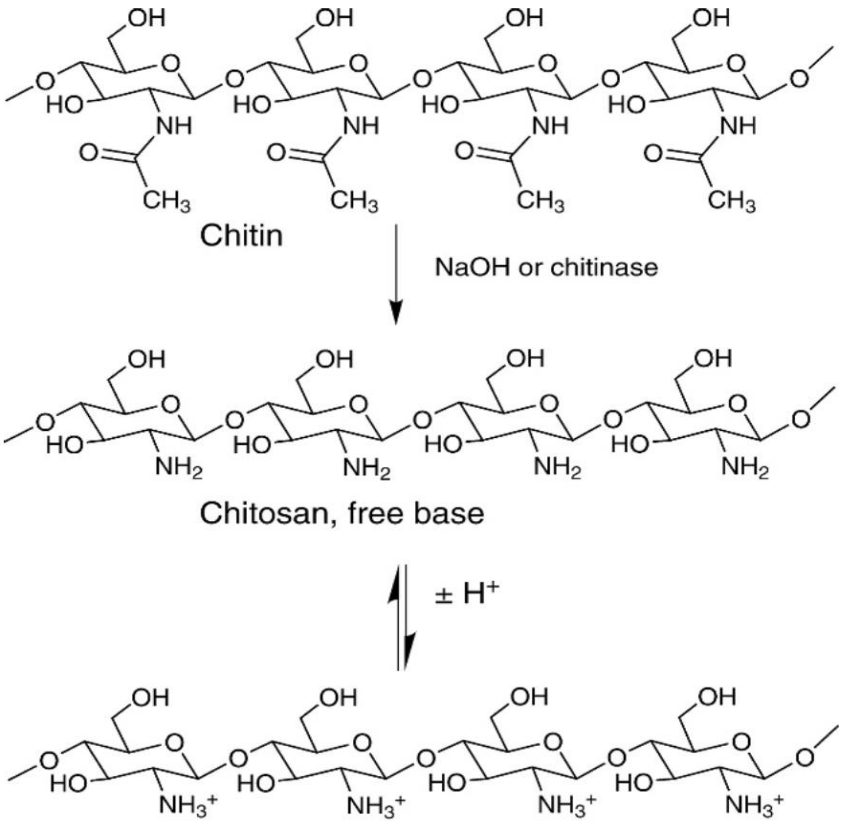

Figure 11: Synthesis of Chitosan from Chitin (Friedman and Juneja, 2010).

3.3.2. Properties

In an acidic microenvironment whose pH is lower than the pKa of chitosan (pKa ~ 6.5), chitosan exists in protonated form leading to positive charge. The reaction is driven by the following equation:

chitosan–NH_2 (unprotonated) + H^+ ⇔ chitosan–NH_3^+ (protonated); pKa = ~ 6.5 (Eq. 1)

The degree of protonation can be manipulated by varying the pH. At pH = pKa = 6.5, 50% of the chitosan is protonated; at pH = 5.5, 90% of the chitosan carries positive charge. In addition, chitosans also vary greatly in their molecular weights (50-2000 kDa) and viscosity of its solutions (Singla and Chawla, 2001).

3.3.3. Mode of microbial inactivation

Various efforts in elucidating the mechanism of bacterial inactivation of chitosan lead to three possible explanations: (i) in low pH (<6.0) where chitosan is predominantly protonated, the positively charged amino groups (NH_3^+) electrochemically interact with the negatively charged carboxylate residues (-COO) on the bacterial membrane surface, leading to cell wall disruption and consequent leakage of cytosolic materials (Tsai and Su, 1999), (ii) in higher pH (>6.0), the unprotonated amine groups of chitosan chelate with metal cations, such as Ca^{2+} that is essential nutrient for microbial growth (Figure 10) (Wang et al., 2005), (iii) chitosan penetrates into the cell and binds to the bacterial DNA to inhibit mRNA and protein synthesis (Hadwiger et al., 1986; Sudarshan et al., 1992). The general consensus is that the mechanisms driven by electrostatic force prevail over the one involving cell penetration.

