EVALUATION OF PHOTOSENSITIZER CURCUMIN AS A SURFACE SANITIZER ON FOOD CONTACT SURFACE TYPICAL OF THE COLD SMOKED FISH INDUSTRY AND AS A TREATMENT METHOD FOR REDUCTION OF *LISTERIA MONOCYTOGENES* POPULATION IN COLD SMOKED SALMON

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ABSTRACT OF THESIS

Evaluation of Photosensitizer Curcumin as a Surface Sanitizer on Food Contact Surface Typical of the Cold Smoked Fish Industry and as a Treatment Method for Reduction of *Listeria monocytogenes* population in Cold Smoked Salmon

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The low processing temperature of cold smoked salmon poses several risk factors, one of which is the growth of the foodborne pathogen, *Listeria monocytogenes.* The desire for novel, naturally sourced antimicrobial agents is increasing with increased consumer demand and concern of antimicrobial resistance by foodborne pathogens. Photosensitizer curcumin (PSC) was prepared by suspending it in sterile distilled water. The absorption spectrum of PSC was evaluated using a spectrophotometer. A light unit of wavelength 430 nm was set up for the experiments. The antimicrobial efficacy of curcumin was evaluated on *Listeria monocytogenes* planktonic and solid system, followed by optimizing the PSC exposure time versus the light intensity combination.

The first part of the study aimed to evaluate photosensitizer curcumin as a surface sanitizer on food contact surfaces typical of the cold-smoked fish industry. To form a *Listeria monocytogenes* biofilm, 1 mL of working culture of 10^6 CFU/mL was added to 9 mL of salmon exudate in which two 2x2 cm stainless steel (SS) coupons were placed for 72 hr. This was followed by 1 min exposure to 160 ppm curcumin and 76.59 kJ/m^2 light dose. A control and 200 ppm of chlorine treatment were also performed for comparison.

The PSC treatment with light exposure significantly decreased the population of *L. monocytogenes* similar to that of the 200 ppm chlorine treatment with a 3.45 log reduction. These results suggest potential application of the treatment method for control of the pathogen on stainless steel surfaces found frequently in the cold-smoked fish industry.

The second part of this study aimed to evaluate photosensitizer curcumin as a treatment method for cold smoked salmon. Salmon was sectioned into 3 cm x3 cm pieces with a thickness of 0.3 cm and inoculated with 100 μL (10^8 CFU/mL) *Listeria monocytogenes*. The fish were treated with exposure to 320 ppm PSC for 1 min, followed by exposure to 76.59 kJ/m^2 light dose. For comparison, a control with no treatment and treatment with exposure to 25 ppm of chlorine for 10 min was also performed. This was followed by brining, drying, and cold smoking. Another treatment was performed to gain perspective on exposing part of the previously PSC-treated salmon samples to an additional 76.59 kJ/m^2 light dose post cold smoking. All the cold

smoked salmon were vacuum packed and sampled on day 0, day 1, day 5, and day 10. Analyses of water phase salt, pH, moisture content, water activity, and color analyses of raw salmon, and smoked salmon on day 0 and day 10 were performed.

There was a consistent decrease in the *Listeria monocytogenes* population observed over time. The population of *Listeria* on the post smoking light treated salmon was significantly lower compared to control and chlorine treated salmon at each stage of treatment or storage except on day 5.

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Dedication

I would like to dedicate my thesis to my family, especially my parents, Kothai and Vengatesan, who have ensured to give me the best opportunities, and without their support, I would not be who I am today. To my sibling Shre, who has been a source of inspiration for me. A special dedication to my grandma Sornavalli who was a constant source of wisdom in my life. My grandparents, Visalakshi, Sevugan, and Murugappan, who have pushed me to give my best and been there when I needed them the most. I would like to express my appreciation to my friends Marina, and Kalsang who have helped me through tough times and have inspired me to be my best. I also want to acknowledge my dear friend Suha who has been a source of strength and encouragement throughout writing my thesis and presenting my defense. I would like to thank Dr. Ponnusamy, who has been like family to me in the U.S. Dr. Schaich, for believing in me and providing me several opportunities to work with her in the Principles of Food Science lab. I would also like to express my gratitude to my advisor Dr. Matthews for his guidance and generosity.

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1. INTRODUCTION

Smoking meat and seafood were one of the earliest methods of preservation, where meat or seafood was hung over an open fire. With modern technologies such as refrigeration and vacuum packaging, a shift towards sensory attributes was observed with the invention of cold smoking. The lack of heat treatment and lesser salt content make cold-smoked food a good niche for microorganisms, including pathogens. Aseptic processing, treatment of seafood, reduction of water activity by adding salt and drying, modified atmosphere packaging, and refrigeration helps control the growth of but not eliminate the pathogens and spoilage microorganisms (Arvanitoyannis et al., 2012). However, it can still pose a serious threat to vulnerable populations, including children, pregnant women, their fetuses, and immunocompromised individuals due to *Listeria monocytogenes. Listeria* is a pathogen that is prevalent in nature. Fish caught from a contaminated water body is the primary source of introducing the pathogen in the fish industry. Once introduced, it can contaminate and harbor food contact surfaces by forming biofilms which can further cross-contaminate fish. *Listeria monocytogenes* is a major food-borne pathogen that causes death in the United States (CDC, 2022). Typically, the fish is washed with traditional antimicrobials such as chlorine in the food industry. With the emergence of novel and natural antimicrobials and an increase in antimicrobial resistance to conventional chemicals, there is a shift toward studying the effects of natural treatment methods in combating pathogens. One such method is photodynamic therapy.

Since its accidental discovery by Oscar Raab in 1900, the method of photodynamic therapy with several dyes has been extensively researched, of which curcumin is one. Photodynamic therapy involves the accumulation of photosensitizers in target cells, which, when exposed to the light of wavelength coinciding with the absorption spectrum, enter an excited singlet state from where it can emit fluorescence and heat to return to the ground state or reaches triplet state through intersystem crossing from which it can emit phosphorescence or transfer its energy to form a reactive oxygen species or radical ions that can inactivate pathogens (Kwiatkowski et al., 2018). Curcumin is a phytochemical component of the spice turmeric *Curcuma longa* Linn. Curcumin has been used as a coloring and flavoring agent and is generally recognized as safe with an acceptable daily intake of 0-3mg/kg of body weight (EFSA, 2021). Due to its anti-inflammatory, anti-cancer, anti-mutagenic, and antimicrobial properties, it has been used as a medicinal herb in Asian countries (Hewlings & Kalman, 2017). Studies utilizing curcumin in photodynamic inactivation have proven successful in inactivating or reducing pathogens in food. Some of the pathogens that curcumin has demonstrated to reduce on different foods or surfaces are *Vibrio parahaemolyticus, Listeria monocytogenes, Staphylococcus aureus, Pseudomonas,* murine norovirus-1 (MNV-1) *Salmonella,* and *Escherichia coli.* (Correa et al., 2020, Wu et al., 2015, Chen et al., 2020, Chen et al., 2021, Gao et al., 2020, and Gong et al., 2020).

This study was proposed to study the inactivation of *Listeria monocytogenes* by photosensitizer curcumin in the presence of light on food contact surfaces typical of the

smoked fish industry, and as a treatment method to inactivate *Listeria monocytogenes* on cold smoked salmon.

2. HYPOTHESIS AND OBJECTIVES

The hypothesis of this research is that the photodynamic therapy (PDT) in the presence of photosensitizer curcumin has the potential to negatively affect *Listeria monocytogenes* biofilm on food contact surfaces typical of the cold smoking industry and on cold smoked salmon.

The objectives of this research are-

1. To evaluate the absorption maximum of the photosensitizer curcumin and construct a light source with appropriate wavelength to activate the photodynamic properties of curcumin.
2. Identify the minimum inhibitory concentration and minimum bactericidal concentration of curcumin for the *Listeria monocytogenes* strains used in this study.
3. Evaluate the optimum combination of incubation time of *Listeria monocytogenes* in curcumin and light dose to inhibit the pathogen in media, stainless steel coupons, and salmon.
4. Evaluate the formation of *Listeria* biofilm on SS coupons.
5. Determine the antimicrobial effect of PDT with curcumin on SS coupons with *Listeria* biofilm and on salmon inoculated with *Listeria* followed by cold smoking.

3. LITERATURE REVIEW

3.1 Cold Smoking-

3.1.1 Introduction-

Historically, smoking is one of the earliest methods of preserving meat and may have been around for over 10,000 years (Arvanitoyannis et al., 2012). It is speculated that meat was hung over fire to protect against canines when smoking was adopted for its preservative qualities against enzymatic and microbial degradation and its organoleptic properties (Šimko, 2005). Later, smoking was employed as a preservation method during seasons of surplus harvest in wet, humid weather.

Smoke is an emulsion with two discrete phases – liquid-phase particles of smoke and gas-phase smoke vapors in a continuous phase of air and vapors (Foster et al., 1961, Horner, 1997). Depending on the type of wood used, the component of smoke differs. Moreover, depending on the temperature of smoke generation, the fraction of composition of smoke components can also vary. The major compounds in smoke vapors are acids, phenols, carbonyls, alcohols, and hydrocarbons (Horner, 1997, Adeyeye, 2019).

A combination of surface drying, salting, deposition of phenolic antioxidants, and antimicrobial substances like nitrites, phenols, and formaldehyde attains the preservation action of smoking. These compounds are products of the pyrolysis of wood compounds. Surface drying due to smoking and drying steps can physically block the

pathway of microorganisms and aerobic microbial proliferation, whilst salting reduces water activity and hinders the proliferation of pathogenic and spoilage microorganisms. Phenolic antioxidant substances delay the oxidation and rancidity of unsaturated fish lipids (Sampels, 2015; Horner, 1997). Besides its preserving qualities, smoke also has an important role in imparting flavor and color. Polymerization of phenols, Malliard reaction, and deposition of smoke particles, resulting in the characteristic tint of light lemon to dark brown color of the smoked fish. The chemical reactions rate increases with temperature and results in a darker-colored product. Phenols such as syringol, 4-methylsyringol, 4-allylsyringol, guaiacol, 4-methyl guaiacol, and trans-isoeugenol deposited on the fish during smoking impart the characteristic smoky flavor. Though the exact proportion of concentrations contributing to the flavor profile is unknown, carbonyl compounds and furans also play a role in the taste of smoked food (Hui, 2014).

With the invention of refrigeration and novel packaging methods, a shift towards improving sensory attributes occurred to obtain a moister and less salty product (Busta et al., 2001). Thus, cold smoking was introduced. Cold smoking aims to partially coagulate the fish proteins by employing smoke at an appropriate time and temperature combination that would also reduce water activity (Codex Alimentarius, 2013).

3.1.2 Cold Smoking Process-

Once fish is received in the processing plant, it is thawed if frozen, washed, and eviscerated. Depending on the size of the fish and desired product, it is either filleted,

cut or processed as a whole accordingly. The fish is thoroughly rinsed in chlorinated water (25 to 50 ppm) prior to salting or brining.

Sodium nitrite is combined with salt to inhibit the outgrowth and toxin production by *Clostridium botulinum.* The desired product, if modified air packed (MAP), is fish with at least 3.5% water phase salt. In comparison, the air-packaged product is 2.5% water phase salt and with a sodium nitrite concentration within the lower and upper limit of 100 ppm and 200 ppm, respectively, in the loin muscle (21 CFR 172, and Busta et al.,2001).

$$\%\ Salt\ in\ water\ phase = \frac{\%Salt}{\%Salt+\%Moisture} x100$$ (Moody, 1990).

Dry salting is usually carried out for a duration of time, in the context of the weight of fish, for the desired product. The salt-fish ratio varies from 1:8 for light salting, 1:3 for split fish, and 1:1 for heavy salting. A brine of 30-50 °S brine is usually used. Temperature is maintained under 3°C. (Moody et al., 1990).

After draining the brine, the fish is rinsed and subjected to drying at 20°C to 28°C for 1 to 6 h. The drying process should be fast enough to avoid enzyme and microbial degradation of fish but slow enough to prevent case hardening, which would lead to poor absorption of smoke. (Busta et al., 2001, Horner, 1997, Moody et al., 1990)

After drying, the fish undergoes the process of smoking. The best temperature to maintain for the cold smoking process is <30°C. Traditional smoking methods utilize smoke from burning hardwoods like maple wood, oak, alder, and hickory. The typical

time-temperature combinations for smoking fish are 10°C for 24h, 32°C for 20h, and 49°C for 6 h, after which it is cooled to <3°C, packed, and distributed (Busta et al., 2001, and Moody et al., 1990).

3.2 ***Listeria monocytogenes***

3.2.1 Physiology and Pathogenesis

Listeria species include *L. monocytogenes, L. seeligeri, L. innocua. L. ivanovii, L. grayi, L. welshimeri, L. marthii, and L. rocouratiae* of which *L. monocytogenes* is the only pathogenic species to humans (Zunabovic et al., 2012). The first documented discovery of *Listeria monocytogenes* was in 1924 when small gram-positive bacilli were first isolated from the heart's blood of an acute case of listeriosis in a pregnant rabbit. This discovery was followed by the sudden deaths of the rabbits. The organism was initially named *Bacterium monocytogenes* due to the production of strikingly large mononuclear leukocytosis by the host (Murray et al., 1926).

