Effect of Cetyl-Pyridinium Chloride (CPC) Treatment on Inactivation of *Listeria monocytogenes* on Selected Foods

By

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ABSTRACT

Food-borne pathogens such as *Listeria monocytogenes* are a major concern for the food processing industry. As the consumers' demand for fresh fruits and vegetable supply has increased in past few years, it is essential that the microbiological safety of these supplies be adequately controlled and monitored. Cetyl pyridinium chloride (CPC) has been shown to have some antimicrobial effect against *L. monocytogenes* and has been used to decontaminate raw poultry. The main objective of this study was to determine the effectiveness of CPC treatment against a cocktail of pathogenic strains of *L. monocytogenes* on selected foods.

In the first study, the inactivation of a *L. monocytogenes* cocktail suspended in an aqueous medium with varied concentrations of CPC was evaluated at two temperatures (23°C and 1°C) with a constant contact time (15 s). As the concentration of CPC increased in the solution, the inactivation effect of *L. monocytogenes* also increased. In the second study, the exposure time was varied at each CPC concentration. Experiments were carried out separately at 23°C and 1°C. An increase in exposure time of CPC resulted in enhanced inactivation of *L. monocytogenes* populations in the cell suspension. Thus, CPC concentration, temperature and treatment times influenced the inactivation level of *L. monocytogenes*. Up to 6 logarithmic reductions was observed with as low as 10 ppm (or 0.001%) CPC was added to the cell suspension containing *L. monocytogenes* cocktail (10^7 CFU/ml).

The second phase of the study was to treat selected foods with CPC surface decontamination of *L. monocytogenes*. Prepared potato strips, red pepper strips, broccoli florets and boneless chicken thighs were inoculated by dipping in to an aqueous suspension containing 10^7 CFU/ml of *L. monocytogenes* and treated with three different concentrations (0.2 to 0.7% for potato and red pepper; 0.4 to 1.0% for broccoli and chicken) of CPC for three different treatment times (15, 30 and 60 s). Extent of inactivation levels were

enumerated by serial dilution plate count. Results had showed that, CPC was more effective against *L. monocytogenes* when the pathogen is present in suspension form where CPC contact with the pathogen is most effective. The study revealed that the microbial counts on the products tested could be reduced by CPC treatment at concentration levels higher than 0.4% with a 60 s treatment at room temperature. Weibull model demonstrated a good fit for the destruction kinetics.

Overall, results indicated that, many factors such as concentration of CPC, treatment time, temperature during treatment, type of food surface have vital role in destruction of *L. monocytogenes* and CPC treatment can be utilized for decontamination if concentrations higher than 0.4% are permitted for application.

RÉSUMÉ

Les pathogènes d'origines alimentaires tels que le *Listeria monocytogenes* sont aujourd'hui une préoccupation majeure pour l'industrie agro-alimentaire. Étant donné que la demande de fruits et légumes frais a augmenté au cours des dernières années, il est essentiel que l'innocuité de ces produits soit contrôlée et surveillée de manière adéquate. Il a été démontré que le chlorure de cétylpyridinium (CPC) a un certain effet antimicrobien contre le *L. monocytogenes* et a été utilisé pour décontaminer la de volaille. Par conséquent, l'objectif principal de cette étude était de déterminer l'efficacité du CPC contre un cocktail de souches pathogéniques de *L. monocytogenes* sur des aliments sélectionnés.

Dans la première étude, l'inactivation du cocktail de *L. monocytogenes* en suspension dans un milieu aqueux avec des concentrations variées de CPC a été évaluée à deux températures (23^{o}C et 1^{o}C) avec un temps de contact constant (15 s). Lorsque la concentration de CPC augmentait dans la solution, l'effet d'inactivation de *L. monocytogenes* augmentait également. Dans la deuxième étude, le temps d'exposition variait à concentration de CPC constante. Les expériences ont été réalisées séparément à 23^{o}C et 1^{o}C. Une augmentation du temps d'exposition de la CPC a entrainé une inactivation accrue de la population de *L. monocytogenes* dans la suspension bacterienne. Ainsi, la concentration de CPC, la température et le temps de traitement ont influencé le niveau d'inactivation de *L. monocytogenes*. Lorsque aussi peu que 10 ppm de CPC a été ajouté à la suspension cellulaire contenant le cocktail de *L. monocytogenes,* une réduction de 6 logarithmi a été observée.

La deuxième phase de l'étude consistait à traiter la surface des aliments sélectionnés avec le CPC pour les décontaminer de *L. monocytogenes*. Des bandes de pommes de terre préparées, des bandes de poivrons rouges, des fleurons de brocoli et des cuisses de poulet désossées ont été inoculées par trempage dans une suspension aqueuse contenant 10^7 CFU/ml de *L. monocytogenes* et traitées à différentes concentrations (jusqu'à 1%) de CPC (15, 30 et

60 s). Le niveau d'inactivation de *L. monocytogenes* a été dénombré par une technique d'énumération totale sur plaques. Les résultats ont montré que le CPC était plus efficace contre *L. monocytogenes* lorsque le pathogène est présent sous forme de suspension puisque le contact de la CPC avec le pathogène y est alors plus efficace. L'étude a révélé que la contamination microbienne sur les produits testés pouvait être réduites par un traitement CPC à des concentrations supérieures à 0,4% avec un traitement de 60 s à température ambiante. Le modèle de Weibull a démontré un bon accord pour la cinétique de destruction.

Dans l'ensemble, les résultats indiquent que de nombreux facteurs tels que la concentration de CPC, le temps de traitement, la température pendant le traitement, le type de surface alimentaire jouent un rôle essentiel dans la destruction de *L. monocytogenes* et un traitement CPC peut être utilisé pour la décontamination.

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CONTRIBUTION OF AUTHORS

This thesis has been written in manuscript style to suitably edit chapters and highlighting research for publication. A part of this thesis research has been prepared to present in upcoming NABEC conference 2018. Some results have also been communicated to the CTAQ partners during the several meetings held in 2017 and 2018. Authors involved in the thesis work and their contributions to the various articles are as follows:

Kshitija Pawar is the MSc. candidate who planned and conducted all the experiments, on the advice and guidance of her supervisor, gathered and analysed the results and prepared drafts for the thesis and the manuscripts for scientific presentations and publications.

Dr. Hosahalli S. Ramaswamy is the thesis supervisor, under whose guidance the research was completed. He coordinated and supervised the candidate in planning and conducting the research, as well as in correcting, reviewing and editing the thesis.

ABBREVIATIONS

L	Litre
ml	Millilitre
µl	Microliter
µm	Micrometer
cm	Centimetre
mm	Milimeter
h	Hour
min	Minute
s/sec	Second
^{o}C	Degree Celsius
%	Percent
g	Gram
ppm	Parts per million
Std dev.	Standard deviation
~	Approximately
/g	Per gram
CFU	Colony forming unit
RT	Room temperature
CT	Chilling temperature
QAC	Quaternary ammonium compounds
CPC	Cetly-pyridinium chloride
TSA	Tryptic soya agar
TSB	Tryptic soya broth
RTE	Ready-to-eat

CHAPTER 1

INTRODUCTION

Food is an edible and portable substance obtained from plant or animal, that consist of nutritive components like carbohydrates, fats, proteins, essential minerals and vitamins that generates energy when ingested and digested, and helps to grow and maintain the health of the body (Jacobs et al., 2012).

Foods can be contaminated with microorganisms such as bacteria, viruses or toxic substances or any other toxins. Consumption of such contaminated food can cause illness and may lead to death in many cases. Bacteria such as *Bacillus cereus, Campylobacter jejuni, Clostridium botulinum, Listeria monocytogenes, Salmonella spp., Shigella spp., Staphylococcus aureus* are pathogenic bacteria in foods that can cause serious public health concerns (Hussain & Altaf, 2016). Among these, *L. monocytogenes* is considered important because various strains of *L. monocytogenes* have been linked with outbreaks through raw and processed meats, seafood as well as fresh vegetables (AFSSA, 2000). *L. monocytogenes* is a gram-positive ubiquitous bacterium which is widely distributed in the environment. It has been isolated from a variety of sources including soil, vegetation, fodder, faecal material, sewage and more importantly water (AFSSA, 2000). However, food has been designated as a primary mode of transmission of *L. monocytogenes* (Schlech et al., 1983).

Consumption of food contaminated with *Listeria* is a major mode of this bacterial transmission (Low & Donachie, 1997). Foods that are contaminated with *Listeria* may look, smell and taste normal. Unlike most bacteria, *Listeria* can survive and sometimes grow on foods stored in the refrigerator (Acha & Szyfres, 2003). These contaminants can enter food supply chain through multiple routes. For example, various food factories receive multiple food ingredients from different parts of the world. As a result, contaminants can easily travel

through such food supply chain from one place to another place. Hence food contamination is a global challenge (Fukuda, 2015).

Many antimicrobials and preservatives have been approved for the use on foods to overcome contamination problems (Podolak et al., 1996). Cetyl-pyridinium chloride ((1-hexadecylpyridinium chloride, CPC) is one such chemical. It is classified under quaternary ammonium chloride compounds (QAC). CPC has tendency to interrupt bacterial respiration by creating pores in cell membrane and causing cell death (Kim & Slavik, 1996). Hence, in this study, the effect of CPC on *L. monocytogenes* was determined in detail, by altering the treatment conditions like, temperature, CPC exposure time and CPC concentration. The main aim of this study was to understand the most effective treatment conditions to get maximum reduction in *L. monocytogenes* populations.

In recent years, the fresh cut vegetable sector has shown the highest occurrence of *L. monocytogenes* (Venturini et al., 2011). Many times, it leads to *L. monocytogenes* food-borne outbreaks (Table 2). Therefore, it is important to overcome *L. monocytogenes* contamination associated with vegetables and other food stuff. Vegetables have an important role in human diet but currently the use of CPC has only been approved on poultry by FDA (presently, up to 1% CPC concentration cab be used on the surface of poutry). Hence, it was important to check the effect of CPC on vegetables too. So that, further steps can be taken to approve its use even on vegetables. Therefore, in this study three commonly used vegetables (potato, red pepper, broccoli) and chicken were selected, to determine the most effective CPC concentration and treatment time on each of them.

Therefore, the following objectives were proposed in this study,

1. Determine the effect of different concentrations of CPC on inactivation of *L. monocytogenes* cocktail at room temperature (23^{o}C) and 1^{o}C

2. Determine the effect of varying CPC treatment times on inactivation of *L. monocytogenes* at room temperature (23^{o}C) and chilling temperature (1^{o}C)
3. Study the inactivation kinetics of *L. monocytogenes* cocktail inoculated on food samples treated with CPC.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

According to Codex Alimentarius, “food” means any substance, whether processed, semi-processed or raw, which is intended for human consumption, and includes drinks, chewing gum and any substance which has been used in the manufacture, preparation or treatment of “food” but does not include cosmetics or tobacco or substances used only as drugs (Codex alimentarius, 2007). There are several contamination problems associated with food safety.

2.1.1 Food contamination: A global concern

Food containing microorganisms such as bacteria, viruses or toxic substances or any other toxins are considered to be contaminated. Consumption of such contaminated food can cause illness and lead to death in many cases. Food contaminants are categorised into biological, chemical and physical types. Among these, biological contaminants are more common, such as *Bacillus cereus, Campylobacter jejuni, Clostridium botulinum, C. perfrigens,* Pathogenic *Escherichia coli, Listeria monocytogenes, Salmonella spp., Shigella spp., Staphylococcus aureus, Vibrio cholera, V. parahaemolyticus, V. vulnificus and Yersinia enterocolitica* (Hussain & Altaf, 2016).

2.1.2 Microbiological Contamination of Food

In the food production chain there are many stages from the farm to the dining table, where food can get contaminated (Lynch et al.,2009). Microorganisms can enter in to the crop and food animals in the farm at the time of primary production or harvest and during pre-slaughter or/and post-slaughter of animals. Post-harvest consists of food processing, distribution and marketing, storage, preparation and serving. At any of these steps, pathogens can get in to food either directly or indirectly. Plant foods can get contaminated at pre-harvest

stage due to unhygienic practices of workers in the field or through improperly cleaned harvesting equipment. (Alum et al., 2016).