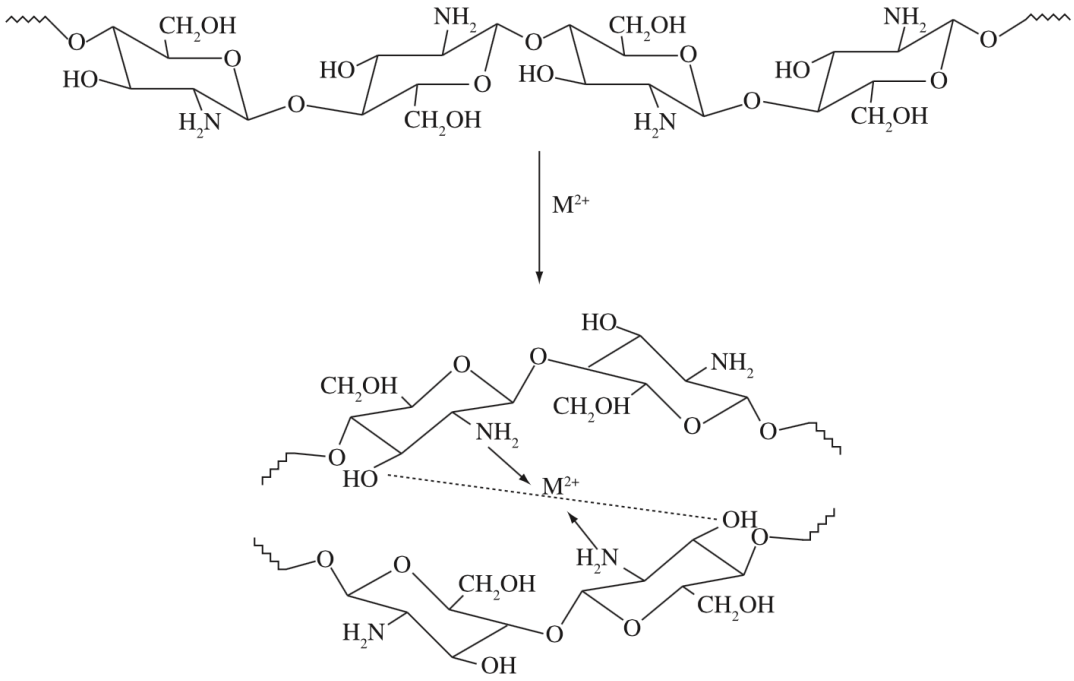

Figure 12: The chelation of cationic metal by chitosan (Wang et al., 2005).

3.3.4. Factors affecting the bacterial inactivation by chitosan:

3.3.4.1. pH:

As pH determines the mechanism of inactivation, the bactericidal effect of chitosan was found to be optimal at pH 6.0, the pH at which protonated amine groups predominate to alter the membrane permeability and at which the unprotonated groups are still present in an adequate amount to chelate metal cations from bacterial uptake for nutrition (Sudarshan et al., 1992).

3.3.4.2. Temperature and Time:

Chitosan solutions decreased their antibacterial activity after 15-week storage. Chitosan solutions stored at 4°C gave equal or better antibacterial activity than those at 25°C (No et al., 2006). Experiments with chitosan on Escherichia *coli* showed increased lethal effects when the temperature was increased from 4°C to 37°C. The stress caused by exposure to low temperature might have altered the cell membrane in a manner that reduces the binding sites for chitosan (Tsai and Su, 1999).

3.3.4.3. Molecular Weight (Mw) of Chitosan:

Chitosan with lower molecular weight exhibits higher antimicrobial activity as the low molecular weight enhances mobility, which facilitates for effective binding to the membrane surface (Vishu Kumar et al., 2005).

3.3.4.4. Deacetylation:

Lower degree of acetylation resulted in more effective antimicrobial activity of chitosan as deacetylation reveals free amino groups, which interact with bacterial cells (Andres et al., 2007; Tsai et al., 2004).

3.3.4.5. Cell age:

Age of the cells with regards to bacterial species affects the effectiveness of chitosan. For *Staphylococcus aureus,* late exponential phase was the most sensitive to lactose chitosan derivatives (Chen and Chou, 2005); whereas for *Escherichia coli*, mid exponential phase was the most susceptible (Yang et al., 2007). Stationary and late stationary phases were generally least sensitive to chitosan derivatives.

3.3.4.6. Microbial species

In several studies, Gram (-) appeared to be more susceptible to chitosan than Gram (+). The outer membrane in Gram (-) carries more negative charge than does the cell wall of Gram (+). Higher negative charge leads to more interaction with the positively charged biopolymer of chitosan.

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4. THE SYNERGISTIC EFFECT OF CHITOSAN ON PHOTOSENSITIZATION BY SODIUM CHLOROPHYLLIN

4.1. Abstract:

Microbial photoinactivation is a sensitizing process where a photosensitizer inactivates microorganisms by generating reactive oxygen species in the presence of light. Sodium copper chlorophyllin (Na-Chl) is a green dye approved by the Food and Drug Administration as generally recognized as safe (GRAS) in dry beverage mixes. Na-Chl is a hydrophilic anionic photosensitizer, which is known to be less effective than cationic photosensitizers due to repulsive electrostatic forces with the negatively charged membrane of the bacterial cells. Chitosan is an antimicrobial polysaccharide with high positive charge. In this study, we investigated the synergistic effect of chitosan on photosensitization of *Staphylococcus aureus* and *Escherichia coli* O157:H7 by sodium copper chlorophyllin.

In the experiment with *S. aureus*, the result showed a synergistic effect specific to the sequence of treatment. Concurrent incubation of both antimicrobial agents and sequential incubation with chitosan added first did not yield significant difference in log reduction (concurrent incubation had 2.65 log CFU/ml reduction, sequential incubation with chitosan added first gave 2.53 log CFU/ml reduction) compared to the control sample treated with photosensitizer alone (2.43 log CFU/ml reduction). The sample group sequentially incubated with Na-Chl first increased the log reduction to 4 log CFU/ml. Pre-incubation with Na-Chl prior to treatment with chitosan was necessary for the synergistic effect.

In the experiment with *E. coli* O157:H7, chitosan did not improve the effectiveness of photosensitization by sodium chlorophyllin. On the contrary, samples treated with both antimicrobial agents had reduced log reduction compared to the control sample treated with chitosan alone (1.4 log CFU/ml reduction). The presence of Na-Chl impeded the antimicrobial activity of chitosan. The results emphasized the importance of intracellular localization of photosensitizer in photosensitization.