This gram-positive, non-spore-forming, coccobacillus, facultatively anaerobic bacteria found abundantly in nature, was first isolated from human subjects showing symptoms of mononucleosis-like syndrome. One of the earliest pieces of evidence of *Listeria monocytogenes* being foodborne was found in East Germany when the serotypes of *Listeria monocytogenes* isolated from a cow with atypical mastitis matched with that of a stillborn twin, the mother of whom had consumed raw milk from the cow (Gray & Killinger, 1966).

The *in vivo* studies conducted by Armstrong et al. (1966) suggests that *Listeria monocytogenes* enters the host cell through phagocytosis, forming phagosomes that break down, releasing its contents into the cytoplasm, thus damaging the host cell. It is capable of multiplying within the monocyte-macrophage series and spreading among cells.

Listeria monocytogenes is the third most common cause of death the result of foodborne illness. Usually, individuals with underlying conditions such as immunocompromised, the elderly, and fetuses are more susceptible. In pregnant women, once this pathogen crosses the placenta, it can cause abortion, stillbirth, or premature birth. Pregnant women, in general, are 10 times more likely to acquire the infection, but this is shown to vary depending on race and cultural ethnicity. For example, Hispanic pregnant women are 24 times more likely to get infected by listeriosis. The infection can also cause serious illness to death in newborns (CDC, 2022). In the elderly, *Listeria* can cause encephalitis, meningitis, and infection in the blood. Though it is infrequent, *Listeria* can cause gastroenteritis and fever in healthy individuals as well (FDA, Buchanan, 2017).

3.2.2 Prevalence and Outbreaks

Early evidence of the occurrence of listeriosis was documented to be through oral consumption with successful infection in rabbits and mice. In fact, the first documented foodborne outbreak of listeriosis in humans was the consumption of raw milk from the udder of a cow infected with *Listeria*. Even though the earliest outbreaks

were identified in 1949, owing to long, variable periods of disease on-set, food samples for analysis post clinical symptoms were unavailable. Outbreaks of listeriosis in the 1980s linked to the consumption of coleslaw and Mexican-style cheese suppressed any doubts of *Listeria* being foodborne (Slutsker & Schuchat, 1999).

Listeria monocytogenes' natural habitat is soil, water, and decaying vegetation which is the source of *Listeria* infection for farm animals and the root cause of contamination associated with spread through the food chain (Fenlon, 1999). *Listeria* is omnipresent in food processing, agricultural, and aquacultural environments. It is also an inert, transitory member of the human intestinal tract (Strawn et al., 2013, Buchanan et al., 2017).

Even though the improved control measure beginning in the 1990s reduced the prevalence of *Listeria* in different foods, the rate of listeriosis remained constant, with severe, systemic forms occurring more frequently (Buchanan et al., 2017). Around 1600 people are estimated to have listeriosis each year, of which 260 die. The case-fatality rate is estimated to be 20% (CDC, 2021). A recent meta-analysis shows that the prevalence of *Listeria monocytogenes* in deli meat is 2.9%, in soft cheese is 2.4%, and 2% in packaged salads (Churchill et al., 2019). Another meta-analysis conducted by Liu et al. (2012) showed that there is a prevalence of 8.5% of raw meat harboring *Listeria,* whilst it was 3.2% in ready-to-eat meats (Liu et al., 2020). Another prevalence study in Iran reported 9.2% of ready-to-eat foods, 5.1% of seafood, 5% of poultry, 4% of traditional dairy, 2.6% of raw meat, 1.4% of commercial dairy, and 0.2% of eggs

(Hamidiyan et al., 2018). *Listeria* ranks as the second most expensive foodborne illness, with an estimated loss of $2.8 billion annually (Hoffman et al., 2015).

Moreover, *Listeria monocytogenes* can accumulate and form biofilm on common food contact surfaces (Blackman and Frank, 1996). A biofilm is an accumulation of microbial cells that are enclosed in a matrix consisting mainly of self-secreted polysaccharide material that cannot be removed by gently rinsing the surface. They are more resistant to chemical intervention, like sanitizers and disinfectants, than their planktonic counterparts (Donlan, 2002). *Listeria monocytogenes* form niches in favorable growth conditions in cold and wet environments such as refrigerated rooms in food processing facilities. Persistence of the presence of certain *Listeria* strains pertains to tolerance to disinfectants (Carpentier et al., 2011). Cleaning surfaces prove to be more arduous when *Listeria* strains form biofilms and act as a source of contamination in foods (Beresford et al., 2001). But increase in biofilm formation is not correlated to an increase in foodborne diseases (Borucki et al., 2003).

3.2.3 *Listeria monocytogenes* in Cold Smoke Seafood Industry-

The two major pathways for *Listeria monocytogenes* to enter the fish processing plants are through the surface of fish harvested from contaminated waters and the gastrointestinal mucosa and gills that harbor the pathogen. The bacteria proliferate through the spread of intestinal bile to other tissues. With insufficient hygiene, there is a high risk of cross-contamination, especially with equipment that is difficult to clean and sanitize (Skowron et al., 2018). Prevalence data suggests up to 39.4% of cold-

smoked salmon are positive for *Listeria* (Rotariu et al., 2014). The extensive processing steps that include filleting, salting, drying, and smoking require a lot of handling by employees and could be a contributing factor in the spread of the pathogen.

Research suggests that the highest occurrence of *Listeria monocytogenes* contamination in processed seafood was found in cold-smoked fish (Fallah et al., 2013). A 2019 report indicated that a *Listeria monocytogenes* outbreak affected 22 people in five countries was traced back to Estonia-produced cold-smoked trout and salmon (EFSA, 2019). Currently, the UK health security agency and food standards Scotland are investigating a *Listeria* outbreak linked to smoked fish that has affected 12 people since 2020, six of whom have become infected since January of 2022 (FSA, 2022). *Listeria monocytogenes*, being psychotropic and halotolerant; the temperature maintained during the cold-smoking process and the salt content of the product are ideal for its growth (Busta et al., 2001).

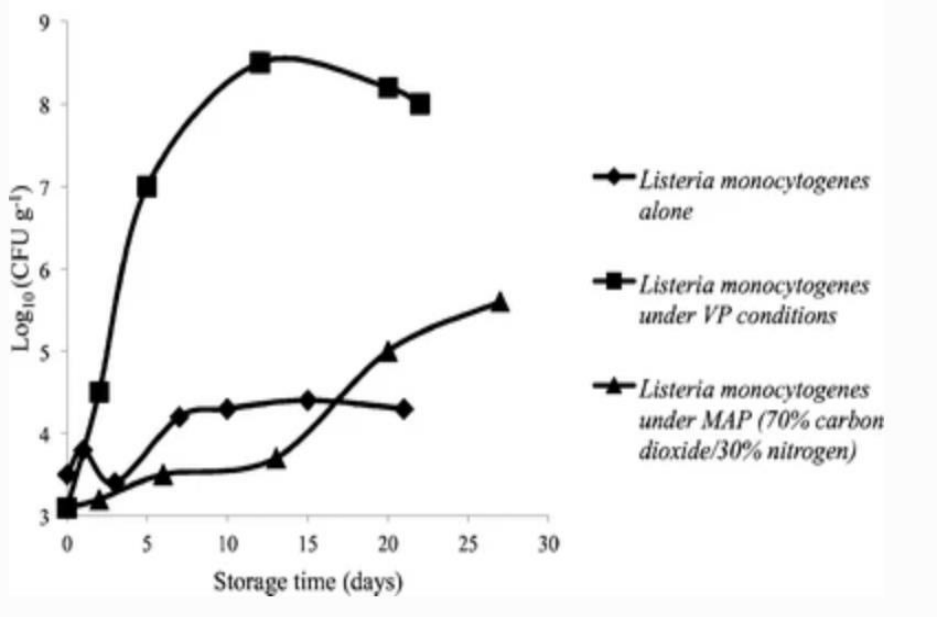

Fig 3.1. Growth of *Listeria monocytogenes* in cold smoked salmon products stored in air at 4°C, vacuum packaging, and modified atmospheric packaging (70% CO2 and 30% N2) at 5°C. Adapted from Arvanitoyannis, I. S. and K. V. Kotsanopoulos (2012). "Smoking of fish and seafood: history, methods and effects on physical, nutritional and microbiological properties." Food and bioprocess technology 5(3): 831-853. Copyright © 2011, Springer Science Business Media, LLC

Cold smoking should be conducted below 90°F (Belichovska et al., 2019). At lower temperatures (63-70 °F), a 10 to 25-fold decrease in the population of *Listeria monocytogenes* was observed, whilst if the temperature is higher (72 – 87 °F), a 3-fold decrease was observed. When contaminated brine was injected into the flesh of fish, a 2 to 20-fold increase in the *L. monocytogenes* population was observed, regardless of applied smoke. Populations of *L. monocytogenes* are typically at 100 CFU/g (Eklund et al., 1995, and Jorgensen and Huss, 1998}, but four of 76 positive samples in a study

contained 1,000 CFU/g, while 12 of the 76 samples contained 100–1000 CFU/g, all after ≥14 days of storage (Jorgensen and Huss, 1998).

To determine the strains of *Listeria monocytogenes* in cold smoked salmon that are associated with outbreaks, it should be evaluated if the processing plant, environment, equipment, and product are ecological niches for certain serotypes and find if the same is found in patients with listeriosis (Rørvik, 2000). In the study, 72 isolates of *Listeria monocytogenes* from 10 different imported fish products from 12 different producers and 47 isolates from clinical cases of listeriosis in humans were typed by serotyping and multilocus enzyme electrophoresis. Analysis of the isolates showed 47 fish isolates and 17 human isolates belonged to serotype ½ a, 12 fish isolates belonged to serotype 7, 4 to serotype atypical 7, 4 to serotype ½ c, 2 to serotype 3a, 2 fish isolates and 8 human isolates to type ½ b and 1 fish isolate and 21 human isolates to serotype 4b. There were 9 electrophoretic types (ET) found among the 72 isolates. Among the 9 ETs five belonged to ET4 (½ b), ET9 (½ a), ET11 (½ c), ET22 (7) and ET26 (1/2 a) and were found in more than one fish product. The major subtype found in fish was ½ a. There was no significant association between specific fish products and electrophoretic types (Boerlin, 1997). In a different study, 245 strains were isolated from human and animal clinical cases of listeriosis and raw and processed foods, which included samples from fisheries, dairies, cheese, cattle with mastitis, raw meat, and meat products. The ET1 (4b) was most frequently isolated in clinical listeriosis, while the ET4 (1/2 b) was predominant in raw and processed food products. Among the fishery samples (raw salmon, smoked

salmon, raw salted salmon, shrimp, Greenland halibut, and smoked cold roe), 35% of the strains belonged to either ET1 or ET4 (Nørrung, 1993).

3.2.3.1 Food contact surfaces-

Cleaning food contact surfaces in the food industry involves removing debris, dismantling, or removing equipment to expose the surfaces, applying cleaning agents with mechanical energy, and rinsing with water. Finally, sanitation checks are done after cleaning and sanitation to make sure the process is successful. Since water is the solvent used for both cleaning and disinfection, it should be potable and free of metal ions. Most industries use cleaning in place (CIP) and cleaning out of place (COP). Disinfection after cleaning food contact surfaces and equipment plays a critical role in preventing cross-contamination of food contact surfaces and food. Where it is feasible in energy, steam is commonly used for disinfecting surfaces (Huss, 1994). Other common disinfecting agents are chlorine, chlorine, iodophors, quaternary ammonium compounds (Quats), and ampholytic compounds (Phillips, 2016).

Chlorinated sanitizers with free chlorine concentration of 200 ppm are potent, while iodophors with 25 ppm free iodide are lethal to a broad spectrum of foodborne microorganisms. Quats have a poor ability to eradicate gram-negative bacteria (Huss, 1994). Peracetic acid's mode of action involves oxidizing the outer cell membrane of vegetative cells, endospores, yeast, and mold spores by transfer of electrons, destroying the cell enzymes, which in turn inactivates the pathogens. (Gawande et al., 2013).

Failure to properly disinfect leads to cross-contamination, foodborne outbreaks, and diseases.

The presence of organic material, regular exposure to sublethal concentrations of disinfectants, and biofilm formation may result in resistance or tolerance of strains to disinfectants. Evaluation of 200 *Listeria monocytogenes* isolates recovered from fish processing plants in Norway and European countries revealed that 10% were resistant to benzalkonium chloride, while 13% were resistant to quaternary ammonium compounds (Aase et al., 2000).