Food processing are different methods or processes that can make changes in food substances to alter their quality and shelf life (Truiswell & Brand, 1985). In food processing industries, pathogenic bacteria or microbial toxins can enter in to food through cross-contamination. For example, *L. monocytogenes* can be transferred from processing surfaces to foods (Kaarina, 2007). Table 1 shows the list of microbial contaminates associated with varies foods and the symptoms observed after ingestion of the respective contaminated food.

Table 1: Selected microbial contaminants and their commonly-associated foods and symptoms (Alum et al., 2016)

Microbial Contaminant	**Associated foods**	**Symptoms**
Clostridium perfringens	Raw meats, poultry, fish, stews, cooked turkey and beef, casseroles, gravy dressings, food that sits for extended periods and dried foods such as spices and vegetables	Perfringens food poisoning (Intense abdominal cramps, watery diarrhea)
Staphylococcus aureus	The red meats, especially ham, poultry, potato, macaroni and tuna salads, custard and cream-filled bakery product, the sandwich sauces.	Staphylococcal food poisoning (Sudden onset of severe nausea and vomiting. Abdominal cramps. Diarrhea and fever may be present.)
Clostridium botulinum	Vegetables, improperly or home-canned or bottled foods, including canned meats, corn beef, canned fish, smoked fish and vegetables, honey, mushroom, improperly processed peppers, asparagus, soup, spinach.	Botulism (Vomiting, diarrhea, blurred vision, double vision, difficulty in swallowing, muscle weakness. Can result in respiratory failure and death)
Listeria monocytogenes	Dairy (soft cheeses and coleslaw), meat products (pate, sausages), cold-smoked	Listeriosis (Fever, muscle aches, and nausea or diarrhea.

	and gravid rainbow trout products, sliced cold cuts, soft cheese, butter, ice-cream and coleslaw raw vegetables, fermented raw-meat sausages, raw and cooked poultry, raw meats (all types), and raw and smoked fish	Pregnant women may have mild flu-like illness, and infection can lead to premature delivery or stillbirth. The elderly or immune-compromised patients may develop bacteremia or meningitis.)
Escherichia coli OI57:H7	ground beef, raw milk, chicken, vegetables and fruits, and any food exposed to raw faecal matter is at risk of being contaminated	Haemorrhagic colitis, Severe (often bloody) diarrhea, abdominal pain and vomiting, little or no fever. Can lead to kidney failure.)
Salmonella	Raw meats, eggs, fish shellfish, poultry, Milk and dairy products, fish, shrimp, frog legs, yeast, coconut, sauces, salad dressing, cake mixes, cream-filled desserts and toppings, dried gelatine, peanut butter, cocoa, chocolate, pork. In general, beef is less often contaminated with salmonella than poultry and pork.	Acute gastroenteritis, painful abdominal cramps, diarrhea that may be sometimes bloody, fever, vomiting, headache and body aches
Vibrio vulnificus	Undercooked or raw seafood, such as shellfish (especially oysters)	Vomiting, diarrhea, abdominal pain, blood-borne infection. Fever, bleeding within the skin, ulcers requiring surgical removal. Can be fatal to persons with liver disease or weakened immune systems.)
Hepatitis A	Raw produce, contaminated drinking water, uncooked foods and cooked foods that are not reheated after contact with an infected food handler; shellfish from contaminated waters	Hepatitis (Diarrhea, dark urine, jaundice, and flu-like symptoms, i.e., fever, headache, nausea, and abdominal pain)

2.2 *Listeria monocytogenes*

Listeria monocytogenes is a facultatively anaerobic, gram-positive, rod-shaped coccobacillus bacteria. It measures about 0.5 to 2 μm long and 0.5 μm in diameter. It can reproduce at temperatures between 1 to 45°C. *Listeria* can grow in the pH range between 4.3 to 9.6. *L. monocytogenes* is classified into 11 sero-types; out of which, three serotypes (1/2a, 1/2b and 4b) are associated with the majority of listeriosis cases (Acha & Szyfres, 2003; Low & Donachie, 1997; Rocourt & Bille, 1997).

Epidemiological data on foods involved in listeriosis outbreaks, show that even a single surviving bacterial cell has the potential to multiply in food and reach significantly higher in number (>1000 CFU/g) and further responsible to cause disease (Ross et al., 2002). Therefore, many countries have established low tolerance levels of *L. monocytogenes*. For instance, the USA practices 'zero tolerance' (no organisms found in 25 g of a food product) (Shank et al., 1996). Canada and France apply different standards for different foodstuff. The zero tolerance is applied to foods which support *L. monocytogenes* growth, and which also have extended shelf-lives. However, a level of not more than 100 CFU/g is tolerated in certain foodstuffs because the probability of getting listeriosis is thought to be low when food contamination with *L. monocytogenes* fell below 100 CFU/g (AFSSA, 2000).

L. monocytogenes has ability to grow and replicate even at near freezing temperatures in refrigerated foods. Hence, it is a big concern for the products with a long shelf life stored under refrigeration (Rychli et al., 2014). Research also has shown that *L. monocytogenes* can grow and survive between -0.5 to 9.3^{o}C under laboratory conditions. Moreover, many strains of *L. monocytogenes* show resistance towards a number of environmental factors like, high salt or acidity in food, low humidity or low oxygen in food environments (Walker et al., 1990). Food rheology or structures inside the food matrix can influence the microecological

conditions thus have an indirect effect on the ability of *L. monocytogenes* to multiply (Buchanan et al., 2017).

2.2.1 Pathogenesis of *L. monocytogenes*

In the 1920s *L. monocytogenes* was first described as a human pathogen. It causes listeriosis, listeriasis, listerellosis, and circling disease in animals. In humans, *Listeria* can cause listeriosis and it can be very severe with up to 50% mortality rate (Low & Donachie, 1997). *L. monocytogenes* is widely spread in nature including in agricultural, aquaculture and food processing units. Domestic animals can get infected with *L. monocytogenes* and develop listeriosis by the consumption of contaminated feed. For example, listeriosis in domestic ruminants is usually caused by the ingestion of poor-quality silage (EFSA, 2017).

Listeriosis is categorised into many types; for example, listeriosis in pregnancy, listeriosis of the central nervous system (CNS), febrile gastroenteritis, glandular listeriosis, local listeriosis and typhoid listeriosis. In case of pregnant women, listeriosis occurs mostly during the third trimester and is characterised by a flu like symptoms that includes fever, chills, malaise, arthralgia, back pain, and diarrhoea. *L. monocytogenes* can transmit from mother to child during the birth of child through the birth canal. In most of the cases, this infection is not very severe. However, intrauterine infection of the foetus can lead to foetal death, spontaneous abortion, premature delivery or in some cases the foetus dies soon after the birth. *Listeriosis* of the Central nervous system(CNS) can lead to Meningitis (Doganay, 2003).

L. monocytogenes has ability to form biofilms on food processing equipment. Through contaminated food or water, it can enter the human body. Inside the body it can pass through the epithelial barrier of the intestinal track. It can also cross blood-tissue barrier and infect body organs like brain or uterus, that leads to severe infections such as meningitis, encephalitis and bacteremia (presence of bacteria in blood). *L. monocytogenes* is mainly

responsible to cause listeriosis that shows food poisoning such as symptoms including abdominal cramps, nausea and diarrhea. It can also cause spontaneous abortion or miscarriage, pre-term delivery in pregnant women (EFSA, 2014). Immuno-deficient, pregnant women, infants and elder people are at high risk (Cartwright et al., 2013; EFSA, 2017).

For a healthy human population, foods in which *L. monocytogenes* levels of less than 100 CFU/g are considered to have a negligible risk. Therefore, the EU has set microbiological criterion for *L. monocytogenes* in Ready-to-eat (RTE) food to ≤100 CFU/g (EFSA, 2014). However, the risk of developing listeriosis in humans depends on multiple factors that are interrelated between the pathogen, food and the host. Such influential factors include, the initial level of contamination, the ability of the contaminated RTE food to support the growth of pathogen, change in temperature throughout production and distribution of RTE foods, the consumer food safety practices, portion size of food etc. Furthermore, the disease incidence depends on variety of factors such as, virulence potential of *L. monocytogenes*, host susceptibility or immune status of the host, dose (the number of pathogens ingested) and the choice of treatment (Buchanan et al., 2017; Evans & Redmond, 2016).

2.2.2 Food-borne Outbreaks due to *L. monocytogenes*

Listeriosis is spread across the world (Table 2) and the first outbreak of food-borne listeriosis was observed in 1979 at Boston hospital (Acha & Szyfres, 2003; Gahan & Hill, 2005). An increasing trend in listeriosis cases have been seen in the EU/EEA during the overall period 2008–2016. Since 2010, United states has seen number of listeriosis outbreaks that are associated with vegetables and fruits such as celery, lettuce, cantaloupe, sprouts, stone fruit and caramel apples (Buchan et al., 2017).

In Canada, the first *L. monocytogenes* outbreak was reported in 1981, in the Maritime Provinces. It occurred due to consumption of contaminated cabbage in coleslaw (Gahan & Hill, 2005). Recently, on 22nd January 2016 the Canadian Food Inspection Agency (CFIA) issued food recall warning, to recall packed salad products (chopped salads, salad blends and kits) produced at Dole processing facility in Springfield, Ohio suspected of *L. monocytogenes* contamination.

Table 2: List of some important *L. monocytogenes* food-borne outbreaks in various countries

Type of food	Year	Country	Factors leading to outbreak	Reference(s)
Coleslaw	1981	Canada	Cabbage was fertilized with manure from sheep with listeriosis and held in cold storage for months, allowing possible *L. monocytogenes* growth before the cabbage was used to make coleslaw.	(Schlech et al., 1983)
Shrimp cooked	1989	United States	10 people who consumed contaminated shrimps served during one party in Connecticut state (US) developed listeriosis. Contaminated Shrimps acted as vehicle of *L. monocytogenes* in this outbreak	(Riedo et al., 1994)
Salad, rice	1993	Italy	The salad was stored overnight at ambient temperature in June	(Salaminal & Dalle Donne, 1996)
Salad, corn and tuna	1997	Italy	A blend of canned corn and canned tuna prepared was evidently contaminated during preparation. Subsequent tests suggested that the time and temperature would have	(Aureli et al., 2000)

			allowed growth.	
Pre-cut celery	2010	United states	Listeriosis was associated with machine cut, diced celery served in five different hospitals in Texas. The outbreak strain of *L. monocytogenes* was detected at the processing facility and in several bags of diced celery retrieved from the manufacturing facility.	(Buchanan et al., 2017)
Caramel apples	2014	United states	Research has showed insertion of the holding stick into apple may have created a local microenvironment at the apple-caramel interface that supports rapid growth, whereas neither the apple nor caramel alone support its growth	(Glass et al., 2015)

The most recent *Listeria* food-borne outbreak is ongoing South African listeria outbreak 2017-2018. It is considered as the world's largest *Listeria* food-borne outbreak ever. In January 2017, the first laboratory case of this outbreak was confirmed. According to the report published in March 2018 by World Health Organization (WHO), 978 cases of listeriosis were confirmed with 183 deaths. Figure 1 shows that, newly born were most susceptible among entire population (Centre for Enteric Diseases (CED) & National Institute for Communicable Diseases (NICD), 2018). On 4 March 2018, the Ministry of Health, announced that ready-to-eat processed meat product called "Polony" was the source of this outbreak. According to the WHO report, different strains of *Listeria* were involved in this outbreak, hence it was indicated that more than one outbreak is ongoing. To control the situation, 15 South African countries issued recalls for the concerned products.