4.2. Introduction:

In developed countries, consumers have gradually shifted their interests towards the nutraceutical values of foods more than just the sensory pleasure. Research has shown that consumption of fruits and vegetables is essential for good health (Drewnowski and Gomez-Carneros, 2000). Leafy vegetables and fresh fruits are an excellent source of phytonutrients, which include ascorbic acid (vitamin C), carotenoids, anthocyanins, phenols and vitamins (Goldman, 2003) . The most notable health benefit offered by these phytonutrients is their exceptional anti-carcinogenic activity (Hollman, 2001). However, most phytonutrients, such as vitamin C and thiamin, are sensitive to heat. Conventional thermal processing methods can degrade the functional compounds in plant and vegetables, attenuating the benefit of fruit and vegetable intake (Fennema, 1982). In order to provide a reliable supply of fresh produce without compromising on safety, the food industry demands a method of non-thermal sanitization that is simple and effective.

Microbial photoinactivation is a sensitizing method where a sensitizer requires the energy from a light source to inactivate microorganism. Despite the fact that photosensitization has been widely employed in the medical field, its application in the food industry remains an open field to explore. Chlorophyllin is a derivative of chlorophyll, light absorbing molecules that impart green pigment to plants. Chlorophyllin was scientifically proven to inactivate a number of food related microorganisms that are gram-positive (+) bacteria, such as *Staphylococcus aureus* (Wang et al., 2013a), *Bacillus cereus* (Luksiene et al., 2010), and *Listeria monocytogenes* (Luksiene et al., 2010) when exposed to light at ~400 nm wavelength. However, its effectiveness on food pathogens that are gram-negative (-) has not yet been investigated. Due to the intrisic difference in the membrane structure of Gram (+) compared to Gram (-) bacteria, Gram (+) bacteria are more susceptible to photosensitization than are Gram (-) bacteria (Malik et al., 1992). With emphasis on applications to the food industry, this study aims to contribute a foundation to the development of a non-thermal sensitizing method that is effective against a broad array of microorganisms related to food.

Chitosan has recently generated substantial interest due to its ability to extend the shelf life of food. Chitosan is synthesized from deacetylation of chitin, the main component of the exoskeleton of insects and crustacae, such as shrimps and crabs. Chitosan is a polyshaccharide with high positive charge (at pH <6.5), whose degree varies along with the degree of deacetylation during the synthesis of chitosan from chitin. The bacterial cell membrane carries negative charge due to its structural

components. The binding of the positively-charged NH_3^+ groups of chitosan to the negatively-charged phosphoryl groups of phospholipid components of cell membranes increases permeability of the cell membranes and ultimatey disrupts the bacterial membranes (Liu et al., 2004).

This present study attempted to evaluate the synergetic effect of chitosan on photosensitization of representatives of Gram (−) and Gram (+) bacteria; i.e., *Escherichia coli* O157:H7 and *Staphylococcus aureus*, respectively, by Sodium Chlorophyllin. In order to elucidate the impact of the treatment sequence with respect to effectiveness, the study investigated the results from three different methods of treatment: co-incubation of both chitosan and photosensitization followed by illumination (concurrent incubation), sequential incubation of chitosan first followed by Na-Chl, and sequential incubation of Na-Chl first followed by chitosan.

4.3. Materials and Methods

4.3.1. Chemicals and stock solution preparation

Low-molecular-weight chitosan with ≥75% deacetylation and Sodium Copper Chlorophyllin (Na-Chl) were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. A stock solution of 1% w/v chitosan was prepared by dissolving appropriate amount of chitosan in sterile 1% v/v glacial acetic acid (pre-filtered through 0.2 μm membrane filter). Two different concentrations (0.25% and 0.5% w/v) of chitosan solutions were prepared by diluting the stock solution with sterile

water. Na-Chl 750 µM stock solution was prepared in sterile water and filter sterilized using a 0.2 µm membrane filter. Different concentrations (5, 25, 50, 75, 100, 150, 200 µM) of Na-Chl solution were obtained by diluting the stock solution with sterile water. All the chemical solutions were kept in the dark at 4°C and used within three weeks of storage.

4.3.2. Bacterial strains and bacterial culture preparation

Staphylococcus aureus (ATCC 10832) and *Escherichia coli* O157:H7 (86.42) were grown overnight (~18 hours) in tryptic soy broth (TSB) at 37°C and maintained on tryptic soy agar (TSA) at 4°C for subsequent culturing within one month of storage. Aliquots (~ 20 µL) of overnight cultures were transferred to 10 mL of fresh TSB and incubated at 37°C to late logarithm phase (~10^8 CFU/ml). The cells were washed two times by centrifugation (20 min, 5000 RPM) followed with resuspension in equal amounts of sterile water. The fresh bacterial suspension was immediately used for the photosensitization experiments.

4.3.3. Light apparatus set up:

The light source for photosensitization was a 41 light-emitting diode-based (LED) lamp emitting blue light at λ=400 nm (Generic brand, USA). The lamp was positioned 2 cm directly above the surface of samples being tested. The intensity was calculated to be 20 $mW.cm^{-2}$ at the surface of samples.