3.2.4.2. Fish-

Controlling contamination is difficult due to the conditions of the harvesting environment in the fish processing industry. The indigenous or commensal microflora of fish widely varies and is associated with diversity in aquatic environments such as fresh, polluted, salt, pristine, cold, tropical, temperate, open ocean, and estuarine-. The commensal microbiota and the microflora of the processing environment influence the microflora of the fish product. Usually, fish is chilled soon after harvest, which slows down the growth of the microflora. Fish species that are known to carry parasites that are harmful to humans are frozen before further processing to kill the parasites. In addition, cleaning, smoking, salting, drying, and packaging processes change the flora of fish (Busta et al., 2001)

The spoilage microflora in lightly preserved fish products including CSS is usually dominated by *Lactobacilli* predominantly associated with gram negative fermenting bacteria *Photobacterium phosphorium* and psychrotrophic *Enterobacteriaceae*. Other bacteria isolated from spoiled CSS include *Brochotrix thermospacta, Aeromonas* spp., yeast, and mold. The flora also depends on the type of processing; marine *Vibrio* and *Photobacterium phosphorum* dominate dry salted salmon, while LAB and *Enterobacteriaceae* dominate salmon injected with brine. The foodborne pathogens often associated with CSS include *Listeria monocytogenes* and *Clostridium botulinum*. (Løvdal, 2015).

Several factors can inhibit the growth of microorganisms in cold smoked salmon. The shelf life of CSS is influenced by fluctuations in temperature and salt content. The addition of nitrates or nitrites can affect the bacteria. While 3 or 5% wps is not enough to combat the growth of microorganisms, 6% wps significantly inhibited the growth of *Listeria monocytogenes* at low storage temperatures. Due to health concerns, the latter salt content is not practical for human consumption. Though the antimicrobial effect of heat in cold smoking is minimal, the smoke from wood has several volatile components, including carboxylic acids, phenols, aldehydes, and alcohols that have antimicrobial effects on the vegetative form of microorganisms on the surface of the fish. The dry pellicle that forms on the surface during smoking inhibits the lethal effects of smoking. (Løvdal, 2015).

Widely practiced methods to inhibit the growth of *Listeria monocytogenes* in cold-smoked salmon involve different combinations of concentrations of NaCl and Nitrite and processing temperatures (Jahncke, 2007). Nitrite at 200 ppm with dry salt has an inhibitory effect against *Listeria monocytogenes* at 5°C, whilst there was no effect at 10°C (Pelroy et al., 1994). 2% sodium lactate, on the other hand, showed total inhibition to *Listeria* growth in combination with 3% water phase salt (Pelroy et al., 1994). It was recommended that the CSS that is air packaged has >2.5% WPS, and at least a 3.5% WPS or a 3.0% WPS with at least 100, but no more than 200 ppm of sodium nitrite for MAP products, and the temperature is maintained at 3.0-3.3°C (Løvdal, 2015).

One of the emerging technologies ithe n treatment of cold smoked salmon include high-pressure processing (HPP). It was demonstrated that a pressure of 700-900 MPa for 10 s reduced the population of *Listeria innocua* from 4.5 log CFU/g to nondetectable levels (Gudbjornsdottir et al., 2010). Montiel et al. (2012) established that a combined antimicrobial effect of high hydrostatic pressure (HHP) of 450 MPa on a lactoperoxidase system (LPS) treated cold smoked salmon reduced *Listeria monocytogenes* population by 3.8 log units, preventing pathogen recovery. A complete growth inhibition of *Listeria monocytogenes* on sliced cold smoked salmon with treatment by 1% Verdad N6 (a buffered vinegar fermentate) was observed when stored at 4°C (Heir et al., 2019).

3.3 Photosensitization-

The photosensitization or photodynamic treatment (PDT) phenomenon was first observed in 1900 by Oscar Raab when acridine orange inactivated paramecia in the presence of sunlight {Raab, 1900}. Photosensitizers are non-toxic dyes that in the presence of light and oxygen, become excited to a singlet state. Since this is not a stable state the photosensitizer adopts the following pathways to attain stability.

- The excited photosensitizer from the singlet state emits light (fluorescence) and heat generation to return to the ground state.
- Alternatively, it attains a triplet state through intersystem crossing from where the molecule can emit photonic radiation (phosphorescence). Owing to the slightly longer lifetime of the triplet state (μs), the energy is transferred to an acceptor organic molecule resulting in very reactive metastable species such as radicals or ions, including $HO^{\cdot}$ (type I mechanism) or the energy of the triplet state is transferred to an oxygen molecule to form reactive oxygen species (type II mechanism).

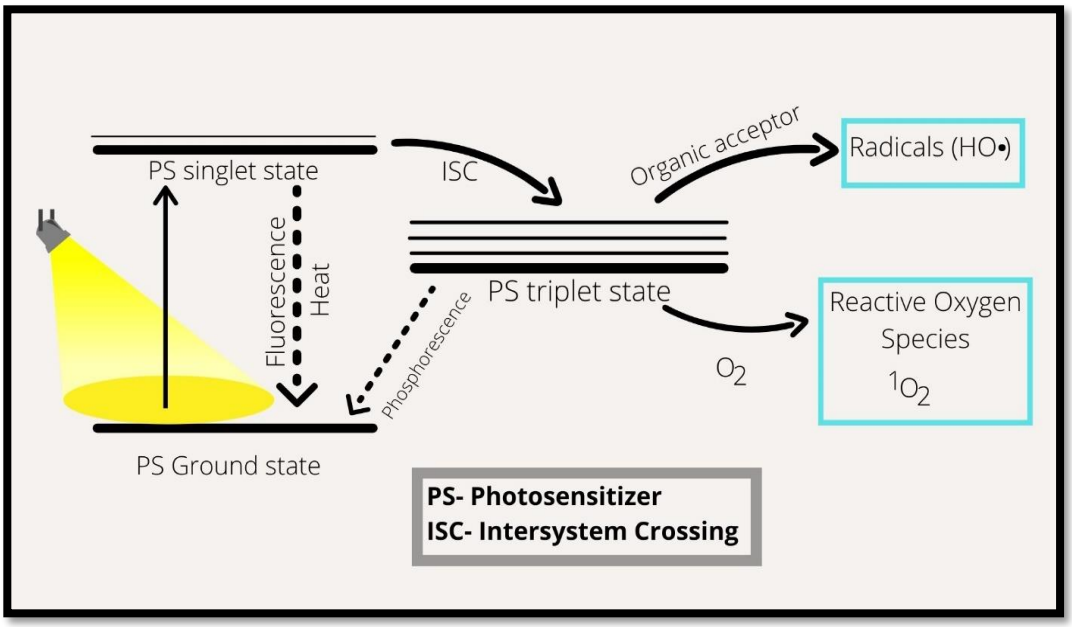

Fig. 3.2 Mode of action of photosensitizers

Though it was predominantly postulated that the singlet oxygen species are responsible for antimicrobial activity, research suggest that both singlet oxygen species and $HO^{\cdot}$ are involved in inactivating both Gram-positive and Gram-negative bacterial cells (DeRosa and Crutchley, 2002, Luksiene and Brovko, 2013, Huang et al., 2012).

PDT can effectively deactivate bacteria, fungi, and viruses including planktonic, biofilm, and spore states of bacteria (Wang et al., 2021). The reactive oxygen species reacts with proteins, lipids, and nucleic acids of microbial and malignant cells, resulting in spatially limited cellular inactivation and death that is selective to the vicinity of its production (Sharma, 2012). They are known to target aromatic amino acid sidechains and sulphur containing amino acids to form sulfoxides (Davies, 2003) and lead to protein carbonylation (Silvester et al., 1998). Furthermore, $HO^{\cdot}$ diffuses through

biological membranes and combine with organic substrates or abstract electrons from these substrates like lipids, fatty acids, and cholesterols in the eukaryotic cells, setting off radical chain reactions and causing extensive damage to the bacterial cell.

3.3.1 Photosensitization in Food Industry-

Light in the visible spectrum has been extensively employed for its non-thermal, easily controllable, and feasible properties in the rearing of poultry, and fish, cultivation of crops, controlling ripening of fruits, spoilage prevention, and pathogen inactivation (Wang et al., 2021). Light of wavelength 460 nm effectively reduced *Salmonella enterica* serovars in orange juice and *Escherichia coli* in milk (Ghate et al., 2016; Srimagal et al., 2016). LED lights in the range of 460-470 nm inhibited the growth of *L.monocytogenes* and *Pseudomonas fluorescens* in packaged cheese at 4°C (Hyun et al., 2020).

Aponiene et al. (2015) reported that after incubating apricots, plums, and cauliflower in 1.5x10^{-5}M hypericin, followed by exposure to 585 nm of light irradiation, a reduction in the population of *Bacillus* by 1.1, 0.7, and 1.3 log CFU/g were observed respectively. The hypericin treatment proved to be non-thermal, environment-friendly, and feasible. Curcumin-mediated PDT (1 .0 μM with 1.14 J/cm^2) was effective in inactivating planktonic cells of *Vibrio parahaemolyticus* below detectable levels. At the same time, 20μM of curcumin at 13.68 J/cm^2 was effective in eliminating the biofilm of the same organism. The treatment also down-regulated virulence and biofilm formation genes (Chen et al., 2020). Curcumin-mediated PDT at 420 nm also proved effective against *Aspergillus flavus* spores in maize kernels and reduced the population by 2 log

CFU/g (Temba et al., 2016). Photoactivated curcumin demonstrated bactericidal effects against *E. coli* on food surfaces under 465 nm LED lights (Aurum et al., 2019). Photosensitized curcumin reduced *L. monocytogenes* and *Salmonella* on chicken skin (Gao & Matthews, 2020). Under the irradiation of 470 nm, curcumin inactivated murine norovirus in oysters (Wu et al., 2015). Since the color of the photosensitizer can affect the color of food products, impacting the sensory qualities, it should be chosen carefully. For example, darker-colored photosensitizers can be used in dark-colored foods (Wang et al., 2021).

Irradiation of *Listeria monocytogenes* biofilm at 405 nm on stainless steel and acrylic coupons at 25°C for 8 h showed a significant reduction in biofilm-associated cells (Li et al., 2018). LED irradiation at 405 nm exhibited the inactivation of *Cronobacter sakazakii* biofilm on stainless steel (Huang et al., 2020). Curcumin bound to polyvinylpyrrolidone mediated PDT against *Staphylococcus aureus* demonstrated a mean reduction of 2.6, 2.5, and 1.7 log for cucumbers, peppers, and chicken, respectively (Tortik et al., 2014). The anti-biofilm effect of PDT is postulated to be associated with the disruption of microbial community physiology by rapid ROS generation and photocatalytic oxidation that can lead to biofilm cell lysis and matrix structure destruction (Wang et al., 2021).

3.3.2. Photosensitiser Curcumin-

Curcumin is a phytochemical derived from the rhizome of the plant *Curcuma longa* Linn., also known commonly as turmeric. It has been utilized as a flavoring and

coloring agent in food and a topical medicine for centuries. Curcumin has been extensively researched for its anti-inflammatory (Chainani-Wu, 2003), antioxidant, chemo-preventative, and chemotherapeutic properties (Sharma et al., 2005). Curcumin has been rendered generally recognized as safe (GRAS) by the FDA (Gupta et al., 2013).

The largest producer of turmeric is India. 2-9% of turmeric comprises the curcuminoids, which are curcumin, demethoxycurcumin, cyclic curcumin, and bisdemethoxycurcumin, of which the major component is curcumin, whilst the minor is cyclic curcumin. Soxhlet, ultrasonic, and microwave extractions are the more commonly used extraction methods. The most effective method to extract curcumin from turmeric is solvent extraction followed by column chromatography. Polar and non-polar organic solvents such as hexane, ethyl acetate, acetone, methanol, and ethanol have been used, of which ethanol is the most effective solvent. Curcumin can be extracted from curcuminoids using column chromatography with silica gels and dichloromethane/acetic acid or methanol/chloroform. It can be further purified using silica gel and eluent mixtures of chloroform/dichloromethane and ethanol/methanol. Detection and estimation can be made using high-performance liquid chromatography techniques (HPLC) (Paramasivam et al., 2009; Priyadarsini, 2014).

Curcumin is a bis-α,β-unsaturated β-diketone and exists in equilibrium with its enol tautomer (Sharma et al., 2005). The molecule has two chromophores of aryl buten-2-one connected by a methylene group. These chromophores give the yellow color to curcumin. In contrast, the conjugation between the two chromophores provides more

photochemical pathways for interaction with oxygen which is not available to a single chromophore (Chignell et al., 1994). The nomenclature of curcumin according to IUPAC is ((1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione). (Diaset al., 2020)

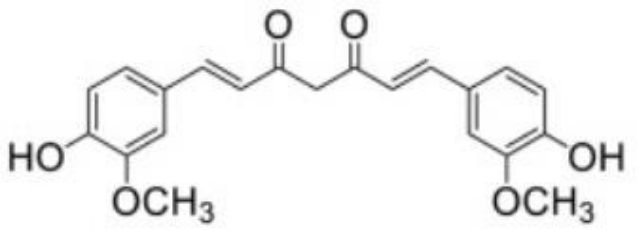

Fig. 3.3 Structure of curcumin molecule. Adapted from Dias, L. D. et al. (2020). "Curcumin as a photosensitizer: From molecular structure to recent advances in antimicrobial photodynamic therapy." Journal of Photochemistry and Photobiology C: Photochemistry Reviews: 100384.