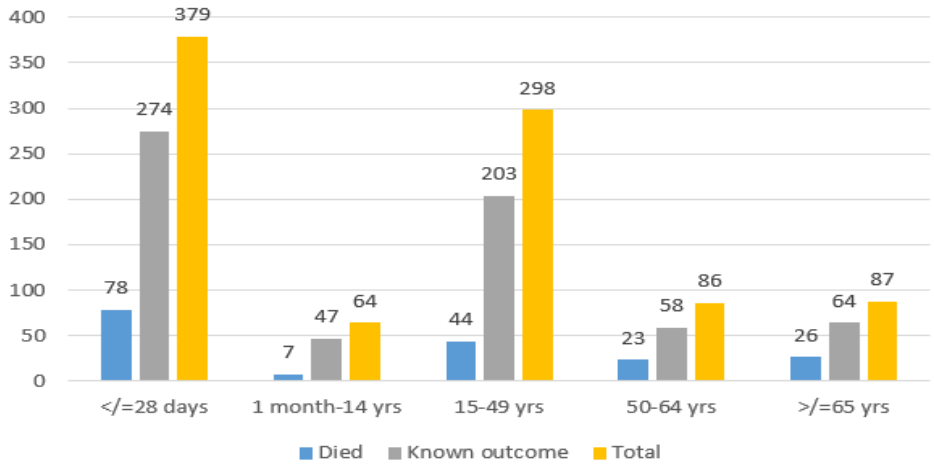


Figure 1: Age distribution and outcome of laboratory-confirmed cases of listeriosis identified from January 2017 to March 2018 (n=914 where age was reported)

2.3 Antimicrobial agents in food industry

Food industries follow various cleaning and disinfecting processes. Cleaning steps are usually applied to remove organic matter, because organic matter can reduce the efficiency of disinfectants used and prevent the disinfectant from reaching certain parts of the equipments (Gibson et al., 1999). In such situations, the bacteria can survive, whereas sometimes the bacteria like *L. monocytogenes* get exposed to cleaning solutions but they still survive and remain attached to the equipment. However, such bacterial cells become very sensitive to the subsequent treatments, due to the prior chemical exposure (Taormina & Beuchat, 2002). Hence, in various situations after the use of sanitizing chemicals, certain subsequent treatments can be given to ensure complete removal of the bacteria. Table 3 shows the list of few antimicrobials and the effective conditions to use them on specific foods.

Table 3: Antimicrobial processing aids for which Health Canada has issued a Letter of No Objection (LONO) (Health Canada, 2015).

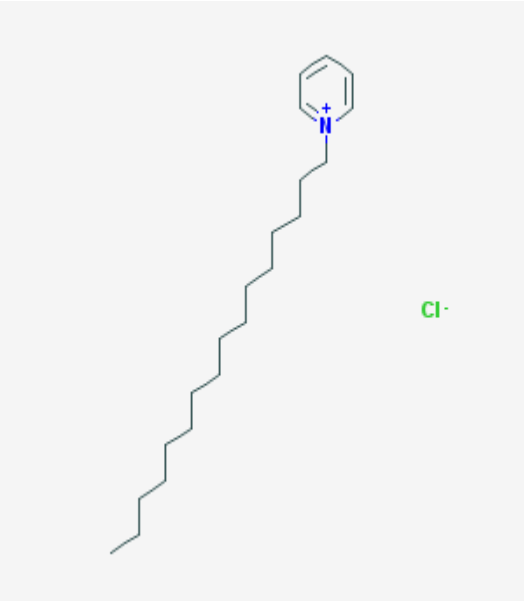
Processing Aid Substance that is the subject of LONOs	Uses	Specific Conditions
Acidified sodium chlorite	1) Red meat carcasses, parts and organs 2) Poultry carcasses, poultry parts, organs and trim applied prior to immersion in a pre-chiller or chiller tank	Applied as spray or dip at levels between 500 and 1200 ppm of sodium chlorite prepared by acidifying the sodium chlorite solution with food-grade acid (i.e., citric acid, phosphoric acid or hydrochloric acid) to achieve a pH of 2.2 to 3.0, equivalent to 50 to 266 ppm chlorous acid formed when prepared in the same manner as above to achieve a pH of 2.5 to 2.9
Calcium hypochlorite	Red meat carcasses down to a quarter of a carcass	Applied as a spray at a level not to exceed 20 ppm calculated as free available chlorine measured prior to application followed by a potable water rinse. NOTE: In the case of pork carcasses, potable water rinse not required if pork carcasses held for a minimum of 12 hours prior to further processing
Cetyl pyridinium chloride (CPC) containing 1.5 times its (i.e., CPC) weight of propylene glycol	Raw poultry carcasses before or after air or immersion chilling	Not to exceed 1% aqueous solution of cetylpyridinium chloride and not to exceed 1.5% propylene glycol applied to raw poultry carcasses followed by a potable water rinse
Chlorine dioxide	Red meat carcasses and parts	Not to exceed 20 ppm chlorine dioxide followed by a rinse with potable water

	On whole or eviscerated poultry carcasses prior to immersion in pre-chiller and chiller tanks	Applied as a spray at a level not to exceed 50 ppm chlorine dioxide, without subsequent potable water rinse
Peroxyacetic acid, hydrogen peroxide, acetic acid, sulphuric acid (optional), and 1-hydroxy-ethylidene-1,1-diphosphonic acid (HEDP) (an aqueous mixture)	1) Red meat carcasses, parts, trim, and organs 2) Poultry carcasses, parts, and organs	1) The level of use in water that yields a concentration no greater than 220 ppm peroxyacetic acid, a concentration of hydrogen peroxides no greater than 150 ppm, and a concentration of HEDP no greater than 13 ppm 2) The level of use in water that yields a concentration no greater than 220 ppm peroxyacetic acid, a concentration of hydrogen peroxides no greater than 110 ppm, and a concentration of HEDP no greater than 13 ppm
Sodium hypochlorite	Red meat carcasses down to a quarter of a carcass	Applied as a spray at a level not to exceed 20 ppm calculated as free available chlorine measured prior to application followed by a potable water rinse **NOTE**: In the case of pork carcasses, potable water rinse not required if pork carcasses held for a minimum of 12 hours prior to further processing
	On whole or eviscerated poultry carcasses prior to immersion in a pre-chiller or chiller tank	Not to exceed 50 ppm calculated as free available chlorine measured prior to application

2.3.1 Cetyl Pyridinium Chloride (CPC)

2.3.1.1 Introduction of CPC

CPC (Figure 2) is soluble in water as well as in chloroform. It is colourless and odourless compound with neutral pH, that has been used in oral hygiene products such as toothpaste, throat lozenges, and mouthwashes. CPC present in such products reduces bacterial attachment and ultimately inhibits plaque formation on tooth surfaces (Cutter et al., 2000; Renton-Harper et al., 1996).



Molecular formula: $C_{21}H_{38}ClN$

IUPAC name: 1-hexadecylpyridin-1-ium;chloride

Melting point: 80°C

Molecular weight: 339.992 g/mol

Other names: Pristacin; 1-Hexadecylpyridinium chloride;

Figure 2: Chemical structure of CPC (Source: PubChem)

CPC is active and stable over a broad range of temperatures and is tolerant to hard water (Cutter et al., 2000). At room temperature, the pure form of CPC is present in a solid state. The melting point of anhydrous CPC is 77°C and the melting point of its monohydrate form is between 80-83°C. It has a pyridine-like odour and it is combustible. The National Library of Medicine Toxicology Data Network (TOXNET) reviewed the toxicological study on CPC. It was reported that, general population may be exposed to CPC predominantly through ingestion or dermal contact due to its use in products like, mouthwashes, external

deodorants and cough lozenges. According to TOXNET, the substantial toxicity due to CPC is extremely rare after exposure to low concentration products that are usually present in the households mentioned above. The fatal dose in humans ingesting cationic detergents or QAC's (that includes CPC) has been estimated to be 1 to 3 g (TOXNET). Based on risk assessment of human health, the acceptable limit of CPC for an average adult (70kg in body weight) has been determined to be 4.4 mg/day (Bosilevac et al., 2004).

2.3.1.2 Mode of action

CPC is a quaternary ammonium compound and QAC's has ability to get adsorb to the bacterial cell surface and destroy the cell wall and cell membrane of bacteria and fungi. As a result, it shows direct or indirect lethal effect on the cell. They are positively charged polyatomic ions containing NR^{+4} group and R-group (an alkyl group or an aryl structure) (IUPAC, 1997).

The antimicrobial activity of CPC is due to an interaction of positively charged cetyl pyridinium ion with the negatively charged cell surfaces. This interaction subsequently inhibits bacterial metabolism by forming weak ionic compounds that interfere with bacterial respiration (Kim & Slavik, 1996; Cutter et al., 2000). CPCs have tendency to bind the phospholipids and proteins of the membrane irreversibly, disrupting the permeability of the microbial cell membrane, which also contributes to cause cell death. In addition, bacterial cells also have the tendency to absorb certain molecules, which influences the activity of such molecules towards bacterial cells (Daoud et al.,1983).

In case of gram positive bacteria like *L. monocytogenes*, cell membrane shows high affinity towards quaternary ammonium compounds such as CPC, due to its positive charged ion. As a result, the membrane gets disintegrated. However, it should be noted that, presence of organic matter can decrease the activity of QACs. Concentration of QACs, has an influence on its antimicrobial effect. At medium concentrations, QACs are effective against

gram-positive microorganisms, viruses, fungi, and algae. At low concentrations, QACs may show bacteriostatic effects (growth of microbes is inhibited but they are not necessarily killed) (Talaro & Talaro, 1993).

It was also reported that CPC showed higher bactericidal effects towards gram-positive bacteria than gram-negative bacteria. This could be because of differences among the types and contents of phospholipids present in cell membrane of these bacterial groups (Robinson, 1970).

2.3.1.3 Use of CPC in food industry

In United States, CPC has been approved to treat the surface of raw poultry carcasses prior to immersion in a chiller, however it has been not yet approved for use on beef carcasses (FDA 2003). Health Canada has also issued the letter of no objection (LONO) for CPC, to its use on poultry carcasses (Table 2). CPC treatment can be administered by dipping method or spraying method (Xiong et al., 1998).

CPC has ability to bind and precipitate acidic mucopolysaccharides that are found on chicken fascia. Hence, CPC treatment on fascia prevents the collagen fibres network formation and reduces the number of bacteria (Thomas & McMeekin, 1991). According to the study conducted in 2000, residual-CPC has proved to extend antimicrobial activity of CPC (Cutter et al., 2000). These results are supported by another study, which found that CPC-residual activity on poultry surfaces minimizes the cross contamination of *Salmonella* species between carcasses (Breen et al., 1997).

2.3.1.4 Factors influencing CPC treatment

CPC can be applied onto the food sample by using fine mist, spray, or a rinse. Some foods can be dipped into CPC solution. Research has shown that spray-washing of beef fat with 1% CPC solution immediately reduced the inoculated *E. coli O157:H7* and *S. typhimurium* to undetectable levels (Cutter et al., 2000). However according to another

research, when CPC treatment was given to food samples in the form of spray, low spray pressures do not significantly reduce the pathogen present on the food samples. The effectiveness of treatment was not enhanced even after increasing the spray pressure from 20 to 35 psi or increasing the spray temperature from 25 to 55^{o}C (Singh et al., 2005).

CPC spray treatments at concentrations of 2 mg/ml was used to kill *Salmonella* cells on chicken skin (Breen et al., 1997). This study however implied that increase in exposure time (from 1 to 3 minutes) of CPC spray treatment did not show any increase in log reduction of *Salmonella* populations on chicken skin (Breen et al., 1997). Studies also showed the effectiveness of CPC washes against *Salmonella typhimurium* on poultry. They observed that up to 2.5 log reduction in *S. typhimurium* at 0.5% concentration (Kim & Slavik, 1996).

Recent study evaluated three different temperatures (10, 35, and 60^{o}C) and five pressures (from 30 to 150 psi) with 0.1% CPC spray for reducing *Salmonella* population on chicken skin. They observed that, spray pressure had negligible effect on the treatment effectiveness at low temperatures. However, it greatly impacted treatment effectiveness at high temperatures (Wang et al., 2016). Hence, temperature was the influential factor during CPC spray treatments. Singh et al., 2005 observed formation of foam when CPC solutions were prepared at high temperatures and under pressure. The foam can have negative impact on decontamination of bacteria present on food surface, because foam hurdles the contact between the CPC solution and the food surface.

Furthermore, studies reported the use of CPC for removal of *L. monocytogenes* on fresh beef and fresh-cut vegetables was found that, CPC concentration had a huge impact on destruction of *L. monocytogenes.* The most effective concentration was 0.5% CPC and showed approximately a 3.25 log reduction in case of fresh beef and 3.70 log reduction in fresh-cut vegetables (Lim & Mustapha, 2004; Wang et al., 2001).

2.3.1.5 Impact of CPC treatment on quality of food

According to Safe Foods Corp., CPC does not show any adverse effects (like taste, smell, colour, texture) on treated food when it is applied with specific conditions mentioned in Table 3.