4.3.4. Preparation of samples

The addition of each compound to the bacterial culture sample followed specific sequential order. Two methods of treatment were carried out in this study: (i) concurrent incubation method where the bacterial suspension was simultaneously incubated with both chitosan and Na-Chl for 30 minutes at 37°C before being exposed to light irradiation, (ii) sequential incubation method where the bacteria suspension was incubated with one agent for 15 minutes before the addition of the second agent for another 15 minutes incubation. There are a total of three sample groups, one sample with concurrent incubation and two samples with sequential incubations. Regardless of the incubation methods before light irradiation, all three sample groups contained the same amount of each component in the final solutions: 500 µL of bacterial solution (~10^8 CFU/ml), 100 µL chitosan, and 400 µL Na-Chl.

Final concentrations of chitosan tested were 0.025%, 0.05 %, and 0.1% (%w/v). 200 µL of each sample was transferred to 96 well plate to be irradiated with light for 2 minutes. The treated samples were serially diluted and plated to assess the bacterial log reduction. The data were statistically analyzed with ANOVA test for significance at $p<0.05$.

4.3.5. Statistical analysis:

The results were analyzed using analysis of variances (ANOVA) test followed by post-hoc t-test to confirm the significance of different sample groups.

4.4. Results:

4.4.1. Identification of optimal concentration for Chlorophylin:

S. aureus suspension was incubated with different concentrations of Na-Chl solution for 15 minutes prior to illumination. The illumination time was fixed at 10 minutes with varying concentrations of Na-Chl to assess the correlation between photosensitizer concentrations and photosensitization effectiveness. The final concentrations of Na-Chl in the samples tested were: 2, 10, 20, 30, 40, 50, 60, 80 µM. The results indicated that photosensitization was optimized at Na-Chl concentration of 30 µM (Figure 4.1). Below 30 µM, increasing concentration corresponds to increasing effectiveness of photosensitization. Above 30 µM, concentration and effectiveness have inverse correlation. This phenomenon aligns with other studies, where high concentration of photosensitizer was reported to have decreasing effectiveness due to inner filter effect, i.e., self-shielding of light (Barr et al., 1990).

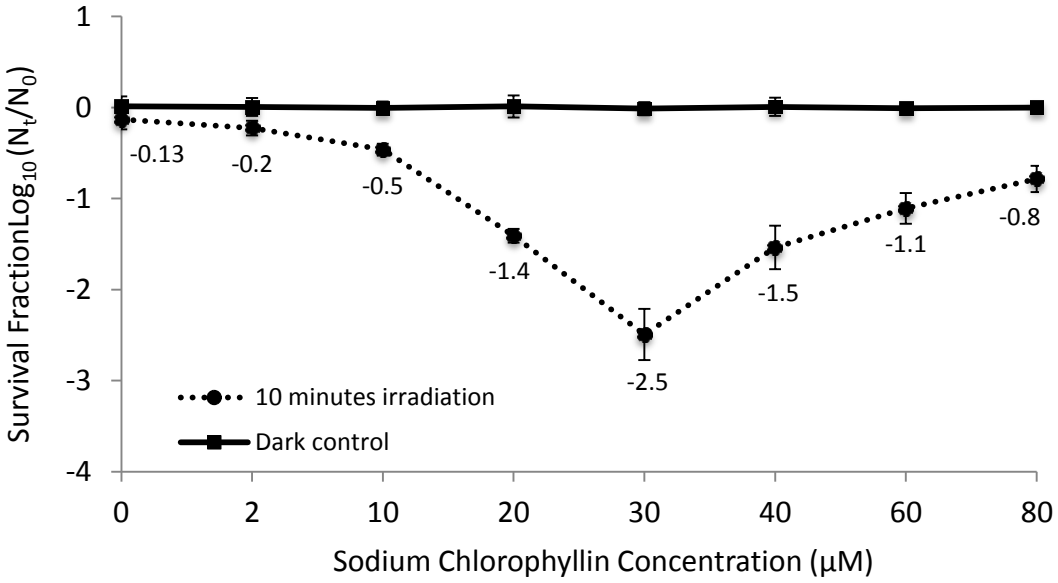

Figure 13: Survival fraction $Log_{10}(N/N_0)$ of *S. aureus* with respect to concentration of sodium chlorophyllin after 10 minutes illumination

4.4.2. Identification of optimal period of time for irradiation during photosensitization:

S. aureus suspension was incubated with Na-Chl for 15 minutes prior to illumination. The final concentration of Na-Chl in bacterial suspension was fixed at 30 µM while the illumination time was varied (2 minutes, 10 minutes and 20 minutes) to identify the optimal minimum illumination time length for photosensitization. A reduction of 2.1 log CFU/ml was observed with 2 minutes illumination, 2.6 log CFU/ml reduction with 10 minutes illumination, and 2.9 log CFU/ml reduction with 20 minutes illumination (**Figure 14**). Comparing between 2 minutes and 20 minutes, 10 fold increase in time gives less than 1 log difference in reduction.