Curcumin has shown inhibition of biofilm formation by uropathogenic organisms by interfering with the Quorum Sensing (QS) system, thereby decreasing cell density and the amount of extracellular polymeric substances (EPSs). The decrease of the latter reduces the nutrient availability for further cell growth and the surface conditioning for further attachment. Furthermore, curcumin has toxic effects on planktonic cells, hindering biofilm formation. Curcumin in bacterial cells has also been shown to downregulate gene expression, inhibit response to DNA damage, and manipulate the molecules of DNA to exert bacteriostatic effects. Curcumin disrupts the RNA to inhibit the synthesis of proteins by binding to microtublins, thereby interrupting bacterial cell

division. Curcumin has been proven to increase the cell permeability of pathogens such as *Staphylococcus aureus* and *Escherichia coli* by disrupting the localization of membrane-associated proteins. (Zheng et al., 2020)

Penha et al. (2017) demonstrated that curcumin is a natural photosensitizer that absorbs blue light in the absorption spectrum of 400-500 nm. Gram-positive bacteria are more susceptible to photosensitizers than Gram-negative bacteria owing to their more porous cell membrane structure (Zheng et al., 2020). The bacteriostatic effect of curcumin is due to the autoxidation mechanism and oxygen-free radicals that disrupt the cell integrity (Zheng et al., 2020). Photodynamic treatment with curcumin negatively influenced the biofilm formation in Listeria monocytogenes and downregulated the pathogen's virulence genes by targeting cytoplasmic DNA and proteins. Huang et al. (2020) demonstrated that the CUR-D-Tyr co-crystal formed by co-crystallization of curcumin using D-Tyrosine had 16.5 times better solubility in water than pure curcumin, thereby increasing the photodynamic inactivation of *Vibrio parahaemolyticus* due to increased bioavailability (Gu et al., 2022). Current research using PSC is summarized in Table 3.1

Table 3.1 Summary of literature review on recent research on photosensitizer curcumin

Pathogen	Concentration of Curcumin	Light Dose	Time	Log reduction	Reference
V. parahaemolyticus	1.0 μM 20 μM	1.14 J/cm^2 13.68 J/cm^2	5 min 60 min	non-detectable (planktonic) complete eradication (biofilm)	(Chen et al., 2020)
	50 μM 100 μM	9.36 J/cm^2 1.56 J/cm^2	30 mins 5 min	1.5 log CFU/g (Oyster) 3.4 log CFU/g (Oyster)	(Chen et al., 2021)
	1.6 μM	13.68 J/cm^2	60 min	3.91 Log CFU/mL (Packaging Film)	(Chen et al., 2021)
Listeria spp.	0.2 μM 1.0 μM	0.54 J/cm^2 0.54 J/cm^2	5 min 5 min	4 log CFU/mL Complete inactivation	(Huang et al., 2021)
	1.2 μM	6.84 J/cm^2	30 min	4.51 log CFU/mL (Packaging Film)	(Chen et al., 2021)
	3.7 mg/L 3.7 mg/L	270 J/cm^2 270 J/cm^2	30 min 30 min	4.9 log CFU/mL (biofilm) 6.1 log CFU/mL (planktonic)	(Bonifácio et al., 2018)
	1 uM	420 nm 20 W	1 min	3 log CFU/mL (Pears)	(Chai et al., 2021)
	300 ppm	32.1 kJ/m^2	5 min	2.9 log CFU/cm^2 (chicken skin)	(Gao et al., 2020)
S. aureus	0.75 mg/mL	16.47 J/cm^2	>5 min	4.34 log (Pasteurized cheese)	(Ferreira dos Santos et al., 2019)

S. putrefaciens	1.6 μM	13.68 J/cm^2	60 min	3.37 log CFU/mL (Packaging Film)	(Chen et al., 2021)
S. saprophyticus	50 μM	8.64 J/cm^2		4.5 log CFU/mL (biofilm)	(Yang et al., 2021)
Pseudomonas	30 μM	470 nm 15 W	90s	3.19 log CFU/mL (Sturgeon)	(Gong et al., 2020)
E. coli	20 μM	3.6 J/cm^2		3.5 log CFU/g (Oyster)	(Gao et al., 2019)
Salmonella	300 ppm	32.1 kJ/m^2	5 min	1.5 log CFU/cm^2 (Chicken skin)	(Gao et al., 2020)

4. MATERIALS AND METHODS

4.1 Preliminary Studies-

4.1.1 Preparation of bacterial culture and inoculum

In this study, six strains of *Listeria monocytogenes,* obtained from Dr. Martin Weidman, Cornell University, the origin of which are indicated in Table 2.1, were used. The strains are resistant to 100 μg/mL nalidixic acid (Alfa Aesar™, England); media was supplemented with 100 μg/mL nalidixic acid. The nalidixic acid stock was prepared fresh each time by dissolving in 0.3 N NaOH (VWR, Solon, OH), followed by filtering through a 0.2 μm filter (Ascrodisc® Syringe Filters, Pall Corporation, Ann Arbor, MI).

Stock cultures of each strain were prepared by isolating a colony from TSA plates and incubating in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) for 18 h at 37 °C. The stock cultures were stored in 20% glycerol at -80°C. For preparing working cultures, the frozen stocks were thawed and streak plated individually onto tryptic soy agar (TSA; Difco, Becton Dickinson, Sparks, MD) supplemented with 100 μg/mL nalidixic acid, and the plates were incubated at 37 °C for 20 h. A colony was then isolated, transferred to 10 mL TSB, and incubated at 37 °C for 20 h. Cells of each strain were harvested by centrifuging at 4000 x g for 15 min (AllegraTM 21R, Beckman Coulter with S4180 rotor) at 4 °C and the pellets were washed with 10 mL 0.1% sterile peptone water (SPW; Difco, Becton Dickinson, Sparks, MD) until TSB was removed, and the final pellet

was resuspended in 1 % SPW. A cocktail of *Listeria monocytogenes* inoculum was prepared by combining 1 mL of each strain.

Table 4.1. *L. monocytogenes* strains used in this study

Bacterial strain	**Isolate No.**	**Ribotype**	**Origin**	**Reference**
L. monocytogenes	T2-063	DUP-1043A	Drain, cold smoking oven	Thimothe et al., 2004
	T1-085	DUP-1062A	Drain, slicing area	
	T1-131	DUP-1052A	Norwegian Salmon	
	M6-171	1045A	Whole Salmon	Malley et al., 2013
	N1-63	1039C	Cold smoked Norwegian Salmon	Norton et al., 2001

	N1-347	1039C	Brine, Atlantic Salmon	

4.1.2 Preparation of photosensitizer curcumin

Photosensitizer curcumin (PSC; Sabinsa corp.) was freshly prepared in the dark, by suspending in SDW, and cooled to 4°C prior to use. The absorption spectrum of the photosensitizer curcumin was determined by UV/Vis spectrophotometer (Agilent Cary 50 Spectrophotometer, Agilent Technology).

4.1.3 Spectroradiometer reading

The light device used in this research consists of six solderless LED lights (7.2 W, LED Group Buy, Saint Louis, MO) spread in an arched stainless-steel hood (20 cmx17 cmx13.8 cm). The diodes were accompanied by LED strip lights of 420-430 nm (Lighting Next, Kowloon, Hong Kong). The light unit's power density (W/m^2) was measured using a spectroradiometer (Apogee Instruments model PS – 300) at a distance of 12 cm. The length was chosen based on the position at which the salmon and stainless-steel coupons were treated. The power density was measured at 15 positions, 5 in each position (75 measurements). Figure 1 shows the setup of the light unit and the spectroradiometer detector.

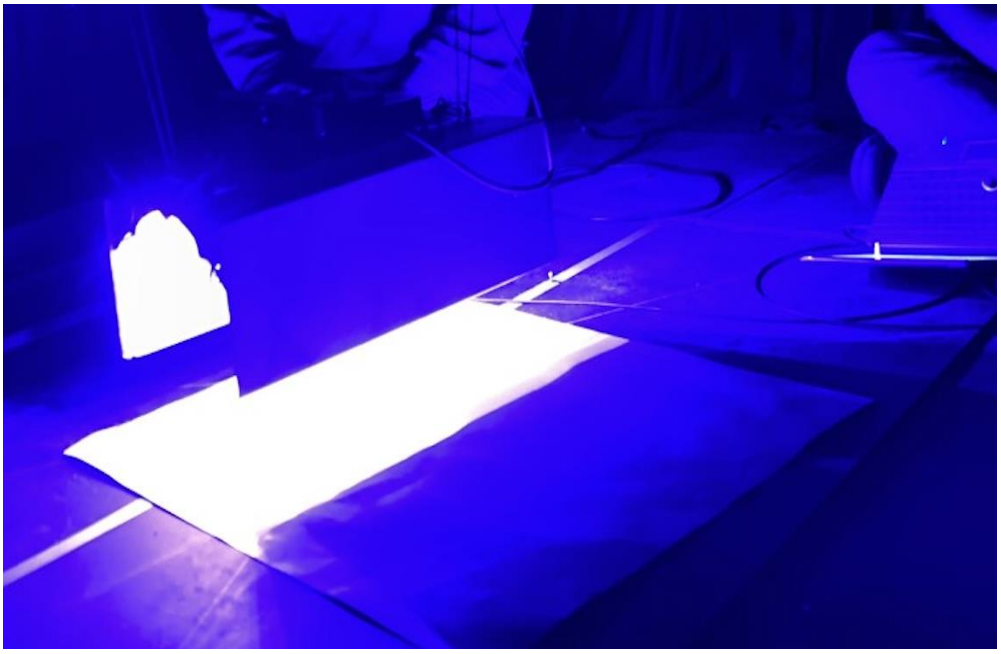

Figure 4.1. Image of spectroradiometer setup to measure the light intensity of the light apparatus

4.1.4 Evaluation of the antimicrobial effects of curcumin on a solid system

The modified Kirby Bauer method was used to evaluate the antimicrobial effects of PSC on a solid system. The working cultures were diluted with TSB to achieve an OD625 in the range of 0.08 to 0.1, resulting in a bacterial population of approximately 8.0 log CFU/mL. The surfaces of the TSA plates were swabbed with the working cultures and allowed to air dry for 5 min, after which 20 μL of different concentrations (0 ppm, 100 ppm, 1000 ppm, and 10000 ppm) of curcumin were dropped onto the surface of the plate. After 1 min incubation in the dark, the TSA plates were exposed to 10 mins of illumination. The distance between the LEDs and plates was 12 cm. Dark control

experiments were also performed. The illuminated and dark control plates were incubated at 37 °C for 18h. The presence or absence of inhibition zones was recorded.

4.1.5 Evaluation of antimicrobial effects of curcumin in a planktonic system

The broth microdilution assay (CLSI, 2016) was used to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of PSC. A 100 μL volume of Mueller-Hinton broth (MHB Difco, Becton Dickinson, Sparks, MD) was added to each well of a 96-well plate (Grenier Bio-one). Subsequently, 100 μL of PSC with varying test concentrations of 2x strength was added, after which a 100 μL volume of curcumin-MHB was removed from each well. The concentration of inoculum of each strain was adjusted to OD625 0.08 to 0.1 with an approximate population of 8.0 log CFU/mL, which was then further diluted by adding 0.1 mL of this inoculum 9.9 mL of TSB. 100 μL of the culture of each strain was eventually added to different wells. Simultaneously, light controls (wells without curcumin) and dark controls (without light exposure) were also conducted. After 1 min of incubation, the 96-well plate was exposed to 76.59 kJ/m^2. The plates were incubated at 37 °C for 16 h. The lowest concentration of curcumin with no visible turbidity in the well was recorded as the MIC for each strain. A 100 μL volume was removed from wells with no visible turbidity and spread plated on TSA plates and incubated at 37 °C for 18 h. The lowest concentration of curcumin that exhibited no bacterial growth on TSA plates was recorded as the MBC.

4.1.6 Optimization of exposure time in the planktonic system

Working cultures were diluted to attain 10^5 CFU/mL, and 1mL of each strain was added to a sterile test tube to make a cocktail. Each well of a 96-well plate contained 100 μL of SDW and 100 μL of curcumin. A 100 μL volume of curcumin-SDW was removed from each well and 100 μL of the prepared microbial culture of the *Listeria* cocktail was added to appropriate wells. Simultaneously, light controls (wells without curcumin) and dark controls (without exposure to light) were prepared. The wells were exposed to curcumin for 0 min, 1 min, 2.5 min, and 10 min, after which the 96- well plate was exposed to a light dose of 76.59 kJ/m^2. A 100 μL sample from each well was diluted and spread-plated on TSA, followed by incubation at 37 °C for 18 h. The exposure time that gave a maximum reduction of *L. monocytogenes* was selected for further experiments.

4.1.7 Optimization of Light dose in planktonic system

This experiment was conducted on 96-well plates. The working cultures were diluted to attain 10^5 CFU/mL, and 1mL of each strain was added to a sterile test tube to make a cocktail. A 100 μL volume of SDW and a 100 μL curcumin were added to appropriate wells of 96-well plates. A 100 μL volume of curcumin-SDW was removed and 100 μL of the prepared *Listeria* cocktail was added to appropriate wells. Simultaneously, light controls (wells without curcumin) and dark controls (without exposure to light) were prepared. The wells were exposed to curcumin for 1 min, after

which the 96- well plate was exposed to 7.66 kJ/m^2, 38.29 kJ/m^2, and 76.59 kJ/m^2 of light doses. A 100 μL volume from each well was diluted and spread-plated on TSA, followed by incubation at 37 °C for 18 h. The light density that gave a maximum reduction of Listeria was selected for further experiments.