Sensory evaluation of beef steaks that were treated with 10 mg/ml CPC and then cooked did not show any unacceptable organoleptic properties like unacceptable flavour, colour and texture (Cutter et al., 2000). Further, Pohlman et al. (2002) used CPC on pork trimmings before grinding and found effective against *Listeria innocua* and aerobic plate counts (APC). In this study, CPC residue in ug/g pork was reported. The results indicated that CPC treatment maintained residual CPC which helped for extending microbial control. Singh et al., (2005) reported that surface treatment of RTE (ready to eat) polish sausages with 1% CPC did not show any adverse effect on its colour. Also, another study have reported no negative effect of CPC on the colour of ground beef produced from CPC-treated beef trimmings (Pohlman et al., 2002).

Study on frankfurter showed that, application of a 1% CPC surface spray on frankfurter prior to packaging reduced *L. monocytogenes* concentrations by 1.4 to 1.7 log CFU/g and it also inhibited growth of the pathogens during refrigerated storage for more than a month (Singh et al., 2005). They also studied the colour analysis of frankfurters after it was treated with 1% CPC. They analysed L* (lightness), a* (redness), and b* (yellowness) values of frankfurters treated by CPC. It was observed that, 1% CPC treatment did not affect the colour (L*, a*, and b* values) of frankfurters stored at 0^0C and 48^0C for 42 days. In addition, the firmness (texture) of the frankfurters was minorly affected by 1% CPC treatment. Similar study on roast beef observed significant ($P>0.05$) difference in lightness (L*), redness (a*), and yellowness (b*) values of roast beef (Singh et al., 2005).

2.4 Inactivation Kinetic Models

During last 15 years various models were developed using predictive microbiology to understand microbial growth in constant and variable environment. It is important to propose useful models to predict microbial decrease in constant and variable environment (Albert & Mafart, 2005).

In thermal and non-thermal inactivation of vegetative microorganisms, there are mainly four types of survival curves which observed as shown in Figure 3. Linear curves (curve A), curves with a shoulder (curve B), curves with a tailing or also called biphasic curves (curves C and D) and sigmoidal curves (curves E and F) (Xiong et al., 1999).

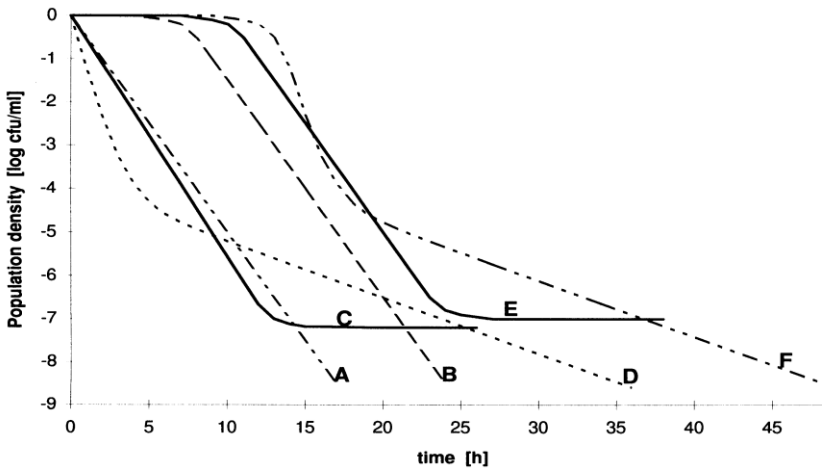


Figure 3: Graphic representations of six different shapes of survival fit all the six different shapes of survival curves (Xiong et al., 1999).

In case of non-thermal inactivation caused by unfavourable environmental conditions, the shape of surviving curves shows more distinct heterogeneity based on the intensity of a stress. Hence, the same bacterial strain can show different shapes of survival curves. When the intensity of the stress changes, often concave curves may become convex or sigmoidal (Buchanan et al., 1994; Glass et al., 2015; Koutsoumanis et al., 1999). The shape of survival curves also varies with other factors such as, the physiological condition of the vegetative cells, the growth phase (exponential or stationary phase) of bacterium. The survival curve

shape also depends on the conditions of adaptations before the application of stress (Lee et al., 1994; Phan-Thanh et al., 2000). A good fit (goodness of fit) of the inactivation curve is important to get good estimates of the model parameters. It also helps to obtain reliable predictions. For example, reliable decontamination times (Albert & Mafart, 2005).

Many primary models have been proposed to model non-thermal inactivation curves. Some models have tendency to describe both growth and the inactivation (Takumi et al., 2000). Some models can fit non-log linear decrease or sigmoidal inactivation curves. The Weibull model was largely used in thermal and non-thermal treatment. It is based on the hypothesis that the resistance to stress of a population follows a Weibull distribution (Peleg & Cole, 1998; van Boekel, 2002). This type of model can describe linear, concave or convex curves (Albert & Mafart, 2005). During the past few decades, the Weibull model has been used to describe bacterial resistance to thermal stress during thermal and also in non-thermal treatment (Peleg & Cole, 1998; van Boekel, 2002).

The inactivation kinetics of the bacterial population decrease (CFU/ml) versus time (sec) is described by the following model:

Weibull equation: $$\boldsymbol{log_{10}}\left(\frac{N}{N_0}\right) = -\left(\frac{1}{2{\cdot}303}\right)\left(\frac{t}{a}\right)^{\beta}$$

where N is the number of survivors, No is the inoculum size, t the time, α is a shape parameter and β is the treatment time for the one decimal reduction.

PREFACE TO CHAPTER 3

Microbial food borne illness is caused by pathogens that enter human body through contaminated food or water. Such pathogens are known as food-borne pathogens, and examples are *Listeria, Salmonella, E. coli, Clostridium* etc. Among these, study on *Listeria monocytogenes* is particularly important due to few reasons. Firstly, it is a psychrophile; means it can grow at low temperatures -5^{o}C to 9.3^{o}C. Hence, it can survive and grow on foods stored in the refrigerator. In addition, foods contaminated with *Listeria* may look, smell and taste normal, hence you cannot see or feel their growth or spoilage. In addition, every year, hundreds of people die due to *L. monocytogenes* infections all over the world. Therefore, it is important to emphasise on effective strategies that can help to inactivate *L. monocytogenes.* One such strategy has been through the use of chemicals. CPC, a quaternary ammonium compound, is one such chemical that has been permitted for use against bacterial pathogens. The focus of Chapter 3 is to evaluate the effect of selected concentrations of CPC directly on the cell suspension of *L. monocytogenes*. A cocktail of three different serotypes of *L. monocytogenes* were used in this study to mimic a more practical approach, as in nature there are multiple strains and serotypes of this pathogen prevalent. The objective of this chapter was to evaluate the effect of different concentrations of CPC by keeping treatment time constant against *L. monocytogenes* cocktail suspension maintained at two temperatures 23^{o}C and 1^{o}C.

All experimental work and data analysis were conducted by the candidate under supervision of Dr. H. S. Ramaswamy. A part of this research was presented in the form of poster at NABEC conference July 2018:

Pawar, K., Ramaswamy, H.S., Goodridge, L. and Cadieux, B., 2018. Effect of CPC on *L. monocytogenes* suspension and on food surfaces inoculated with *L. monocytogenes.* A poster was presented at NABEC conference July 2018

CHAPTER 3

EFFECT OF DIFFERENT CONCENTRATIONS OF CETYLPYRIDINIUM CHLORIDE (CPC) ON INACTIVATION OF *LISTIRIA MONOCYTOGENES* AT TWO TEMPERATURES

3.1 Abstract

The purpose of this study was to evaluate the effect of cetyl pyridinium chloride (CPC) on *L. monocytogenes*. The inactivation effect was evaluated at 23ºC (room temperature, RT) and 1ºC (chilling temperature, CT). The bacterial cocktail was prepared by combining three serotypes (390-1 1/2a, 24-1/2b, 42-4b) of *L. monocytogenes.* A constant treatment time of was applied on the cocktail (cell suspension) for 15 s in treatment solutions with different concentrations of CPC [w/v] between 2 to 10 ppm (0.0002% to 0.001%) at RT and between 2 to 25 ppm (0.0002% to 0.0025%) at CT. Then the suspension was serially diluted and spread plated on-to tryptic soya agar (TSA). Results showed that the 15 s treatment resulted in complete inactivation of *L. monocytogenes* in 10 ppm CTC at RT and 25 ppm at CT. A 10 ppm CPC at room temperature resulted in 6 $\log_{10}$ cycles reduction in *L. monocytogenes* at RT while it only resulted in 2 $\log_{10}$ reduction at CT. Statistical analysis using ANOVA showed significant difference ($P<0.05$) between all the CPC concentrations used for the treatments. Also, a significant difference ($P<0.05$) was observed in inactivation at 10 ppm CPC treatment between 23ºC and 1ºC.

3.2 Introduction

Cetyl-pyridinium chloride (CPC) has antimicrobial properties and it is active against many bacteria such *as Escherichia coli O157:H7, coliforms, Salmonella typhimurium, Campylobacter jejuni, Aeromonas hydrophila, Listeria monocytogenes,* and *Staphylococcus aureus* as well as viruses (Pohlman et al., 2002). CPC is a cationic surface-active agent; hence it has tendency to absorb phosphate ions present in negatively charged bacterial cell

membranes. This reaction interferes in bacterial respiration that ultimately causes cell wall disruption and cell death (Radford et al., 1997). This study was focussed on evaluating the antimicrobial effect of CPC at two different temperatures against a cocktail of three strains of *Listeria monocytogenes* cocktail.

3.3 Materials and Methods

3.3.1 Cultivation and Laboratory Maintenance of *L. monocytogenes* strains

3.3.1.1 Growth of *L. monocytogenes* on Tryptic Soy Agar

For this study, a cocktail of three strains of *L. monocytogenes* (390-1 1/2a, 24-1/2b, 42-4b) were used, which were previously associated with *L. monocytogenes* foodborne outbreaks. *L. monocytogenes* 390-1 1/2a was obtained from the outbreak in Colorado that was isolated from Cantaloupe. *L. monocytogenes* 42-4b and *L. monocytogenes* 24-1/2b were involved in outbreak in Callaway and these strains were isolated from cow and cheese, respectively. All these strains were received from Health Canada.

These strains were activated by transferring a loopful of individual culture on to tryptic soy agar (TSA; Criterion, Hardy diagnostics, USA). Sterile inoculation loop was used to scrape a small quantity of frozen stock and each strain was streaked on to individual TSA plates to obtain isolated colonies. TSA plates were incubated for 24 h at 37ºC. After the incubation period, isolated colonies of *L. monocytogenes* appeared creamy white in colour (as shown in Figure 4) that measured around ~1 mm in diameter with dome-shaped elevation.

Figure 4: Individual colonies of *L. monocytogenes* 390 1 1/2a grown on TSA plate and incubated at 37ºC for 48 h

3.3.1.2. Growth of *Listeria monocytogenes* on Tryptic Soy Broth (TSB)

Single isolated colonies of each strain were picked with a sterile loop from the TSA plate and inoculated separately into three different culture tubes containing 5 m\l tryptic soy broth (TSB; BD Difco, Germany). Then TSB tubes were incubated in rotary shaker incubator at 37ºC for 24 h at 120 rpm speed. After incubation, microbial cells grown in TSB broth (5 ml) were centrifuged at 10000 x g (IEC-Centra® CL2, USA) for 5 min at room temperature and cell pellets were obtained. The cell pellets were re-suspended in 5 mL sterile 0.85% saline and centrifuged again. This washing step was repeated twice, and further serial dilution of each suspension was performed separately. The cell count of each suspension was confirmed by plating 0.1 ml of appropriately diluted suspensions (10^{-4}, 10^{-5} and 10^{-6}) onto TSA plates with the help of sterile spreader. It is important to note that, the content in each microtube was uniformly mixed by vortex mixing (Fisher scientific, USA) before plating. TSA plates were incubated at 37ºC for 24 h in an incubator (Gravity convention incubator, Precision Scientific, Inc).

3.3.1.3 Preparation of a glycerol stock of *L. monocytogenes* (Long term storage*)*

Laboratory stocks of *L. monocytogenes* can be stored indefinitely in 20% glycerol at -40°C. Hence, glycerol stocks were prepared in 1:1 proportion by transferring 500 µL of *L. monocytogenes* suspension (grown in TSB for 24 h) into a sterile cryogenic vial (Fisher Scientific, Denmark) containing 500 µL of 40% glycerol. Therefore, the final concentration of glycerol becomes 20% (v/v). The content was mixed and stored at – 40 °C.