In this study, the inactivation kinetics of *S. aureus* during a photosensitization treatment follows a similar patterns as survival during isothermal inactivations (Marfat et al., 2002; Mattick et al. 2001; Peleg et al., 2005). Instead of following the first-order kinetics, the survival fraction in relation to treatment time shows a a curvilinear semilogarithmic pattern that is appropiately described by a Weibullian inactivation model:

$$\mathrm{Log}_{10}\,(N_t/N_0) = -bt^n \qquad (2)$$

where N_t and N_0 are the momentary ("instantaneous") and initial counts, respectively, and b and n are the parameters of the model. Accodring to this model, if $n>1$, the semilogarithmic survival curve has a downward concavity, and if $n<1$, the curve has an upward concavity, which has been associated with a portion of the

population with increased sensitivity to the treatment that is eliminated fairly rapidly and a portion of the population with higher resistance. When n = 1, the model is linear.

Solver, a standard tool of Microsoft Excel (Microsoft Corp., Seattle, WA) was used to perform the non linear regression and obtained the values for b and n based on the experimental data. The survival fraction of *S. aureus* with respect to different time exposure can be estimated with the following Weibullian model:

$$\mathrm{Log}_{10}\,(N_t/N_0) = -1.90t^{0.14} \qquad (3)$$

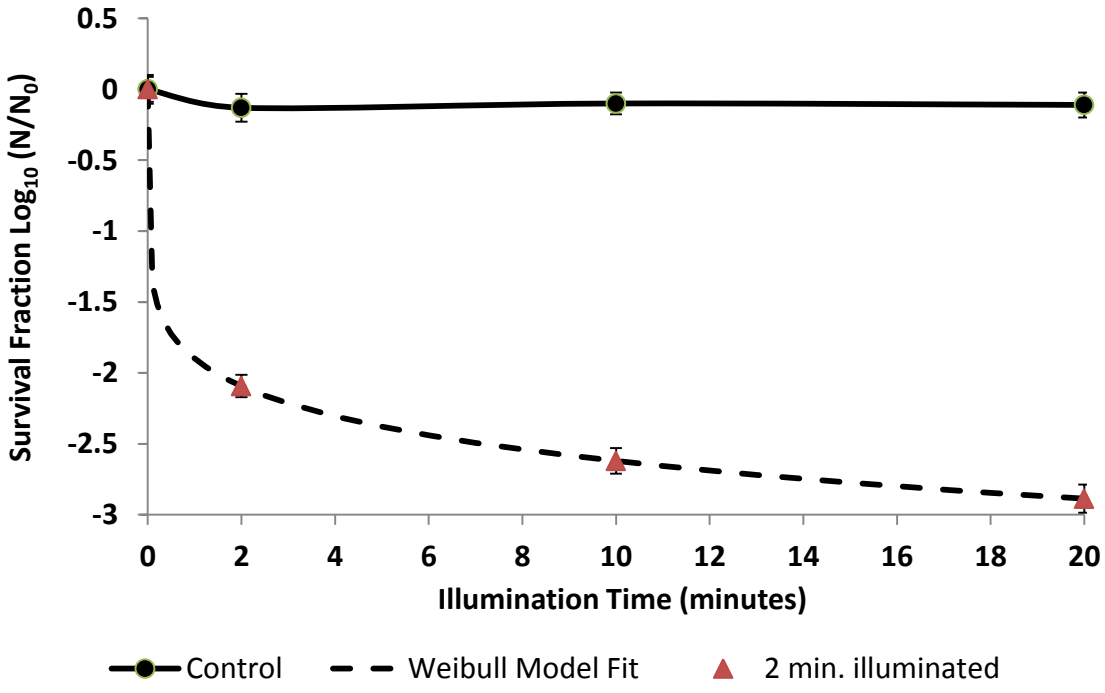

Figure 14: Survival fraction $\mathrm{Log}_{10}(N/N_0)$ of *S. aureus* as a function of illumination time.

4.4.3. Synergistic effect of Chitosan on photosensitization by Chlorophyllin:

4.4.3.1. Gram positive – *Staphylococcus aureus*:

The log reduction of different groups of treatment is shown on the y axis in Figure 17. Na-Chl alone had no lethal effect when incubated in the dark but gave 2.43 log reduction with 2 minutes illumination. Three sublethal concentrations of chitosan were tested: 0.025, 0.05, and 0.1% w/v. Increasing concentration of chitosan increases the lethal effect. There are differences in effectiveness among different treatment groups. Generally, compared to photosensitization by Na-Chl alone, concurrent incubation of both chitosan and Na-Chl had equal (in sample with chitosan at 0.025% and 0.1% w/v) or slight increased effect (in sample with chitosan at 0.025% w/v) on photosensitization; sequential incubation with chitosan first followed by Na-Chl had equal (in sample with chitosan at 0.05% and at 0.1% w/v) or reduced effect (in sample with chitosan 0.025% w/v) on photosensitization; however, when the sample was sequentially incubated with Na-Chl first followed by chitosan, a synergistic effect was observed with killing effect improved by 1.60 log CFU/ml.

Regarding chitosan concentration, both groups of sequential incubations followed the general trend of increasing effectiveness with increasing chitosan concentration. However, when both chitosan and Na-Chl were incubated concurrently, chitosan concentration had an inverse correlation with lethality.