4.2 Stainless Steel Coupons Experiments

4.2.1 Preparation of Salmon Exudate

The salmon exudate method was used to form a biofilm on stainless steel coupons (Li et al., 2018). Salmon exudate was prepared by placing 125 g of salmon in 300 mL of deionized water in a sterile stomacher bag and soaking for 15 mins. The sample was then massaged manually for 1 minute. The liquid was filtered using a Whatman filter paper number 1 (VWR) to remove debris, and the remaining exudate passed through a 0.2 μm filter (Ascrodisc® Syringe Filters, Pall Corporation, Ann Arbor, MI).

4.2.2 Biofilm formation on stainless steel coupons

Stainless steel 304 coupons of dimensions 2 cm x 2 cm were washed and scrubbed in detergent, rinsed with sterile water, and then held in 95% ethanol for 1 h, and removed and autoclaved at 121 °C for 15 mins. After autoclaving, two SS coupons were placed in a 60 x 15 mm (VWR) Petri dish. They were covered with 9 mL of salmon exudate and 1 mL of *Listeria monocytogenes* working culture to achieve a 10^6 CFU/mL population. The plates were incubated at 25°C for 0 h, 24 h, 48 h, and 72 h. At the time

coupons were removed and processed by rinsing with sterile distilled water (SDW), stained with 0.5% crystal violet (CV), rinsed with SDW to remove the excess stain, and destained with 95% ethanol. 100 μL of the ethanol-CV mixture was transferred to 96-well plates, and the OD value was determined at 595 nm (Lee et al., 2015). Based on these results, 72 h incubation was chosen for biofilm formation experiments. Each SS coupon was rinsed with SDW before each treatment. Controls were included where the SS coupons were placed in salmon exudate without *Listeria monocytogenes.*

4.2.3 Pilot to evaluate PSC exposure time and light dose for SS coupons

Stainless steel coupons with biofilms were placed into Petri dishes, and 10 mL of 160 ppm curcumin solution was added, completely immersing the SS coupons. Simultaneously, light controls (no curcumin) and dark controls (no light exposure) were prepared. The SS coupons were exposed to curcumin for 1 min, after which the SS coupons were transferred to dry Petri dishes which were then exposed to a light dose of 76.59 kJ/m^2. Pre-moistened (in 0.1% SPW) sterile swabs were used to swab the surface horizontally, vertically, and diagonally, ten times in each direction. The swabs were dipped and squeezed on the sides of centrifuge tubes that contained 10 mL of 0.1% SPW. The samples were further serially diluted (1:10), and a 100 μL of aliquot was spread-plated on TSA plates and incubated at 37 °C for 18 h.

4.2.4 Stainless Steel Treatments

Stainless steel coupons harboring *Listeria* biofilms were placed into Petri dishes and treated with SDW, 200 ppm chlorine (Clorox), or 160 ppm curcumin. The coupons were treated with 200 ppm chlorine for 6 min and air dried, following the manufacturer's recommendation for treating stainless steel surfaces for *Listeria sp*. The coupons were placed in curcumin for one minute, followed by a 76.59 kJ/m^2 light dose exposure. Control SS coupons were placed in water for 10 min. Sterile swabs pre-moistened in 0.1% SPW were used to swab the surface horizontally, vertically, and diagonally ten times in each direction. The swabs were dipped and squeezed on the sides of centrifuge tubes that contained 10 mL of SPW. The samples were further diluted serially by 1:10 and plated on TSA plates by spread plate method, followed by incubation at 37 °C for 18 h.

4.3 Cold Smoked Salmon Experiments

4.3.1 Preparation and inoculation of Salmon-

Farm-raised Norwegian salmon was used for all experiments. The salmon was placed in the freezer at -17 °C for 1 h to facilitate cutting into pieces of the desired size. The skin was removed, and the edible flesh was sectioned into 3 cm x 3 cm x 0.3 cm. A 100 µL volume of the *Listeria* cocktail was spot inoculated onto the surface of the salmon pieces. The inoculated salmon pieces were left to dry for 30 min in the biosafety cabinet prior to further use.

4.3.2 Optimization of Light Dose and curcumin exposure

The effect of the curcumin exposure and light dose combination on salmon was determined as described. The inoculated salmon pieces were exposed to 320 ppm curcumin for 1 min, after which they were drained and placed on wire mesh and exposed to 76.59 kJ/m^2 of light dose. Light control samples were included with no curcumin dip. The treated samples were placed in Whirl-Pak sample bags with puncture-proof tabs (Nasco, USA) containing 100 mL SPW homogenized for 1 minute. A 1 mL volume of sample from the stomacher bag was then transferred to a 1.5 mL centrifuge tube, which was then serially diluted by 1:10, and 100 μL aliquot was spread plated on modified oxford medium base (VWR) plates and incubated at 37 °C for 18 h.

4.3.3 Set-up of Cold Smoker

The cold smoking chamber (86.36 cm x 76.2 cm x 48.2cm) contained a stainless-steel grill shelf for placing fish pieces during the smoke process. A mixture of pecan and cherry wood chips (Weber Stephen Products Wood, 192 cu. in.) was used for generating the wood smoke. A vent was used to control smoke flow through the chamber.

Fig 4.2. (a) The cold smoker used in this study (b) Set up of the cold smoking experiment.

4.3.4 The cold-smoking Process

The cold-smoke fish process method was described previously (Crapo et al., 2011). Salmon pieces (3 cm x 3 cm x 0.3 cm) were prepared as described. Brine was prepared by dissolving 449 g of salt in 1.5 L of water. Salmon pieces were added to the brine at a ratio of 1:3 by weight. The fish was allowed to soak in brine for 90 min, then placed for 20 min in freshwater containing 25 ppm of chlorine. The salmon pieces were removed from the water and transferred to a wire mesh and dried for 2 h, followed by smoking for 2 h. Before smoking, all processes were performed at or below 7°C. While

smoking, the temperature was maintained below 32.2 °C, and the fish pieces were flipped every half hour to ensure proper exposure to the distribution of smoke.

4.3.5 Treatment Methods

The salmon pieces were non-inoculated or inoculated with 100 μL of *Listeria monocytogenes* cocktail (10^9 CFU/mL) to study the effect of treatment methods on commensal bacteria and *Listeria monocytogenes,* respectively. Samples were collected to determine commensal and *Listeria* populations before treatment and for chemical analysis. Salmon samples used for chemical analyses were not inoculated with *Listeria*. Depending on the treatment method, the fish was either rinsed in 25 ppm chlorine for 10 min or placed in 320 ppm curcumin for 1 min, followed by exposure to 76.59 kJ/m^2 of light dose, while controls had no treatment. For exposure to light, fish dipped in curcumin were placed on wire mesh, followed by exposure to light dose. Samples were collected to determine the effect of chlorine, curcumin, and no treatment on commensal bacteria and *Listeria populations*. The fish was then allowed to soak in brine for 90 min, then in freshwater containing 25 ppm chlorine for 20 min. This was then followed by placing the fish on wire mesh for drying for 2 h. Samples were collected at this stage as a control for smoke treatment. After smoking for two hours, and flipping the fish every half hour, another treatment with a light dosage of 76.59 kJ/m^2 was performed for part of the curcumin-treated salmon. Samples were collected immediately for day 0, and the rest of the pieces were vacuum packed with a Potane precision vacuum sealer machine with a built-in cutter. Microbial samples were

collected and processed on Day 1, Day 5, and Day 10. Samples for chemical analysis were collected on Day 0 and day 10. Refer to Figure 2.8.4.1 for the flow chart of the experiment performed.

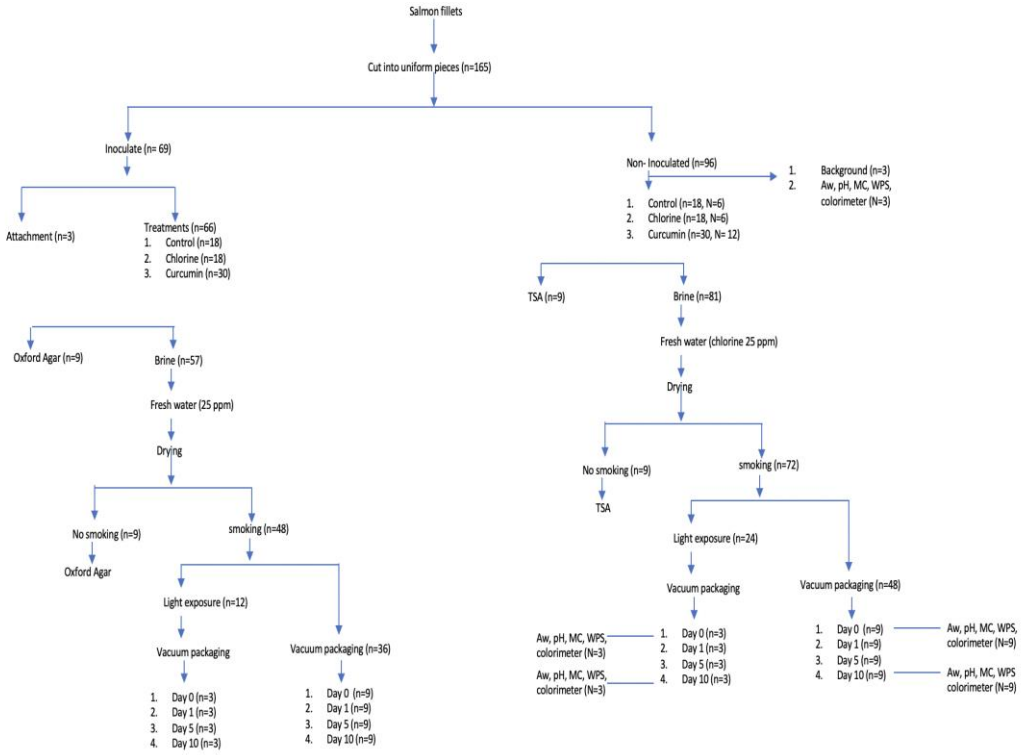

Fig 4.3 Flowchart of cold smoked salmon experiment

Samples for microbiological analysis were placed in Whirl Pak stomacher bags with filters (VWR) containing 100 mL of SPW and homogenized for 1 min. The samples were serially diluted and plated on Oxford media base for enumeration of *Listeria* and TSA for enumeration of commensal bacteria.

4.3.6 Chemical Analyses

4.3.6.1 pH of Fish Samples

Samples of salmon that were collected for pH analyses were homogenized using a tissue homogenizer. 10 g of the ground sample was added to 90 mL of DI water in a conical flask with a magnetic stirrer and mixed to form a homogenous solution. The pH of this solution was measured using an Accumet basic AB15 pH meter (Fisher Scientific) (Lyu et al., 2018).

4.3.6.2 Salt Analyses

The water-phase salt (WPS) content of smoked salmon samples was determined using Mohr's titration method. The concept behind this titration method is that when silver nitrate is titrated against chloride ions in the presence of indicator chromate ions, the endpoint is determined by the formation of red silver chromate. Since the solid yellow color of chromate makes it challenging to detect the initial red of silver chromate, excess silver nitrate was used to enhance detection. To correct this error, a titration with a blank was performed.

To prepare silver nitrate ($AgNO_3$) solution, silver nitrate crystals were dried at 150 °C for two hours. Milli-Q water was boiled to remove carbon dioxide, and 100 mL of this water was poured into a 1 L volumetric flask. 16 g of $AgNO_3$ crystals were added to the flask with continuous stirring. The volume was then made up to 1000 mL with boiled Milli-Q water. Once the $AgNO_3$ solution was cooled down, the solution was made up to

1000 mL as cooling down compresses the volume. The prepared AgNO3 solution was stored in a dark glass bottle and stored in a cool dark place as AgNO3 decomposes in the presence of light. To standardize, the AgNO3 solution was titrated against 1N potassium chloride (KCl) solution in the presence of potassium chromate (KCO3) (indicator).

$$Ag^{+} + Cl^{-} = AgCl\ (s)$$

$$AgNO_3 + KCl = KNO_3 + AgCl\ (s)$$

To determine the WPS content of salmon, samples of fish collected for salt analysis were homogenized using a tissue homogenizer. 2 g of these samples was added to 100 mL DI water in a conical flask and stirred with a magnetic stirrer. The samples were centrifuged at 4000xg for 10 min. The supernatant was transferred back into a conical flask, and a few drops of the indicator potassium chromate was added. The solution was titrated against AgNO3 until the yellow color changed to red, determining the endpoint with the formation of AgCl. The following equation was used to calculate the salt percentage of samples.

$$Salt\ \% = \frac{(V - B) * C * M * 0.1}{W}$$

V- Volume of AgNO3 used in sample titration

B- Volume of AgNO3 used in blank titration

C- Concentration of AgNO3

M- Molecular weight of AgNO3

W- Weight of sample

$$wps = \frac{\%salt}{\%salt + \%moisture} x100$$

WPS- water phase salt

4.3.6.3 Moisture Content of Fish

Samples collected for moisture content analyses were homogenized using a tissue homogenizer. 5 g of sample was placed on the sample pans (Mettler Toledo, Fisher Scientific) and spread to form a thin layer. The sample was, in turn, set in the halogen moisture analyzer (Mettler Toledo HC103, fisher scientific) and moisture content was recorded.