3.3.2 Microbial enumeration and preparation of *L. monocytogenes* cocktail

L. monocytogenes colonies grown on TSA plates (10^{-4}, 10^{-5} and 10^{-6}) were observed to select a plate that contains countable number of colonies (the number of colonies must range between 30-300). The number of counted colonies were recorded for each strain and colony forming units (CFU) per ml were calculated by using following Equation 1.

$$\text{CFU/ml} = \frac{\text{Number of colonies X dilution factor}}{\text{amount of suspension plated in ml}} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(1)$$

The culture of three strains were adjusted to make a final cell concentration of approximately $3x10^7$ CFU/ml. Each strain was mixed to prepare the *L. monocytogenes* cocktail and it was used in the following experiments. The cell count (Table 4) of the cocktail was confirmed by plating 0.1 ml of diluted cocktail on to TSA plates and incubating the plates at 37ºC for 24 h (Figure 5). For each experiment, a fresh cocktail was prepared, and the glycerol stocks were used to grow cells each time.

Table 4: CFU calculations of *L. monocytogenes* strains used to prepare cocktail of approximately 3 x10^7 CFU/ml

Strains of *L. monocytogenes*	Mean of number of colonies	Dilution factor	Amount of suspension plated (ml)	CFU/ml
390-1 1/2a	221	10^{-6}	0.1	2.21 x 10^9
42- 4b	215	10^{-6}	0.1	2.15 x 10^9
24- 1/2b	240	10^{-6}	0.1	2.40 x 10^9

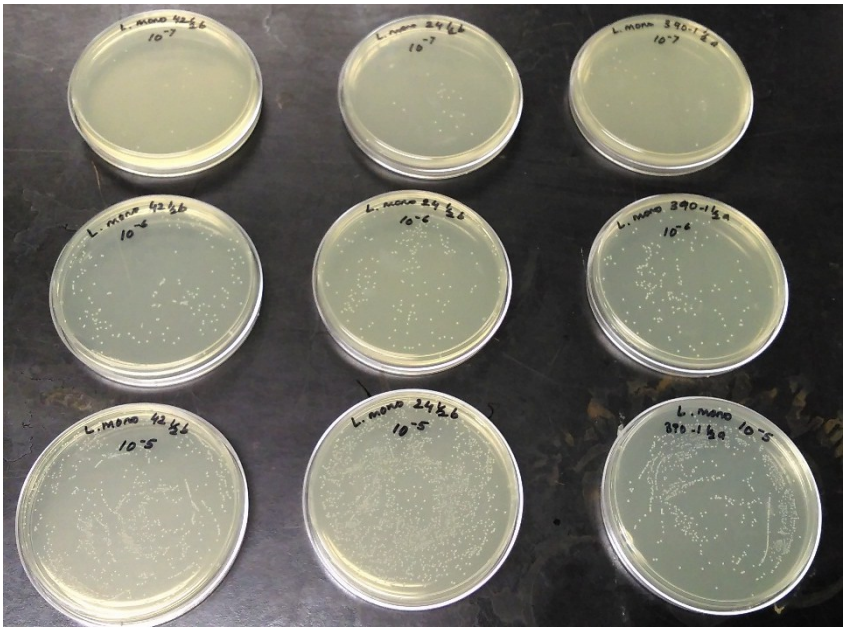


Figure 5: TSA plates containing *L. monocytogenes* colonies. The countable number of colonies (between 30-300) of each strain was observed onto 10^{-6} dilution plate. Hence, these plates were selected to calculate CFU/ml.

3.3.3 CPC (Cetyl pyridinium chloride) treatment

CPC (Alfa-Aesar, England) also known as (1-Hexadecyl) pyridinium chloride monohydrate 98% with molecular formula $C_{12}H_{38}ClN.H_2O$ was used for this study.

3.3.4 Preparation of CPC stock solution

A 1% CPC stock solution was prepared by dissolving 0.1 g CPC in 10 ml distilled water. This solution was filtered sterilized by passing through the 32 mm syringe filter with 0.8/0.2 µm Supor membrane filter (Fisher scientific, Ireland) with the help of a syringe. Further, the required concentrations of CPC (between 2 ppm to 25 ppm) were prepared by diluting the stock with appropriate volume of sterile water (the volume required to add was calculated by following formula: $C_1V_1 = C_2V_2$).

3.3.5 Different concentrations of CPC treatment on *L. monocytogenes* cocktail at RT

In this experiment CPC exposure time (15 s) was kept constant for all five tubes and the concentration of CPC was varied (2, 4, 6, 8 and 10 ppm) at RT. One tube was kept as

control (without adding CPC, 0 ppm). The entire experiment was done at room temperature inside a Bio-Safety cabinet (Class II A/B3 Biological safety cabinet, USA).

1 ml reaction mixture was prepared by mixing sterile CPC solution and *L. monocytogenes* cocktail in a microfuge tube. $C_1V_1 = C_2V_2$ formula was used for making dilution scheme and adjusting final concentrations of CPC and cocktail. For instance, 1 ml of reaction mixture was prepared by mixing 10 µl of 1% CPC stock solution into 990 µl of *L. monocytogenes* cocktail, to get 10 ppm CPC final concentration with 3 x 10^7 CFU/ml of *L. monocytogenes* cells.

Firstly, the cocktail (3 x 10^7 CFU/ml) was added into all microtubes. Then 10 µl CPC stock solution was added into first tube (10 ppm) using a micropipette. As soon as the CPC was added, the contents were mixed by using vortex mixing (Fisher scientific, USA) and the tube was kept aside for 15 s treatment time. Then immediately the content was mixed again and 100 µL content was taken out and followed by serial dilution (10^{-1} to 10^{-5}). All the dilutions were plated onto TSA plates along with undiluted tube (10^0). Similarly, other CPC treatments were carried out. 'Control tube' was prepared without adding CPC. All the plates were incubated at 37^{o}C for 24 h. All experiments were done in triplicates and the mean log CFU/ml were calculated (Appendix I).

3.3.6 CPC treatment on *L. monocytogenes* cocktail at different concentrations at 1^{o}C

In this experiment CPC, again the exposure time (15 sec) was kept constant for all five tubes and the concentration of CPC was varied (10, 15, 20 and 25 ppm). (Below 10 ppm CPC, it showed less than 1 log reduction at CT, hence the concentration range was increased in the experiments carried out at CT). One tube was kept as control (without adding CPC). The entire experiment was done at 1^{o}C temperature under BSC (Class II A/B3 Biological safety cabinet, USA). The temperature of all required solutions was maintained at 1^{o}C by keeping all the tubes and solutions on ice bath. The temperature was monitored using a

thermometer dipped into one tube containing reaction mixture that was placed onto the ice bath.

Firstly, the cocktail (3 x 10^7 CFU/ml) was added into all microtubes placed in the ice bath. Then 10 µl CPC stock solution was added into first tube (10 ppm) using a micropipette. As soon as the CPC was added, the tube was mixed uniformly by using vortex mixing and it was kept again in ice bath for 15 s treatment time. After treatment time, 100 µl content was taken out and followed serial dilution (10^{-1} to 10^{-5}). All dilutions were plated onto TSA plates along with undiluted tube (10^0). Similarly, other CPC treatments (10, 15, 20, 25 ppm) were given. 'Control' tube was prepared without adding CPC. All the plates were incubated at 37ºC for 24 h. All experiments were done in triplicates and the mean log CFU/ml were calculated (Appendix II).

3.3.7 Statistical analysis

After incubation times, the colonies on TSA plates were counted and CFU's were calculated. The results were presented in the form of graphs and data was analysed statistically by using GraphPad prism version 7 (student version). Analysis of Variance (ANOVA) was used to find out the significant difference between the obtained results.

3.4 Results and Discussion

Reduction in *L. monocytogenes* population after treating with various concentration of CPC was evaluated at two different temperatures (23ºC and 1ºC). One-way ANOVA of means $\log_{10}$ CFU/ ml of *L. monocytogenes* recovered after CPC treatments given at room temperature and grown on TSA showed significant difference ($P<0.05$). Further, the Brown-Forsythe statistical test was also applied to the standard deviation observed during various CPC treatments at room temperature. Results showed no significant difference ($P<0.05$)

among SDs of log_{10} CFU/ ml of *L. monocytogenes* at room temperature with a *P*-value of 0.4211.

Results showed that 2 ppm CPC gave <1 log reduction. Comparison of the means indicated no significant difference in the log reduction level between control (non-treated) and 2 ppm CPC treatment, whereas all the remaining CPC concentrations were significantly different (P<0.05) from each other at room temperature. At 4, 6, 8 ppm CPC around 1 log_{10} CFU/ml reduction, 1.5 log_{10} CFU/ml reductions and 3.2 log_{10} CFU/ml reductions were observed respectively (Figure 6). At 10 ppm CPC >6 log_{10} CFU/ml reduction of *L. monocytogenes*.

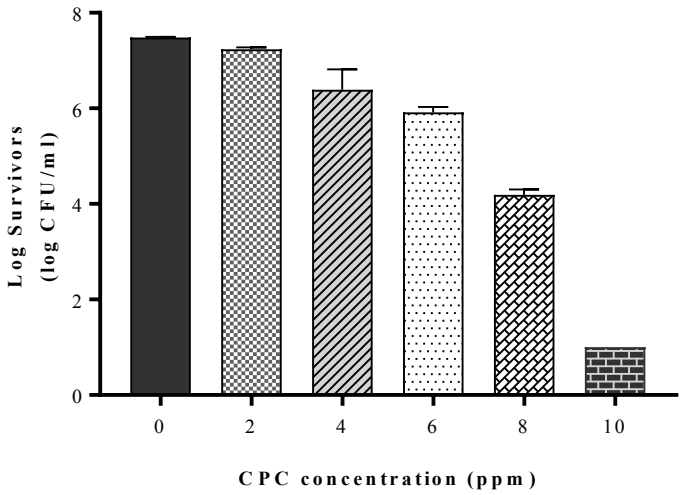


Figure 6: *L. monocytogenes* recovery on TSA after treatment with 2 to 10 ppm CPC at room temperature (23°C). Error bars represents Mean±Std.dev. values.

Table 5: One-way ANOVA analysis of antimicrobial effect of CPC concentration on destruction of *Listeria monocytogenes* cocktail suspension at RT (23°C).

One-way ANOVA summary	
F	514.7
P value	<0.0001
P value summary	****
Significant diff. among means ($P < 0.05$)?	Yes
R square	0.9954
Brown-Forsythe test	

F (DFn, DFd)	1.075 (5, 12)
P value	0.4211
Are SDs significantly different (*P* < 0.05)?	No

ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	***P* value**
Treatment (between treatments)	89.39	5	17.88	F (5, 12) = 514.7	P<0.0001
Residual (within treatments)	0.4168	12	0.03474		
Total	89.81	17			

Note: *P*≤0.05- significant; SS (sum-of-squares value), DF (degrees of freedom), MS (mean squares), SD (significant difference).

Similarly, one-way ANOVA was applied to means $\log_{10}$ CFU/ml of *L. monocytogenes* recovered after CPC treatments at 1ºC showed significant difference (*P*<0.05). Brown-Forsythe test also showed significant difference among SDs of $\log_{10}$ CFU/ ml of *L. monocytogenes* at 1ºC with *P*- value <0.0001 (Table 6).

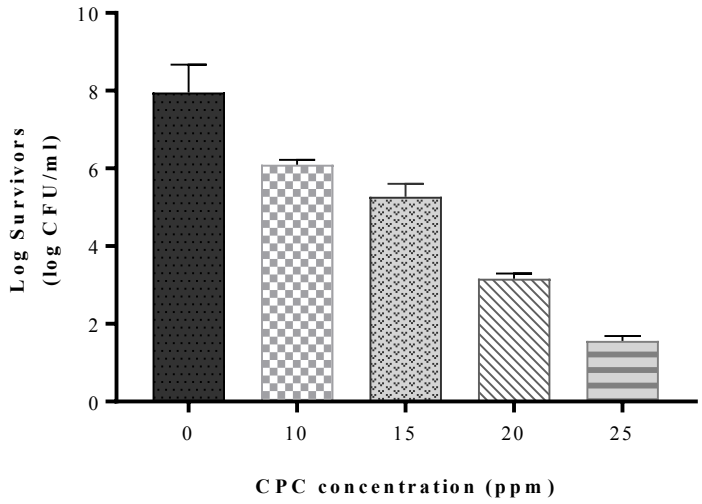


Figure 7: *L. monocytogenes* recovery on TSA after treatment with 10 to 25 ppm CPC at 1ºC. Error bars represents Mean±Std dev. values.