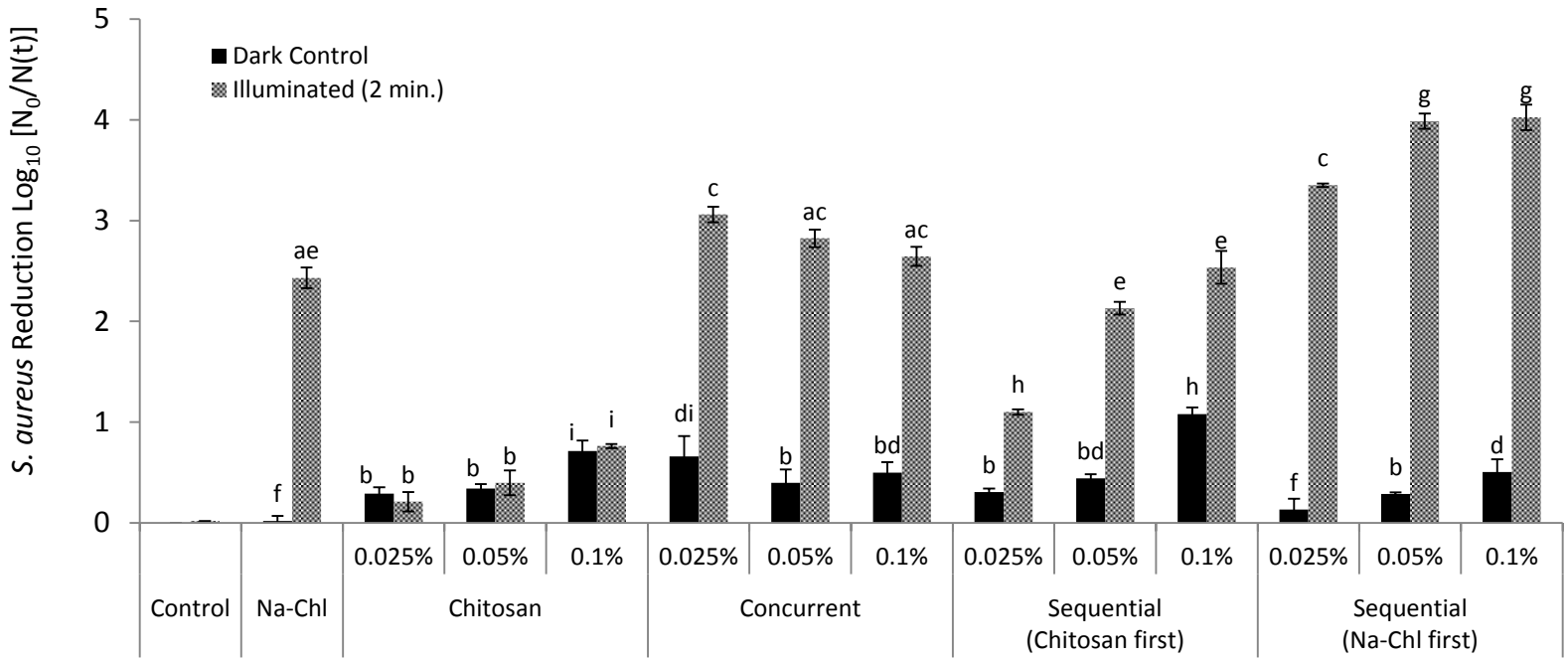

Figure 15: Reduction Fraction $Log_{10}(N_0/N)$ of *S. aureus* in different treatment groups.

Bacterial suspension in sterile water was mixed with antimicrobial solutions in different sequence to examine the synergistic effect of chitosan in relation with sequence of treatment: concurrent incubation of chitosan and Na-Chl, sequential incubation of chitosan first followed by Na-Chl, and sequential incubation of Na-Chl first followed by chitosan. Three different concentrations of chitosan were tested: 0.025%, 0.05%, and 0.1% (%w/v) while chlorophyllin concentration was fixed at 30 µM. Total incubation time was 30 minutes with variation of 15 minutes depending on sequence group. Samples were illuminated for 2 minutes at 400 nm. Log reduction was calculated by serial dilution and plating. Means with different letters are significantly different (t-test, $p<0.05$).

4.4.3.1. Gram negative – *Escherichia coli*:

Chitosan had a more pronounced effect on *E. coli* O157:H7 than on *S. aureus.* When treated with chitosan alone, increasing concentration of chitosan increased the inactivation of *E. coli* O157:H7. It appeared that photosensitization had no lethal effect on *E. coli* O157:H7, and that the presence of Na-Chl impeded the antimicrobial effectiveness of chitosan, regardless of the order of incubation sequence (Figure 18).