4.3.6.4 Color Analysis of Fish

Changes in color of the salmon flesh were determined. Salmon samples were placed on a white dish and a handheld portable colorimeter (Konica Minolta CR-410) was then placed on the samples, and the L, a, and b values were recorded for each sample.

4.3.6.5 Water activity

Salmon samples were placed in the water activity chamber of a handheld water activity meter (Rotronic HygroPalm) and values recorded.

4.4. Statistical Analysis

The experiments were replicated three times for the stainless-steel coupon studies and twice for the cold smoked salmon studies. The average bacterial population post treatments were analyzed using Microsoft Excel for Analysis of Variances (ANOVA), followed by t-test as the post hoc test with a p-value< 0.05 for significance.

5. RESULTS

5.1 Results for Preliminary Studies-

5.1.1 Absorption Spectrum of PSC

According to the absorption spectrum in Figure 5.1, the photosensitizer curcumin has a broad absorption spectrum ranging from 325 nm to 525 nm, with a peak wavelength of 415 nm. The wavelength of the light apparatus of 420 nm to 430 nm was chosen based on the PSC absorption spectra. The peak wavelength did not change with the concentration of curcumin. This was within the range of absorption spectra and not too far from the maximum to have it far from the UV range to address safety concerns to human exposure in an industrial setting.

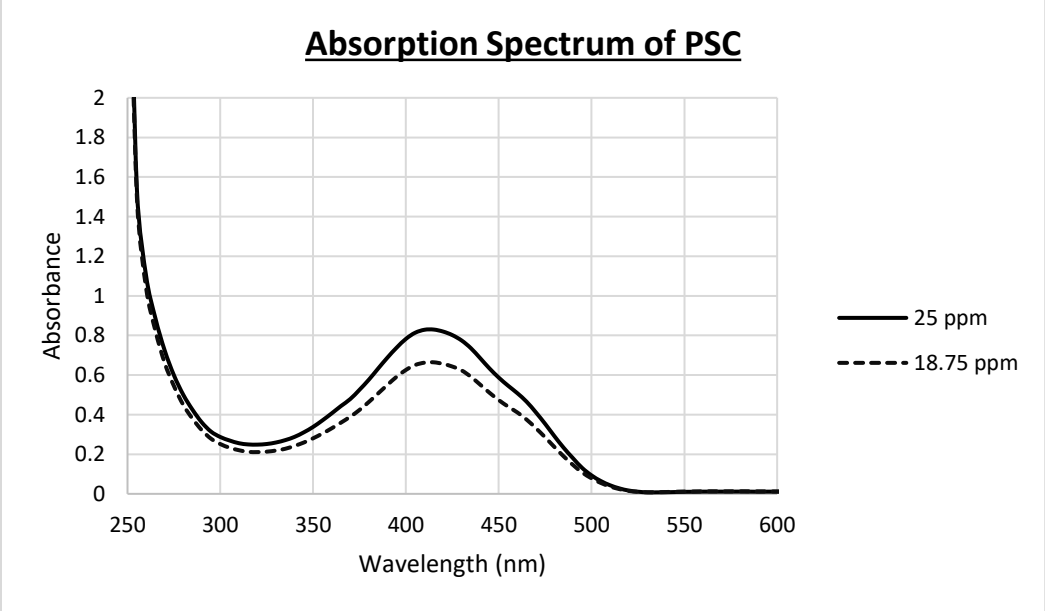

Fig 5.1. Absorption spectra of photosensitizer curcumin at 12.5 ppm and 25 ppm concentration. DI water was used as a diluent. The absorption maxima were found to be 415 nm.

5.1.2 Spectroradiometer results of light apparatus

The spectroradiometer data points were obtained as W/m^2 in 0.5 nm change in wavelength from 226 nm to 1102.5 nm. To get the power density (W/m2) for each position the total output across the full measurement spectrum was summed up and then divided by 2. Then the average power density at all 15 positions was calculated and estimated to be 126.29 W/m^2. The power density against wavelength is plotted in Figure 5.2.

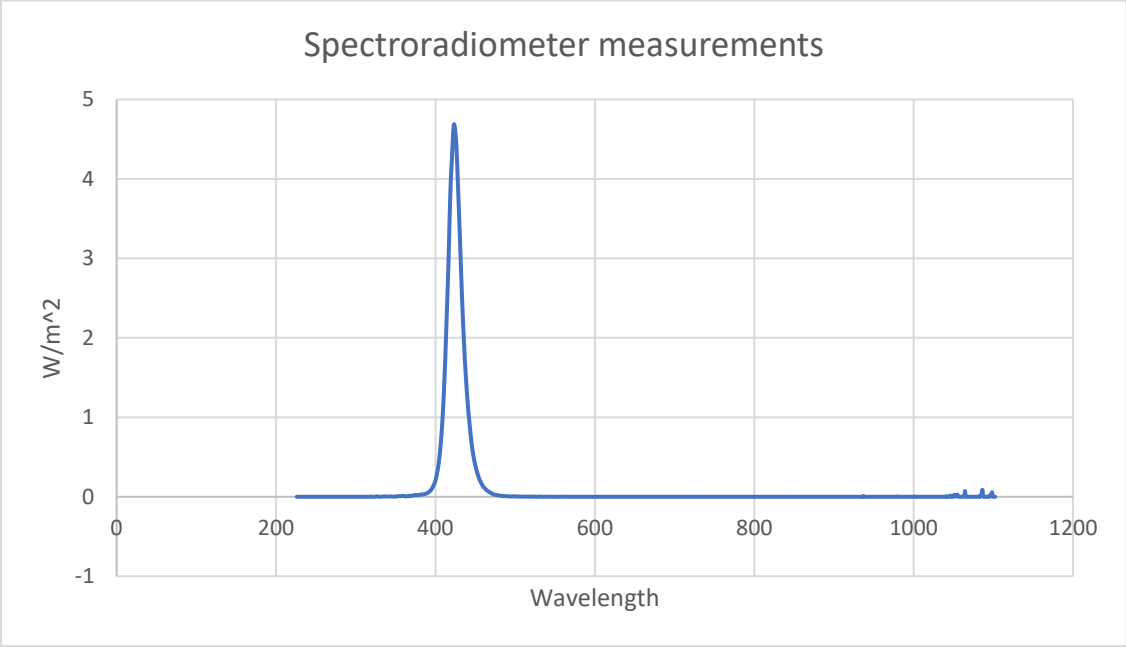

Fig 5.2 Spectroradiometer measurement of light apparatus.

5.1.3 Antimicrobial efficacy of photosensitizer curcumin on solid media

The antimicrobial efficacy of PSC on a solid media against all the *Listeria monocytogenes* strains used in this study were tested. The dark controls had no inhibition zones for all the strains. All the *Listeria* strains had no inhibition zone at 0 ppm, had clear inhibition zones at 100 ppm and 1000 ppm, and had clear inhibition zones with few resistant cells growing in the zones at 10000 ppm. The results are documented in Table 5.1. The '-' indicates no zone of inhibition, the '++' indicates clear zones of inhibition, and the '+' indicates a less clear zone of inhibition with resistant cell growth.

Table 5.1 Summary of results of Modified Kirby Bauer Method

Listeria monocytogenes	**Concentration of PSC**			
	0 ppm	**100 ppm**	**1000 ppm**	**10000 ppm**
T2-063	-	++	++	+
T1-085	-	++	++	+
T1-131	-	++	++	+
M6-171	-	++	++	+
N1-63	-	++	++	+
N1-347	-	++	++	+

5.1.4 Antimicrobial effects of PSC on planktonic system

A MIC could not be determined because there was no visible growth at different concentrations in different trials, but at each trial, there was no visible growth at 80 ppm in the wells, and when plated, there was no growth in the TSA plates as well. Hence it was concluded that 80 ppm of curcumin was the MBC for the *Listeria monocytogenes* strains used in this study. 160 ppm of curcumin was used for our experiments on SS coupons.

5.1.5 Optimization of PSC exposure time in the planktonic system

The initial population of the *Listeria monocytogenes* cocktail was 5.36 log CFU/mL. Without light activation or curcumin, the photosensitizing experiment did not affect the bacterial population. The number of viable cells dropped below detectable levels with exposure to 80 ppm curcumin for 1 min, followed by 76.59 kJ/m^2. Hence one minute of exposure to PSC was adopted as our method for subsequent studies.

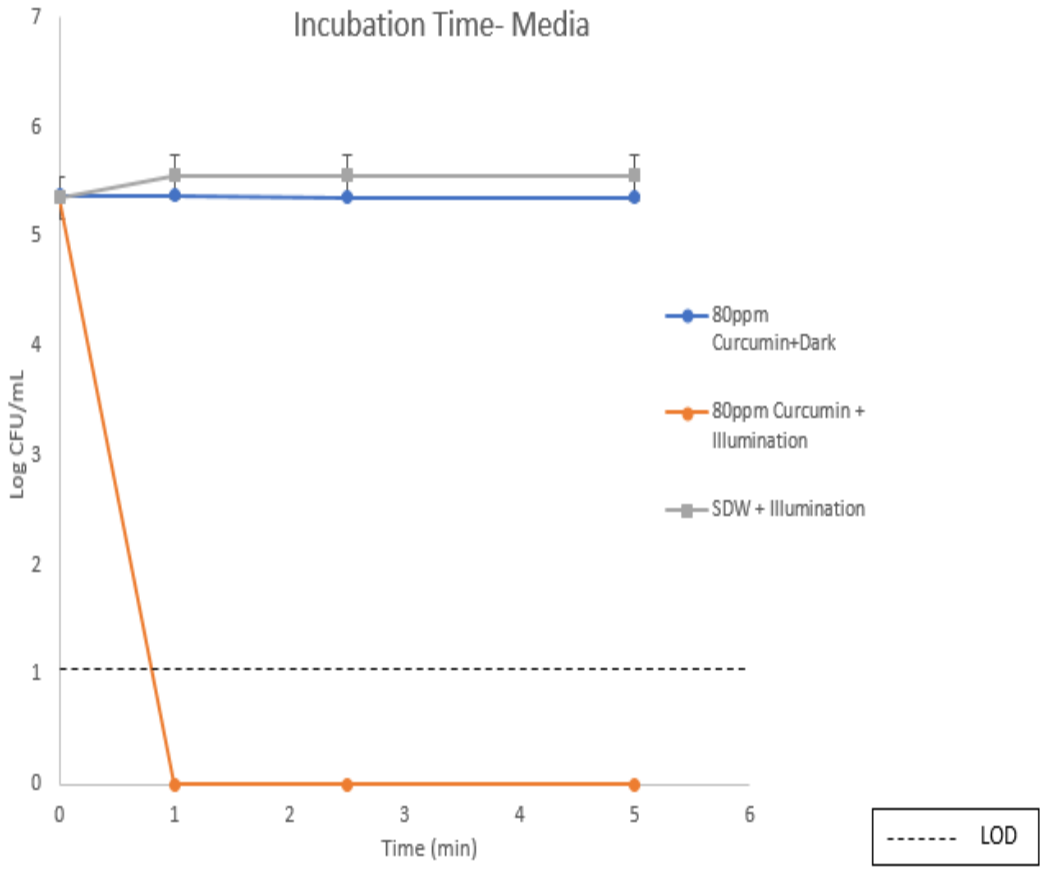

Fig 5.3 Effect of PSC exposure time in *Listeria monocytogenes.* The grey line represents light control, the blue line represents dark control, and the orange line represents exposure to PSC followed by 76.59 kJ/m^2 of light dose. LOD- Level of detection.

5.1.6 Optimization of light dose in the planktonic system

The initial population of the *Listeria monocytogenes* cocktail was 5.35 log CFU/mL. Without light activation or curcumin, the photosensitizing experiment did not affect the bacterial population. The number of viable cells dropped below detectable levels with light exposure of 38.29 kJ/m^2 after exposure to 80 ppm PSC for 1 minute. To be on the cautious side, 76.59 kJ/m^2 of light dose was adopted for further studies.