Table 6: One-way ANOVA analysis of antimicrobial effect of CPC concentration on destruction of *Listeria monocytogenes* cocktail suspension at CT (1°C)

ANOVA summary					
F	92.84				
P value	<0.0001				
P value summary	****				
Significant diff. among means ($P < 0.05$)?	Yes				
R^2	0.9867				
Brown-Forsythe test					
F (DFn, DFd)	8.372e+029 (4, 5)				
P value	<0.0001				
P value summary	****				
Are SDs significantly different ($P < 0.05$)?	Yes				
ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	***P* value**
Treatment (between treatments)	50.03	4	12.51	F (4, 5) = 92.84	P<0.0001
Residual (within treatments)	0.6736	5	0.1347		
Total	50.71	9			

Note: **** represents $P \leq 0.0001$

All the CPC concentrations (10, 15, 20 and 25 ppm) were significantly different (P<0.05) from each other at 1°C by showing 1.8 $\log_{10}$ CFU/ml reduction, 2.6 $\log_{10}$ CFU/ml reduction, 4.7 $\log_{10}$ CFU/ml reduction and CPC >6 $\log_{10}$ CFU/ml reduction of *L. monocytogenes* (Figure 7). Below 10 ppm CPC concentration, the treatment up to 15 s was ineffective and it did not show any reduction in *L. monocytogenes* as shown in Figure 8.

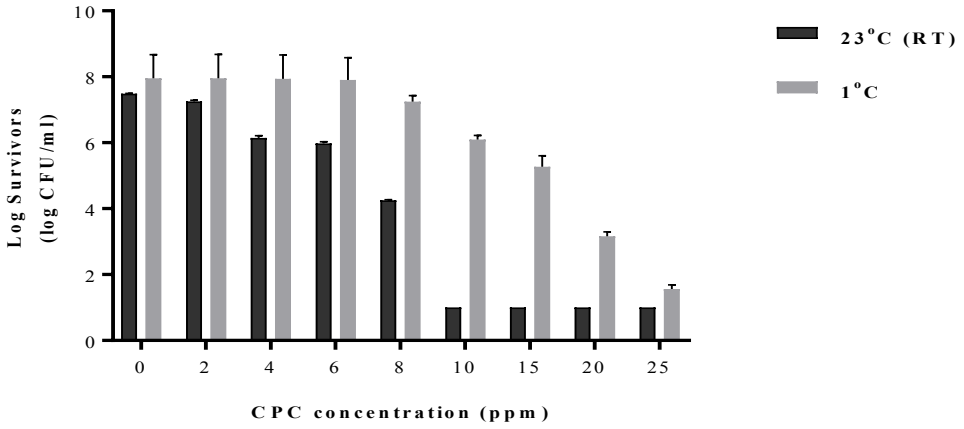


Figure 8: Comparison between log reduction observed after different CPC concentration treatments at 23°C and 1°C. Error bars represents Mean±Std dev. values.

Table 7: Two-way ANOVA analysis of antimicrobial effect of CPC concentration on destruction of *Listeria monocytogenes* cocktail suspension at 23ºC and 1ºC

ANOVA table	SS	DF	MS	F (DFn, DFd)	*P* value
Interaction	21.47	8	2.684	F (8, 18) = 21.8	P<0.0001
CPC concentration	202.4	8	25.3	F (8, 18) = 205.5	P<0.0001
Temperature	44.3	1	44.3	F (1, 18) = 359.8	P<0.0001

Source of Variation	% of total variation	*P* value	*P* value summary	Significant?
Interaction	7.94	<0.0001	****	Yes
CPC concentration	74.86	<0.0001	****	Yes
Temperature	16.38	<0.0001	****	Yes

Note: **** represents $P \leq 0.0001$

Further, reduction in *L. monocytogenes* was compared at both temperatures by applying two-way ANOVA (Figure 8). From results it was observed that temperatures (1ºC and 23ºC) during the CPC treatment and CPC concentration had a significant effect ($P<0.05$) on the recovery of *L. monocytogenes* present in suspension (Table 7). At room temperature, 10 ppm CPC gave >6 $\log_{10}$ CFU/ml reduction, whereas at 1ºC the same amount of CPC showed only 1.8 $\log_{10}$ CFU/ml reduction. To observe >6 $\log_{10}$ CFU/ml reduction of *L. monocytogenes* at 1ºC, the CPC concentration had to be increased up to 25 ppm. It is important to note that the lethal effect of CPC on *L. monocytogenes* populations only increased by 1.8 log after 10 ppm CPC at 1ºC; whereas the reduction increased more than 3 and 6 log after 8 ppm and 10 ppm CPC, respectively, at room temperature (23ºC).

Further, the reduction at CPC concentration (10 ppm) was compared at two different temperatures. Unpaired *t*-test of *L. monocytogenes* populations treated with 10 ppm CPC at 23ºC and 1ºC indicated a significant difference ($P<0.05$) by showing >6 log reduction at 23ºC and 1.8 log reduction at 1ºC (Figure 9).

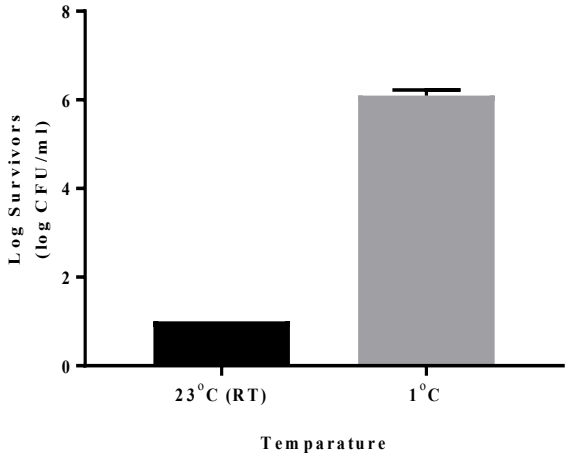


Figure 9: Unpaired *t*-test between 10 ppm CPC treatment at two different temperatures (23°C and 1°C)

Table 8: Unpaired *t*-test to compare the effect of temperature (23°C and 1°C) on CPC treatments against *L. monocytogenes* cocktail suspension

Parameters Analysed	
Column B	1°C
vs.	vs.
Column A	23°C (RT)
Unpaired *t*-test	
P value	0.0003
P value summary	***
Significantly different ($P < 0.05$)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=57.89 df=2
How big is the difference?	
Mean ± SEM of column A	1 ± 0, n=2
Mean ± SEM of column B	6.097 ± 0.08805, n=2
Difference between means	5.097 ± 0.08805
95% confidence interval	4.718 to 5.475
R^2	0.9994

Note: *** represents $P \leq 0.001$

Above all results showed that, number of surviving cells observed at the two temperatures (RT and CT) were different. Temperature has a direct impact on the growth rate of bacteria, its enzyme activity, cell composition, and nutritional requirements. Temperature can also alter the solubility of solute molecules, ion transport and diffusion, osmotic effects

on membranes, surface tension, and density (Herbert 1986). In our study, when CPC stock solution was kept on ice bath, it appeared as a viscous white precipitate liquid. Hence, temperature and solubility of CPC could be the factors for getting higher log reduction at room temperature and less log reduction at chilling temperature. Hence, our study (Figure 8) showed that, temperature is a key factor during chemical interaction with bacterial cell.

Temperature can influence the membrane ion transport system in bacterial cells. Membrane lipid bilayer is the protective layer of bacterial cell and its function mainly depends on its fluidity. Hence the lipid bilayer membrane must be in fluid state to facilitate ion transport (Berry & Foegeding, 1997). Therefore, when the growth temperature of a microorganism is reduced, some of the normally fluid components become gel-like, which prevents the ions transport (Beales, 2004). Some microorganisms also alter their cell membrane fatty acid composition after they sense reduction in temperature, pH, or water activity (aw). (Russell et al., 1995). In addition, according to Berry & Foegeding's study in 1997, low temperature can cause alterations in phospholipids and fatty acid of bacterial outer membrane. This can cause direct or indirect effect on CPC interaction with cell membrane and ANOVA results indicated that bacterial reductions achieved by CPC treatments at 23^{o}C were significantly increased (P<0.05) with increasing treatment time, and significantly greater than those of observed at 1^{o}C (P<0.05). According to study done by Herbert (1986), some enzymes like membrane permeases (membrane transport protein) of psychrophiles are less sensitive to low-temperature inactivation. As *L. monocytogenes* is a psychrophile, its membrane permeases might have disturbed the interaction with CPC, as a result we observed less log reduction at chilling temperature. However, all these could be the possible reasons based on previous findings, but it is important to study the morphology of *L. monocytogenes* to confirm the exact changes that occur at cellular level due to CPC.

Thongbai et al. (2006) studied, the effect of temperature following CPC-nisin treatment on a gram-negative bacterium *Salmonella typhimurium.* They investigated the morphological changes of treated cells using scanning electron microscopy (SEM). They first gave temperature stress by chilling, the cell suspensions of *S. typhimurium* in an ice water-bath (~0.5ºC) for 30 min and then treated with CPC-nisin. They found no difference in the viability of chill-stressed cells and untreated cells (control). This might indicate that chilling stress did not induce any change in the outer membrane of gram negative bacteria which agrees with (Boziaris & Adams, 2001) study that says, chilling did not cause a serious damage to outer membrane of *P. aeruginosa, S. enteritidis PT4* and *S. enteritidis PT7*. However, this study does not confirm the same effect on gram positive bacteria, which lacks outer membrane made up of lipopolysaccharide. Hence, gram positive bacteria like *L. monocytogenes* might act differently to such combined subsequent stress conditions.

In our experiments, morphological changes in *Listeria* cells occur during CPC treatment were not studied. But, study conducted by Thongbai et al. (2006) used SEM image profiling to observe changes in shape and morphology of CPC treated *S. typhi* cells. They also observed epi-fluorescent micrographs, which indicated that nisin-CPC damaged the cell membrane of *S. typhi* by forming pores, that caused leakage of cell materials. Therefore, in our study without any morphological analysis of cells, it is difficult to state the exact reason for difference in number of cell inactivation at two temperatures after CPC treatment.

3.5 Conclusion

The results indicated that, when *L. monocytogenes* cells were present in suspension, 10 ppm CPC was sufficient to give > 6 log reductions at room temperature. But when the same experiment was carried out at chilling temperature, 10 ppm CPC gave ~2 log reductions. Hence according to these results, treatment temperature is the main influential factor in CPC antimicrobial activity.

The overall objective of this study was also to examine, how *L. monocytogenes* reacts to CPC in free state (in bacterial suspension form) and in adhered state (inoculated on food surfaces). Results of this chapter showed that, small CPC is quite effective to inactivate the cells present in free state. 25 ppm at 1^{o}C and 10 ppm CPC at room temperature (23^{o}C) were sufficient to get 5 to 6 log reductions. However, the scenario could be different with the real food inoculated with *L. monocytogenes* which is explored in Chapter 5. Microorganisms that have adhered to food or food-processing equipment have been shown to have more resistance to sanitizing chemicals than free-floating cells (Fatemi & Frank, 1999; Somers et al., 1994). In addition, the antimicrobial compounds that are highly effective in liquid media may not be very active in complex foodstuffs (Norwood & Gilmour, 2000).

PREFACE TO CHAPTER 4

In the previous chapter, the effect of concentration of CPC was evaluated at against *L. monocytogenes* cocktail suspension at two temperatures but at a constant treatment time. This chapter focuses on the study of the effect of CPC treatment time at two temperatures (RT and CT) at a constant concentration against *L. monocytogenes* cocktail suspension.

The combined results of Chapter 3 and Chapter 4 helps to determine the CPC concentration and treatment time required to get complete inactivation of *L. monocytogenes* when present in suspension form at RT (23^{o}C) and CT (1^{o}C). These results are important for designing a CPC treatment protocol for treatment application.

All the experimental work and data analysis were conducted by the candidate under supervision of Dr. H. S. Ramaswamy. A poster from this chapter was prepared for NABEC conference, July 2018.