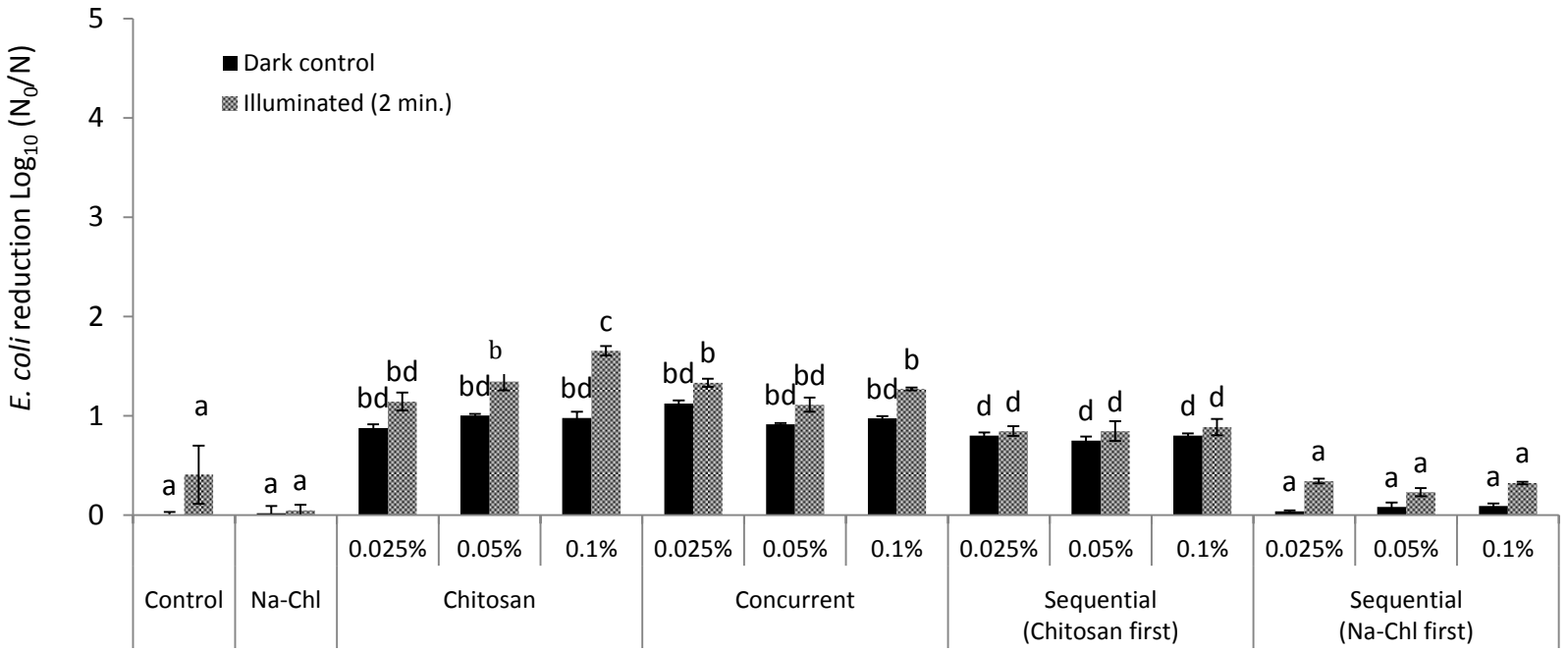

Figure 16: Reduction fraction $Log_{10}(N/N_0)$ of *E. coli* O157:H7 in sample groups of different sequence of treatment.

Bacterial suspension in sterile water was mixed with antimicrobial solutions in different sequence to examine the synergistic effect of chitosan in relation with sequence of treatment: concurrent incubation of chitosan and Na-Chl, sequential incubation of chitosan first followed by Na-Chl, and sequential incubation of Na-Chl first followed by chitosan. Three different concentrations of chitosan were tested: 0.025%, 0.05%, and 0.1% (%w/v) while chlorophyllin concentration was fixed at 30 µM. Total incubation time was 30 minutes with variation of 15 minutes depending on sequence group. Samples were illuminated for 2 minutes at 400 nm. Log reduction was calculated by serial dilution and plating. Means with different letters are significantly different (t-test, $p<0.05$).

4.5. Discussion:

Photosensitization inactivates bacteria by the generation of singlet oxygen ($^1O_2$), a highly reactive species that oxidizes bacterial cellular organs. Many studies showed that the intracellular localization of photosensitizer is necessary for bactericidal effect (Castano et al., 2004). The uptake of photosensitizer by bacteria depends on the physicochemical properties of the photosensitizers and on the types of bacteria.

Gram-positive (+) bacteria differ from their counterparts, gram-negative (-) bacteria, in the structure of the cell membrane barrier. The cell wall of Gram (+) bacteria consists of a peptidoglycan layer facing the extracellular environment and a phospholipid bilayer membrane in contact with the cytoplasm. Embedded within the peptidoglycan layer are lipotechoic acids, which render negative charge to the outer surface of the bacterial cell. Although the porous peptidoglycan layer allows passage of most photosensitizers with molecular weight of lower than 1500-1800 Da (Lazzeri et al., 2004), the simple diffusion through the inner lipid bilayer is selective to small hydrophobic molecules. Hydrophillic molecules with low molecular weight (600-800 Da), such as Na-Chl (724 Da), have to cross the lipid bilayer through the protein transport machinery embedded within the lipid bilayer (George et al., 2009a). For this reason, incubation time before illumination is an important factor deciding the effectiveness of photosensitization, as it allows time for uptake of the photosensitizer into the cells.

E. coli O157:H7 is Gram (-), which is less susceptible to photosensitization. Compared to Gram (+) *S. aureus, E. coli* O157:H7 has an additional layer outside the

peptidoglycan layer: the outer membrane. Unlike the phospholipid bilayer of the inner membrane, this outer membrane has an asymmetric distribution of lipids. The extracellular side contains lipopolysaccharides (LPS), whereas the inner side has most of the phospholipids. LPS carries more negative charge per unit of surface area than does phospholipid at neutral pH. Na-Chl is hydrophyllic and anionic at neutral pH. The uptake of Na-Chl by *E. coli* O157:H7 is made extremely unfavorable by the repulsive electrostatic force from the outer membrane of *E. coli* O157:H7. This explains the absence of photosensitization effect on *E. coli* O157:H7 in our experiment (Beveridge, 1999).