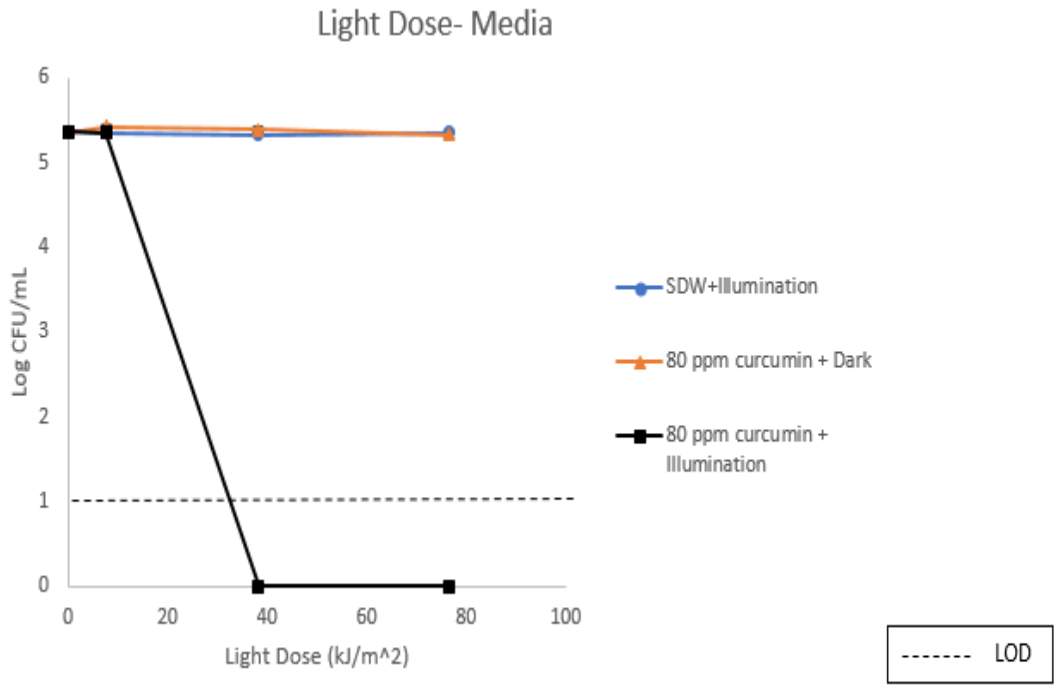

Fig 5.4 Effect of light dose on *Listeria monocytogenes.* The blue line represents light control, the orange line represents dark control, and the grey line represents 1 min exposure to PSC followed by different light doses. LOD- Level of detection.

5.2 Results for SS Coupons studies-

5.2.1 Biofilm formation on stainless steel coupons

The coupons were placed in a Petri dish with 9 mL of salmon exudate and 1 mL of the *Listeria* cocktail for 72 h. Sampling was done at 0 h, 24 h, 48 h, and 72 h to evaluate the biofilm formed. The coupons were washed with SDW to remove unadhered cells, stained with crystal violet, re-washed with SDW to remove excess stain, and de-stained with ethanol. The OD value of the CV-ethanol mixture after the biofilm formation by just salmon exudate on SS coupons was subtracted from the OD

value of salmon exudate and *Listeria monocytogenes* strains. The OD value increased with the time of incubation of *Listeria monocytogenes* and salmon exudate on stainless steel coupons, indicating that the biofilm gets extensive over a period of time.

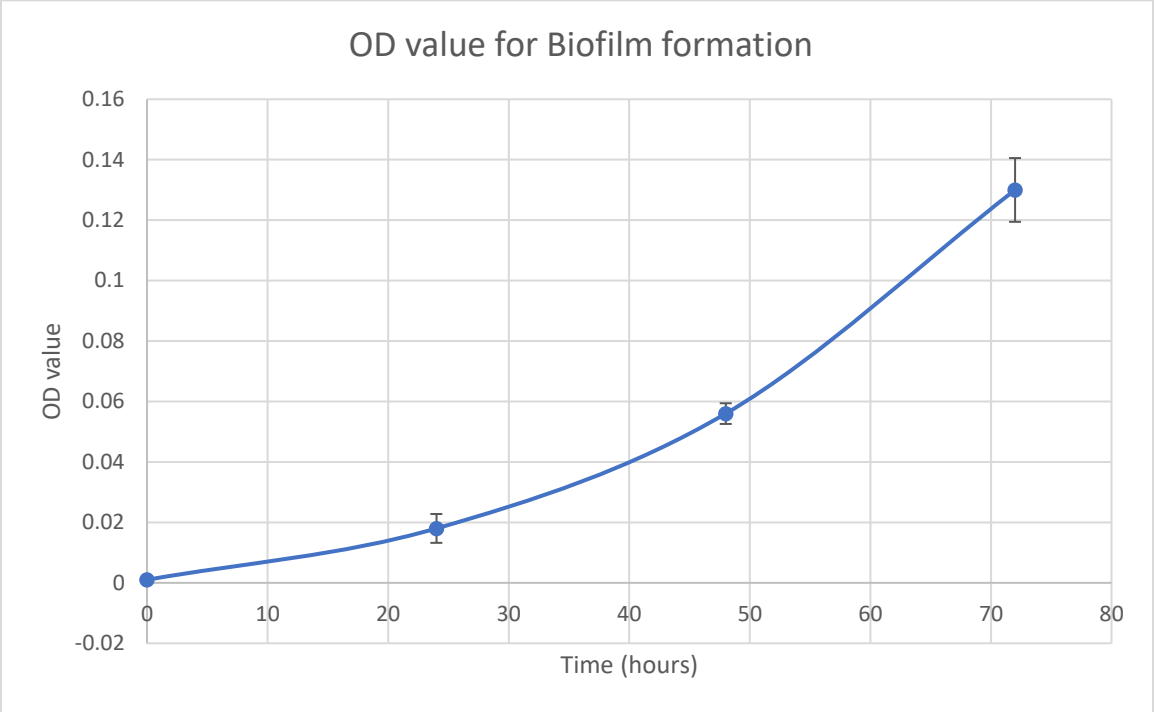

Fig 5.5 OD value of biofilm formed on stainless steel coupons with Salmon exudate and *Listeria monocytogenes* strains over time.

5.2.2 Optimizing PSC exposure time and light dose for SS coupons

To evaluate if the results from the planktonic studies of light dose and curcumin exposure would also work on biofilms on stainless steel coupons, they were incubated in 160 ppm curcumin for 1 minute and then exposed to 76.59 kJ/m^2 of light dose.

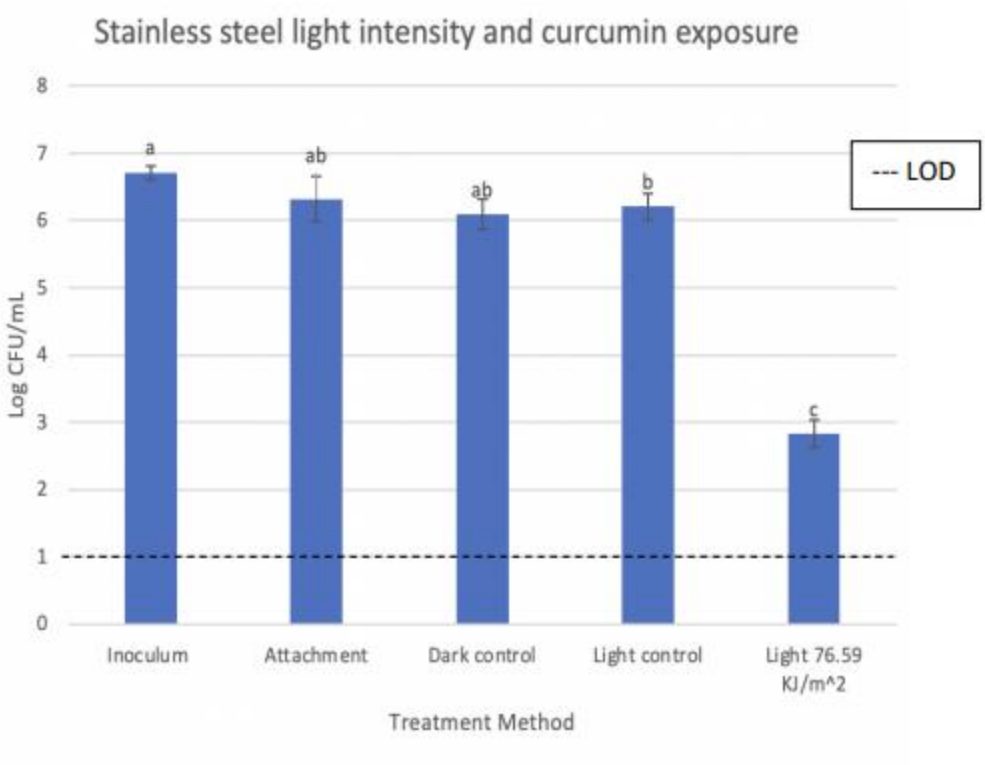

Fig 5.6 Stainless steel light intensity and curcumin exposure. A 3.9 log reduction was observed after 1 minute of curcumin incubation, followed by 76.59 kJ/m^2 of light dose.

5.2.3 Stainless steel coupons treatment

The experiment compared the sanitizing efficacy of curcumin against chlorine. While chlorine had a 3.44 log reduction whilst the PSC treatment had a 3.45 log reduction. The light control, dark control, and water treatment had no statistically significant change from the attachment.

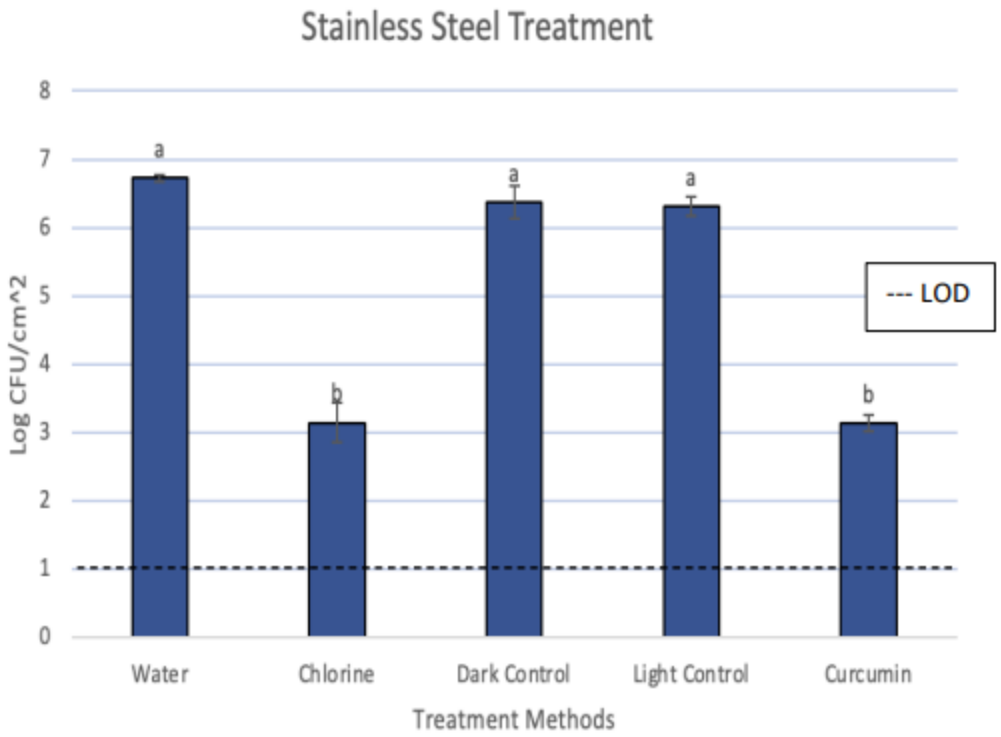

Fig 5.7 Evaluation of curcumin as a sanitizing method, compared to 200 ppm chlorine, and water (treatment control) with light and dark controls.

5.3 Results of CSS Studies-

5.3.1 Optimization of curcumin exposure and light dose on salmon-

Curcumin of concentration 320 ppm was chosen. With curcumin exposure of 1 minute, followed by a light dose of 76.59 kJ/m^2, a 0.83 log reduction in the population of *Listeria monocytogenes* was observed compared to the initial attachment of *Listeria monocytogenes.* attachment population.