Pawar, K. and Ramaswamy, H.S., 2018. Effect of CPC on *L. monocytogenes* suspension and on food surfaces inoculated with *L. monocytogenes.*

CHAPTER 4

THE EFFECT OF VARYING CPC TREATMENT TIMES ON INACTIVATION OF *L. MONOCYTOGENES* AT ROOM TEMPERATURE (23°C) AND CHILLING TEMPERATURE (1°C)

4.1 Abstract

In this study the effect of increased treatment time of CPC was studied on inactivation of *L. monocytogenes* cocktail at room temperature (RT, 23°C) and chilling temperature (CT, 1°C). The cocktail was prepared by combining three serotypes (390-1 1/2a, 24-1/2b, 42-4b) of *L. monocytogenes*. The cocktail (cell suspension) was treated with CPC for various treatment times (from 15 to 180 s) by keeping CPC concentration at 6 ppm at 23°C or 10 ppm 1°C at the initial and resulting concentration of viable cells were enumerated to establish the extent of kill. Results showed that, 180 s treatment time was sufficient to give complete inactivation of *L. monocytogenes* at RT but not at CT. Statistical analysis showed significant difference (P<0.05) between the CPC treatment times at RT and CT. Also, a significant difference (P<0.05) was observed in inactivation of *L. monocytogenes* at 180 s treatment time between RT and CT. The results indicated that, CPC treatment is more effective at RT than CT on *L. monocytogenes* cocktail suspension.

4.2 Introduction

Food pathogens like, *L. monocytogenes* can survive various adverse conditions. For instance, food-processing and sanitizing treatments in industrial facilities may not be adequate to eliminate *L. monocytogenes* completely. It was also reported that exposure of *L. monocytogenes* to harsh treatment by using chemicals can result in survival of the organism in subsequent treatments (Frank & Koffi, 1990). In the previous chapter, the effect of CPC destruction of *L. monocytogenes* at two temperatures was evaluated as a function of CPC concentration. The focus of this chapter is the evaluation of the effect of CPC treatment time

at two temperatures. Evaluation of both concentration and time effect is necessary for the design of effective CPC treatment.

4.3 Materials and Methods

4.3.1 Preparation of *L. monocytogenes* Cocktail

L. monocytogenes strains were grown in TSB for 24 h and cells were enumerated. The cocktail was prepared by mixing appropriate volume of each strain. The detailed procedure was explained in Chapter 3. Same procedure was followed to prepare a cocktail containing $3x10^7$ CFU/ml.

4.3.2 CPC treatments

4.3.2.1 Preparation of CPC stock solution

Same procedure was followed as explained in Chapter 3.

4.3.2.2 Different treatment times of CPC on *L. monocytogenes* cocktail at RT

In this experiment, the concentration of CPC (6 ppm) was kept constant in all tubes and exposure time of CPC was varied (15, 30, 60, 120 and 180 sec). One tube was kept as control (without adding CPC). The experiment was done first at room temperature within a bio-safety cabinet (BSC). To each tube 994 µl cocktail ($3x10^7$ CFU/ml) was added. Then 6 µl of 1% CPC stock solution was added in to the first tube then the content was mixed. This tube was kept aside for 15 s and after completion of exposure time, the content in the tube was mixed again and serial dilutions (between 10^{-1} to 10^{-5}) were followed immediately. From each dilution tube, 100 µl content was plated onto TSA including undiluted tube. All plates were incubated at 37ºC for 24 h in an incubator (Gravity convention incubator, Precision scientific, Inc.). Then, colonies on each TSA were counted, and CFU/ml was calculated. The treated plates were compared with control to find out the log reduction after each exposure time.

4.3.2.3 Different exposure times of CPC on *L. monocytogenes* cocktail at CT

In this experiment the concentration of CPC (10 ppm) was kept constant in all tubes and exposure time of CPC was varied (15, 30, 60, 120 and 180 s). One tube was kept as control (without adding CPC). The experiment was done under BSC (Class II A/B3 Biological safety cabinet, USA) at 1ºC temperature by placing all tubes, solutions and other required contents on ice bath.

Initially, the calculated amount cocktail ($3x10^7$ CFU/ml) was added into all microfuge tubes. Then 10 µl CPC stock solution (1%) was added into first tube (10 ppm) by micropipette. As soon as the CPC was added, the tube was mixed uniformly using vortex mixing (Fisher scientific, USA) and the tube was kept aside for 15 s treatment time. Then immediately the content was mixed again and 100 µl content was taken out to follow serial dilution (10^{-1} to 10^{-5}). All the dilutions were plated onto TSA along with undiluted tube (10^0). Similar procedure was followed for other tubes except the exposure time was increased to 30 s for second tube, 60 s for third tube, 120 s for fourth tube and 180 s for fifth tube. All experiments were done in triplicates. All plates were kept at 37ºC for 24 h in an incubator (Gravity convention incubator, Precision scientific, Inc.).

4.3.3 Statistical analysis

After incubation, the colonies on TSA plates were counted and CFU were calculated. The results were represented in the form of figures and data were analysed statistically by using GraphPad prism version 7 (student version). Analysis of Variance (ANOVA) was applied to find out the significant difference between the obtained results.

4.4 Results and Discussion

L. monocytogenes cells were recovered after treating with constant concentration of CPC for different treatment times and cells were enumerated as CFU/ml. One-way ANOVA of the mean $\log_{10}$ CFU/ml of *L. monocytogenes* recovered at 23ºC after all treatments showed

significant differences (P<0.05). Brown-Forsythe test also confirmed the results by showing significant difference with P-value <0.0001 (Table 9). Increasing the CPC treatment time from 15 to 180 s resulted in greater (P>0.05) destruction of *L. monocytogenes*; with 1.4, 5.6 and >6 log reduction, respectively, after 15, 120 and 180 s at 23ºC (Figure 10).

Similarly, One-way ANOVA of the mean $\log_{10}$ CFU/g of *L. monocytogenes* cells recovered at 1ºC after different treatments times showed significant differences (Table 10). The reduction observed were 3.7 log at 1ºC after 180 s treatment time. In this study, treatments times 15, 30, 60 and 120 s were also significantly different (P<0.05) although the log reduction was increased only from 1.2 to 2.5 (Figure 11).

Figure 12 shows that temperatures (23ºC and 1ºC) of CPC treatment and treatment time together had a significant effect (P<0.05) on the recovery of *L. monocytogenes* present in suspension. Exposure times 60, 120 and 180 s were also significantly different (P<0.05) at both temperatures (Table 11).

The highest log reductions of >6 and 3.7 in *L. monocytogenes* populations were observed after 180 s at room temperature and chilling temperature, respectively. Hence, it can be concluded that, treatment temperature has a huge impact on these treatments when *L. monocytogenes* cells are used in suspension form as explained earlier in Chapter 3.

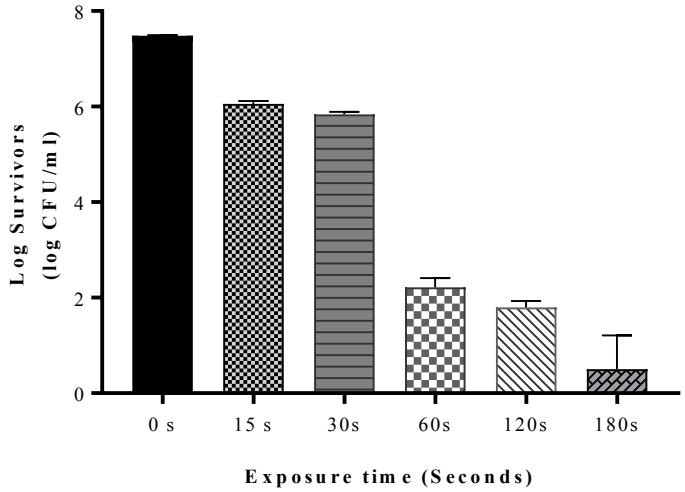


Figure 10: Log survivors (Log_{10} CFU/ml) of *L. monocytogenes* recovered on TSA after different treatment times at 23°C

Table 9: One-way ANOVA analysis of antimicrobial effect of CPC treatment time on destruction of *Listeria monocytogenes* cocktail suspension at 23°C

ANOVA summary	
F	170.5
P value	<0.0001
P value summary	****
Significant diff. among means ($P < 0.05$)?	Yes
R square	0.993
Brown-Forsythe test	
F (DFn, DFd)	9.603e+029 (5, 6)
P value	<0.0001
P value summary	****
Are SDs significantly different ($P < 0.05$)?	Yes

ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	***P*-value**
Treatment (between treatments)	79.99	5	16	F (5, 6) = 170.5	0.0001
Residual (within treatments)	0.5631	6	0.09384		
Total	80.55	11			

Note: **** represents $P \leq 0.0001$

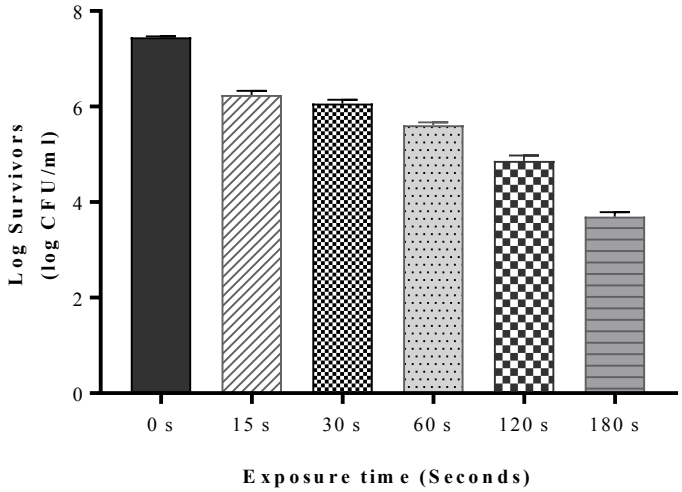


Figure 11: Log survivors (Log_{10} CFU/ml) of *L. monocytogenes* recovered on TSA after different treatment times at 1°C

Table 10: One-way ANOVA analysis of antimicrobial effect of CPC treatment time on destruction of *Listeria monocytogenes* cocktail suspension at 1°C

ANOVA summary	
F	476.1
P value	<0.0001
P value summary	****
Significant diff. among means ($P < 0.05$)?	Yes
R^2	0.9975
Brown-Forsythe test	
	4.961e+027
F (DFn, DFd)	(5, 6)
P value	<0.0001
P value summary	****
Are SDs significantly different ($P < 0.05$)?	Yes

Comparisons of *L. monocytogenes* populations recovered after treatment with 6 ppm CPC over time indicated significant ($P<0.05$) differences between counts recovered at 23ºC as well as at 1ºC. The highest lethal effect of 6 ppm CPC was observed on *L. monocytogenes* suspension after 180 s exposure time at 23ºC. Overall results indicate that the concentration of CPC and treatments times as well as tested temperatures have vital role in recovery of *L. monocytogenes* when present in suspension form. Ahamad & Marth in 1989 also reported that, the type and concentration of acid as well as temperature of incubation affects the behaviour of *L. monocytogenes*.

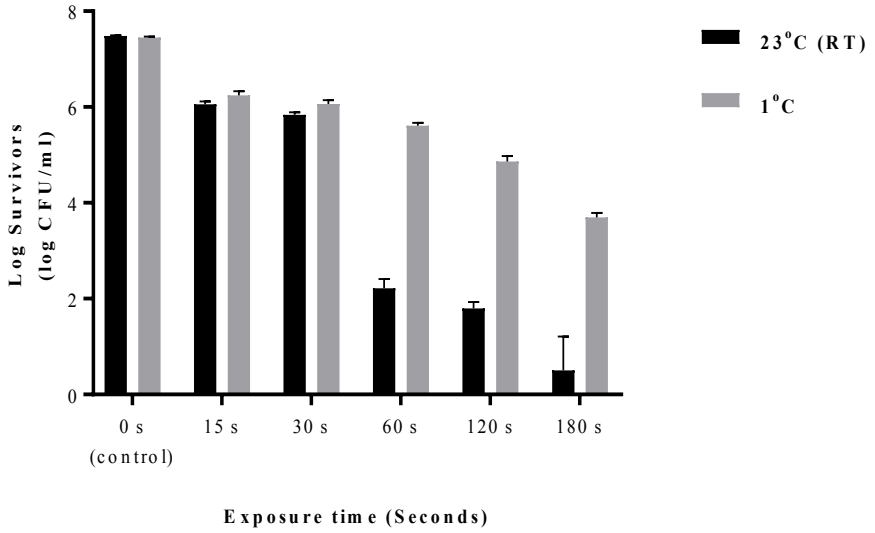


Figure 12: Comparison between log reduction observed after different treatments times at 23°C and 1°C.