Chitosan has a bulky size and carries high positive charge. The consensus agreement on the mode of bactericidal action of chitosan is that chitosan disrupts the cell membrane integrity by binding to the bacterial surface (Liu et al., 2004; Tsai and Su, 1999). In the present study, a greater log reduction was achieved for *E. coli* O157:H7 treated with chitosan (1.4 log CFU/ml) compared to *S. aureus* (0.4 log CFU/ml). The result aligns with other studies, where Gram (-) bacteria were found to be more susceptible to chitosan than Gram (+) bacteria (Friedman and Juneja, 2010; Helander et al., 2001; Tsai et al., 2004). One possible explanation was that the outer membrane of *E. coli* O157:H7 with higher negative charge provided more binding sites for chitosan than did the cellular membrane of *S. aureus* (Andres et al., 2007).

In the present study, the synergistic effect of chitosan on the photosensitization of *S. aureus* by Na-Chl depends significantly on the sequence of treatment and, to a

lesser extent, to the concentration of chitosan. Among three sample groups, one concurrent incubation and two sequential incubations, only the sequential incubation with Na-Chl first resulted in a synergistic effect.

In concurrent incubation, both chitosan and Na-Chl had 30 minutes to interact with the bacterial cells. It was possible that the presence of chitosan interferes with the uptake of Na-Chl by the bacteria. As chitosan carries positive charge, it could bind to both Na-Chl and the bacterial cell surface. The more chitosan is in the solution, the more surface of the bacterial cells is bound by the chitosan, blocking the uptake entrance of Na-Chl into the cells. Furthermore, the co-incubation of chitosan and Na-Chl could allow the complexing of the two entities with opposite charge, resulting in fewer “free” Na-Chl to enter the cells. This would explain why lower concentration of chitosan gives higher bactericidal effectiveness within concurrent treatment.

On the same rationale, in the sequential incubation with chitosan first, the uptake of Na-Chl by the cells would be extremely unfavorable, as most of the cell surface had already been covered by chitosan by the time the Na-Chl was added. The fact that the effect of photosensitization in the second sample group (sequential incubation with chitosan first) was reduced compared to photosensitization alone perhaps indicated that the photosensitizer needed to localize either inside or in very close proximity to the cell to be effective. As chitosan is present in abundance prior to the addition of Na-Chl, excessive amount of chitosan deposited on the surface of the cell membrane might create a barrier between Na-Chl and the bacterial cells.

In the last sample group, in which Na-Chl had 15 minutes of incubation with the bacterial cells prior to the addition of chitosan, a synergistic effect was observed. Sequential incubation with photosensitizer being added first yielded 2 additional log reductions compared to 2.4 log reduction in the sample treated with Na-Chl alone and compared to 2.1 log reduction in the other sequential incubation with chitosan being added first. In this case, the initial incubation of Na-Chl would allow the uptake of a portion of Na-Chl into the cells. Upon the addition of chitosan, the remaining Na-Chl outside the cells could be perhaps pulled closer to the cells by binding to the positively-charged chitosan, which in turn binds to the negatively-charged bacterial cell surface. Because Na-Chl is localized both inside the cells and in close proximity to the cells, more singlet oxygen generated by photosensitization of Na-Chl could damage the bacteria cells compared to the other sample groups.

4.6. Conclusion

This present study demonstrated that chitosan had a synergistic effect on photosensitization of *S. aureus* by Sodium Copper Chlorophyllin; however, such synergistic effect is specific to the sequence of treatment. With respect to photosensitization, the intracellular localization of the photosensitizers is important to its effectiveness. Due to the structural difference of the cell membrane barrier between *S. aureus* and *E. coli* O157:H7*,* only *S. aureus* was susceptible to photosensitization by chlorophyllin.

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5. FUTURE WORK

In this present study, the bactericidal effect of chitosan and sodium chlorophyllin (Na-Chl) was examined in water system at neutral pH. The result showed that chitosan synergistically improved the effectiveness of photosensitization by Na-Chl against *Staphylococcus aureus,* a gram-positive species. However, when tested on *Escherichia coli*, photosensitization by Na-Chl had virtually no lethal effect even in combination with chitosan. Subsequent studies will focus on understanding the uptake pathway of anionic photosensitizer by gram-negative bacteria in the presence of chitosan and ultimately designing an optimal system for effective photosensitization of gram-negative bacteria.

Chitosan exhibits bactericidal activity at pH <6.3 because the NH_3^+ groups on chitosan molecules are protonated at low pH. A study by Helander et al. (2001) revealed that chitosan induced significant uptake of the hydrophobic probe 1-N-phenylnaphthylamine by *E. coli* O157:H7 at pH 5.3 but had no effect at pH 7.3. In future studies, lactic acid will be added as a third treatment to the hurdle system to improve the bactericidal effectiveness in gram-negative bacteria. (Alakomi et al., 2000) reports that lactic acid, which is generally recognized as safe in foods, may potentiate the effects of other antimicrobial substances by acting as a permealizer of the outer membrane of gram-negative bacteria.

Furthermore, the study will be repeated with shortened time of incubation in consideration of practicality in real food manufacturing setting.

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