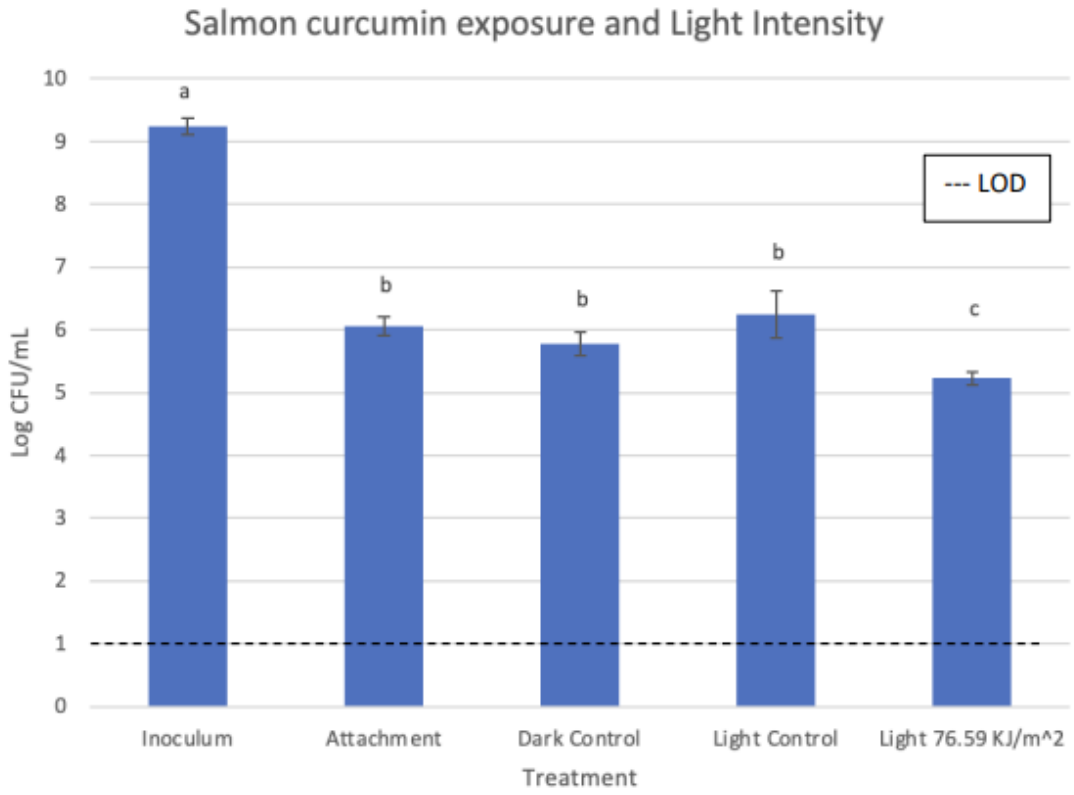

Fig 5.8 Effect of 1 min exposure to 320 ppm curcumin, followed by 76.59 kJ/m^2 of light dose.

5.3.2 Treatment of Cold smoked Salmon

Compared to the control and chlorine treatment, there was a statistically significant change in the *Listeria monocytogenes* population in the cold-smoked salmon exposed to 76.59 kJ/m^2 after smoking.

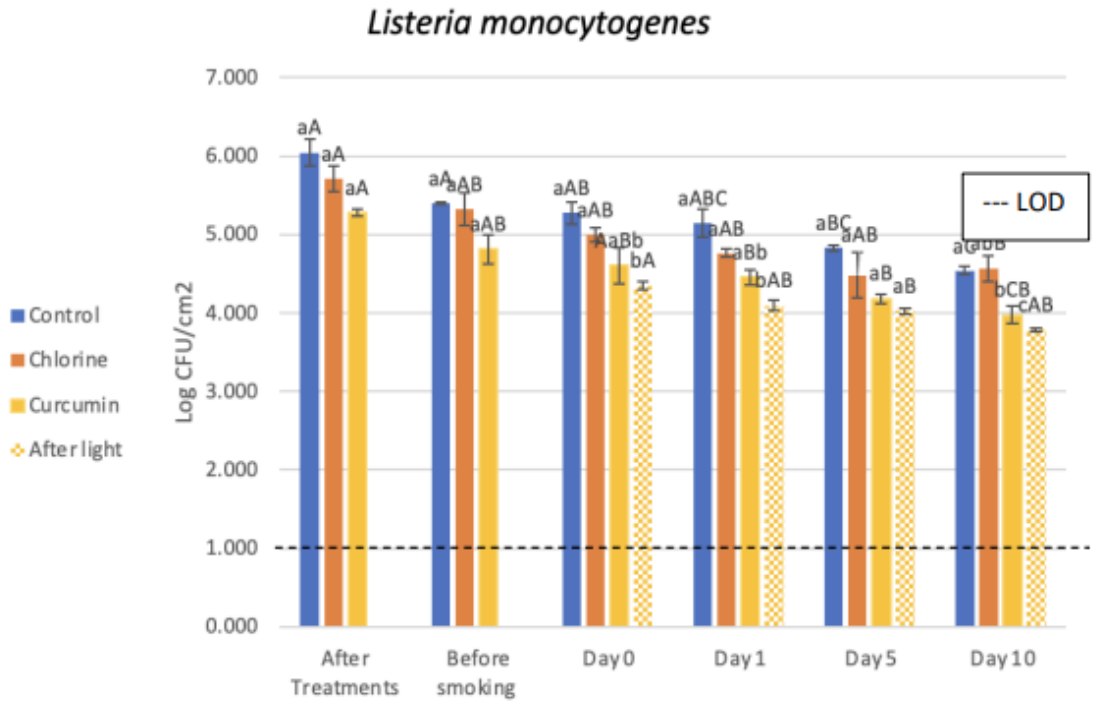

Fig 5.9 Effect of different treatment methods on *Listeria monocytogenes.* There was a consistent decrease in the *Listeria monocytogenes* population over treatments and time. The population of *Listeria* in the light treatment post smoking had a statistically significant reduction compared to control and chlorine at each stage of treatment/storage except day 5.

Table 5.2 Statistical Analysis of *Listeria monocytogenes* population. Lower case letters (a, b, and c) compare the *Listeria* population within each stage/day (within columns), and upper-case letters (A, B, and C) compare the bacterial population within each treatment (rows).

Treatment Method	**Population of *Listeria monocytogenes* (CFU/cm^2)**					
	After Treatment	Before Smoking	After Smoking			
			Day 0	Day 1	Day 5	Day 10
Control	6.0 ^{aA}	5.4 ^{aA}	5.2 ^{aAB}	5.1 ^{aABC}	4.8 ^{aBC}	4.5 ^{aC}
Chlorine	5.7 ^{aA}	5.3 ^{aAB}	4.9 ^{aAB}	4.7 ^{aAB}	4.4 ^{aAB}	4.5 ^{abB}
Curcumin	5.2 ^{aA}	4.8 ^{aAB}	4.6 ^{abAB}	4.4 ^{abB}	4.1 ^{aB}	3.9^{bcB}
After Light			4.2 ^{bA}	4.1^{bAB}	4.0 ^{aB}	3.7 ^{cAB}

6 DISCUSSION

The absorption maxima of curcumin depend on the solvent it is dissolved or suspended in, ranging from 420 nm in toluene to 432 nm in sodium dodecyl sulfate/D2O micellar solution (Chignell et al., 1994). The preferred isomer form of curcumin is the enol form which is more stable than the diketone form owing to the internal H-bond and the extended conjugation of the molecular backbone giving an absorption maximum of 419 nm (Shen and Ji, 2007), which is similar to the absorption maxima of 415 nm obtained in this study. The dissociation of protons shifts the absorption spectrum of curcumin as well, with a shift to 429 nm for deprotonation of the enolic one and to a more significant shift to 531 nm with the dissociation of the phenolic proton (Shen and Ji, 2007). The hydrogen-bonding solvents shifted the absorption spectrum of curcumin toward red, while non-polar solvents shifted it toward blue (Priyadarsini, 2009). The phototoxicity of curcumin is through both oxygen-dependent and oxygen-independent mechanisms. It is moderated by reactive oxygen species and superoxide radicals (do Prado-Silva et al., 2022).

In this study, the duration of interaction between curcumin and planktonic cells prior to light exposure did not affect the reduction in the listerial population like the study by Gao and Matthews (2020). A higher reduction in the *Listeria* population was observed with increased light dose (Fig 5.4). Light dose (kJ/m^2) is calculated by multiplying power density (W/m^2) by illumination time. This was consistent with the decrease in *Bacillus cereus* population with increased illumination time in 5-

aminolevulinic acid (Luksiene et al., 2009) and the decrease in *Listeria monocytogenes* population with an increase in light dose in curcumin (Gao, 2020).

This study demonstrated a 3.45 log reduction in *Listeria monocytogenes* biofilm on stainless steel coupons by photodynamic therapy (PDT) with 76.9 kJ/m^2 and 160 ppm curcumin compared to a strikingly similar reduction in population by 200 ppm chlorine, whilst there was no statistically significant difference when treated with just 160 ppm chlorine or with just 76.59 kJ/m^2 light dose, similar to the results achieved by Huang et al. (2020). Huang's research group also demonstrated a 99.99% reduction in *Listeria monocytogenes* when treated with a 0.2 μM curcumin and 0.5 J/cm^2 light dose. In another study, SS coupons with *Listeria monocytogenes* biofilm were treated with 2% hydrogen peroxide or 2% quaternary ammonium compounds solution for 20 mins (as per manufacturer's recommendations). The results showed a reduction of 5.2 log CFU/cm^2 in hydrogen peroxide treatment and a 4.5 Log CFU/cm^2 reduction with quaternary ammonium compound treatments (Brauge et al., 2020).

The *Listeria* population of control and chlorine samples of cold smoked salmon was significantly larger than that of samples exposed to a light dose of 76.59 kJ/m^2 post-smoking, while the latter was not significantly different from just the curcumin treatment. This was true for the day 0 and day 10 samples but not day 5. Between the control and curcumin samples, there was a significant difference only on day 10. Even though the Graph 5.9 suggests an overall trend in the decrease of the population of *Listeria monocytogenes* over the days within treatment methods or the control samples,

these are not consistently statistically significantly different. Studies conducted by Huang et al. (2021) show an increase in the overall *Listeria* population, including light and dark controls during storage. Al-Holy and Rasco (2015) studied the bactericidal efficacy of acidic electrolyzed oxidized water consisting of 38 ppm free chlorine. A 1.2 log reduction of *Listeria monocytogenes* was observed on trout skin after 10 mins of treatment. Gao and Matthews (2020) demonstrated a 2.9 log CFU/cm^2 with exposure to 300 ppm chlorine and 32.1 kJ/m^2 of light dose.

7 CONCLUSION

This study shows a potential sanitizing effect of photosensitizer curcumin against *Listeria monocytogenes* biofilm on stainless steel surfaces, with a 3.4 log reduction at 160 ppm concentration and 76.59 kJ/m^2 of light dose. The light dose is a crucial component in inactivating the pathogen. This is on par with the 3.4 log reduction of the *Listeria monocytogenes* population by 200 ppm chlorine.

On the other hand, the treatment of salmon with photosensitizer curcumin in combination with 76.59 kJ/m^2 light dose did not have a significant difference in population compared to that of chlorine and control during different stages of the experiment where samples were analyzed. However, there was a significant difference with the reduction in population consistently when the salmon was treated with a second light exposure of dose 76.59 kJ/m^2 compared to chlorine and control.

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9. ANNEX

Table 9.1 Chemical Analyses of cold smoked salmon

	Sample	Avg pH	Avg MC	Avg WPS	Avg aw	Avg Colorimeter		
						L	a	b
Day 0	Fresh	6.454	66.630	0.999	0.957	48.572	14.717	18.445
	Control	6.285	64.792	12.594	0.928	48.904	13.324	17.554
	Chlorine	6.163	62.540	11.284	0.937	50.110	13.087	16.955
	Curcumin	6.115	65.110	12.780	0.935	50.057	13.440	16.630
	After Light	6.102	64.152	10.726	0.937	49.452	12.274	15.590
Day 10	Control	6.277	63.175	13.214	0.921	46.987	13.997	13.514
	Chlorine	6.055	70.754	14.632	0.914	51.160	17.877	16.237
	Curcumin	6.118	66.712	13.131	0.930	49.267	18.920	18.084
	After Light	6.125	62.094	12.684	0.938	46.122	16.240	14.889

Avg- Average; MC- Moisture Content; aw- Water Activity.

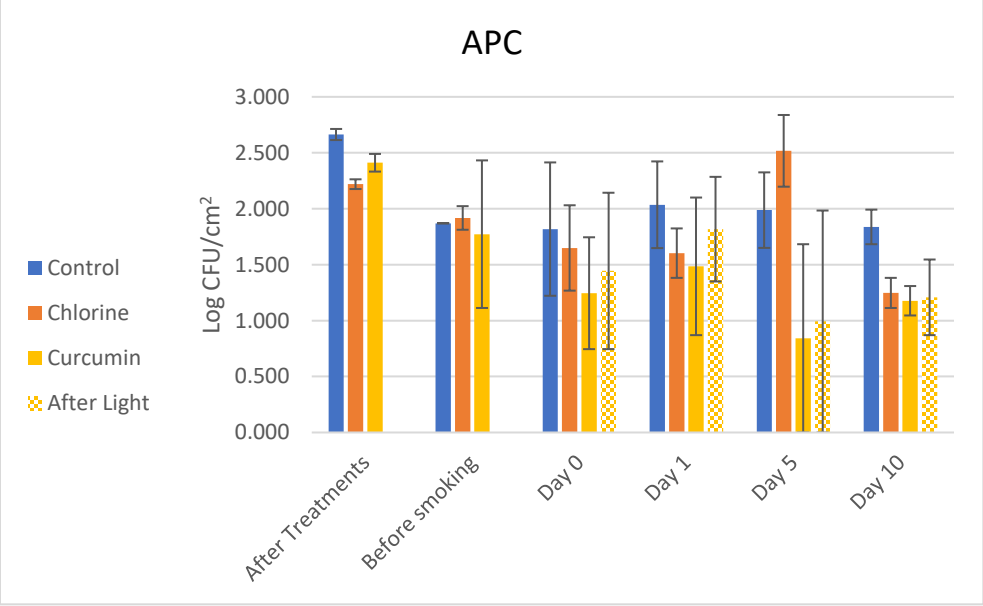

Fig 9.1 Effect of treatment methods on background flora