Table 11: Two-way ANOVA analysis of antimicrobial effect of CPC treatment time on destruction of *Listeria monocytogenes* cocktail suspension at 23°C and 1°C

Source of Variation	% of total variation	*P* value	*P* value summary	Significant?
Interaction	12.66	<0.0001	****	Yes
Row Factor	72.05	<0.0001	****	Yes
Treatment time	14.77	<0.0001	****	Yes
Subjects (matching)	0.2289	0.6275	ns	No

ANOVA table	SS	DF	MS	F (DFn, DFd)	*P*-value
Interaction	14.41	5	2.881	F (5, 6) = 50.27	0.0001
Row Factor	82.01	5	16.4	F (5, 6) = 377.6	0.0001
Treatment time	16.81	1	16.81	F (1, 6) = 293.3	0.0001
Subjects (matching)	0.2606	6	0.04343	F (6, 6) = 0.7578	0.6275
Residual	0.3439	6	0.05731		

Note: **** represents $P \leq 0.0001$

The D-value (decimal reduction time) is another important parameter used to determine the inactivation kinetics. It is the time in minutes that will result in reducing microorganisms by a factor of ten. D values of *L. monocytogenes* cocktail are calculated using slope values (as shown in Figures 13 and 14). The D-values of *L. monocytogenes* cocktail studied was approximately 0.47 min while using 6 ppm CPC at 23°C and 0.93 min

after using 10 ppm CPC at 1°C. In other words, the maximum reduction of *L. monocytogenes* cocktail was obtained after treatment with 6 ppm CPC at 23°C with D-value 0.47 min. It indicates that temperature plays an important role in inactivation of *L. monocytogenes* cocktail.

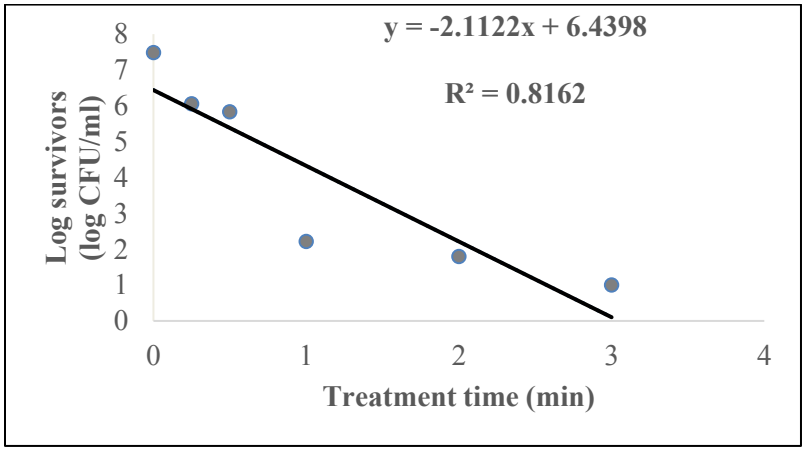


Figure 13: Graph showing slope for calculating D-value of *L. monocytogenes* cocktail treated with CPC at 23°C

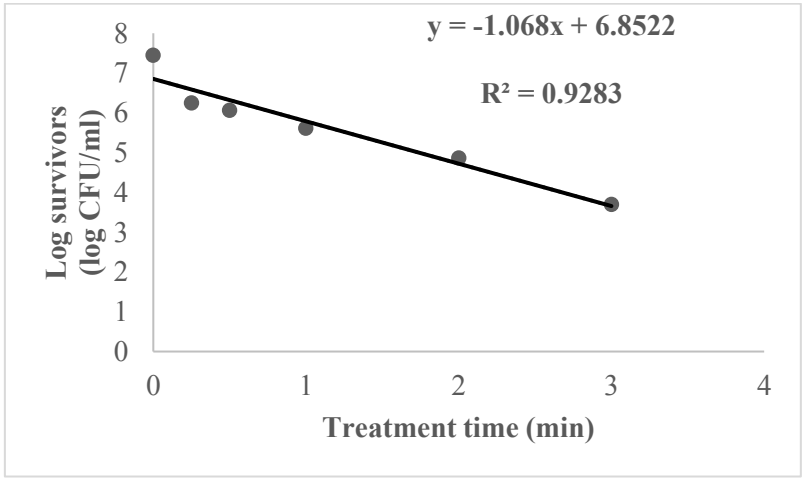


Figure 14: Graph showing slope for calculating D-value of *L. monocytogenes* cocktail treated with CPC at 1°C

4.5 Conclusion

As the treatment time of CPC was increased, inactivation level of *L. monocytogenes* also increased at both temperatures (23°C and 1°C). Along with concentration of CPC, the treatment time has also an important role in inactivation of *L. monocytogenes*. After 180 s

treatment time >6 $\log_{10}$ CFU/ml reduction was observed at 23ºC, while 3.7 $\log_{10}$ CFU/ml reduction was observed at 1ºC. These yielded D values of 0.47 and 0.93 min respectively at 23ºC and 1ºC. Also, comparison between temperatures showed that temperature is the main influential factor in CPC antimicrobial activity against *L. monocytogenes*.

PREFACE OF CHAPTER 5

Food-borne outbreaks of *L. monocytogenes* have increased in the past few years. Since the demand for processed food has increased since the last few decades, it is also important to supply a safe and contamination free processed food. FDA and Health Canada have approved many chemicals that can be used by the food industry to overcome contamination from pathogens.

CPC is one of the quaternary ammonium compounds that has been approved for the use on raw poultry. However, if the use of CPC were to be extended to treating vegetables, it is also important to check its effect on vegetables as compared with chicken. Furthermore, the demand for fresh vegetables is high, hence it is required to understand the effectiveness of CPC against *L. monocytogenes* on fresh vegetables. In the previous chapters, the effect of CPC treatment was explored with direct contact of CPC with the pathogen freely suspended in a liquid medium. This chapter emphasises the CPC treatment on chicken and three vegetables (potato strips, red pepper strips, broccoli florets) that falls under different categories of vegetables based on their classification.

All the experimental work and data analysis were conducted by the candidate under supervision of Dr. H. S. Ramaswamy. A manuscript from this chapter is under preparation for publication:

Pawar, K. and Ramaswamy, H.S., 2018. Antimicrobial effect of CPC on various food surfaces inoculated with *L. monocytogenes* cocktail.

CHAPTER 5

CETYLPYRIDINIUM CHLORIDE INACTIVATION KINETICS OF SURFACE INOCULATED COCKTAIL OF *LISTERIA MONOCYTOGENES* ON FOOD

5.1 Abstract

Cetylpyridinium chloride is one of the chemicals that has been approved to use on poultry. Hence our main objective for this study was to evaluate the efficacy of CPC against *L. monocytogenes* present on chicken along with some vegetables: potato strips, red pepper, and broccoli florets. *L. monocytogenes* inactivation kinetics were also evaluated at some higher CPC concentrations (0.2% to 1%) and varying contact times (15 s to 60 sec) for each food material. It was observed that each food sample gave different inactivation kinetics of *L. monocytogenes*. Overall results indicated that CPC was much more effective against *L. monocytogenes* when treated directly in suspension and it was less effective when *L. monocytogenes* cells were associated with food matrices.

5.2 Introduction

In recent years, consumers' demand for fresh, nutritious, safe, healthy and natural foods has increased enormously. Hence, the market for chilled pre-cut produce has grown significantly. Fresh-cut fruits and vegetables include nominal processes like trimming, peeling and cutting. This fresh supply is then packaged and distributed for consumption (Jennylynd & Tipvanna, 2011). In last decade this section of food industry has grown rapidly (Saftner et al., 2006). Though eating fresh cut fruits and vegetables is considered healthy, bacteria may enter from the external fruit surfaces to the edible portions during cutting and peeling (Selma et al., 2008; Ukuku & Fett, 2002). Hence, it is important to emphasize on various food safety precautions to overcome bacterial contamination.

In the previous chapters, the destruction efficiency of CPC on a cocktail of *L. monocytogenes* was presented. But the pathogen was directly exposed to CPC in a solution at

different concentration levels and two temperatures with some selected treatment times. Treatment like this has merit in understanding the pathogen destruction efficiency of CPC but deviates from reality wherein the pathogens generally are present on food contact surfaces and sometimes even some interior regions below the surface if entry is possible. The CPC activity in such cases could be different due to less efficient exposure of the pathogen to CPC and food surfaces possible protection against the CPC activity. Hence, the objective of the research in this chapter is the evaluation of the effect of CPC on the destruction of *L. monocytogenes* inoculated on food surfaces.

5.3 Materials and Methods

5.3.1 Preparation of 10% CPC stock solution

First 200 ml potable water was measured in a graduated cylinder and transferred into a conical flask. Then the flask was kept magnetically stirred and the temperature was kept at around 70-80^{o}C. Then 20g CPC powder was weighed. Then weighed CPC powder was added into a flask containing the 200 ml distilled water. Continuous stirring was required to get a clear solution of 10% CPC (Figure 15). Once all the CPC powder was completely dissolved, the solution became clear, the flask was removed from magnetic stirring. The prepared 10% stock solution was cooled to room temperature and further diluted to get required concentrations of CPC solution.

5.3.2 Preparation of CPC solutions (0.2%, 0.4%, 0.7% and 1%)

Four aqueous solutions of CPC were prepared at concentration levels of 0.2%, 0.4%, 0.7% and 1% in potable water by dissolving appropriate amount of CPC (as explained below). Potable water was used instead of sterile water because food industry uses potable water for chemical treatment and rinsing of cut vegetables. Hence it gives a more practical approach. All solutions were made fresh prior to conducting experiment and were used at room temperature within 1 h.

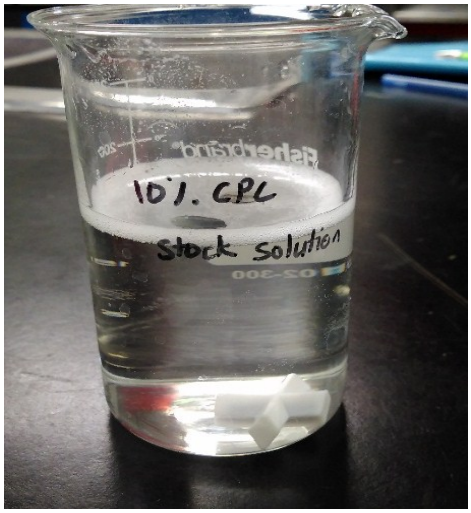


Figure 15: Dissolved 10% CPC stock solution

20 ml of stock solution (10%) was dissolved in 980 ml of potable water to get final 1L of 0.2% CPC solution. 40 ml of stock solution (10%) was dissolved in 960 ml of potable water to get final 1L of 0.4% CPC solution and 70 ml of stock solution (10%) was dissolved in 930 ml of potable water to get final 1L of 0.7% CPC solution.

5.3.3 Preparation of *L. monocytogenes* cocktail for inoculating food samples

Cells were grown, and cocktail was prepared as explained in Chapter 2. For the following experiments, 100 ml of *L. monocytogenes* cocktail was prepared with inoculum concentration of $3x10^7$ CFU/ml.

5.3.4 Inoculation of *L. monocytogenes* cocktail on red pepper

Red pepper strips were received in the frozen state from Bonduelle, Inc., Canada. Each red pepper strip measured around 5 cm in length and approximately 1 cm in width. 25g of frozen red pepper was weighed per sample in sterile petri dish (done aseptically by using sterile forceps in a bio-safety cabinet (BSC)). Then samples were kept in BSC for thawing for 20 min. After thawing, the food sample was dipped into a beaker containing 100 ml *L. monocytogenes* cocktail ($3x10^7$ CFU/ml) for 15 s. The inoculated samples were transferred

into sterile petri dish using sterile forceps. The red pepper samples were kept in BSC itself for 30 min for cell attachment.

5.3.5 Inoculation of *L. monocytogenes* cocktail on potato

Potato sample was received as partially fried 'French fries' from Michel St-Arneault, Canada. Each strip measured around 5-6 cm in length and ~1 cm in width. 25g of frozen sample was weighed and inoculated with *L. monocytogenes* by following same procedure as same as explained above for red pepper.

5.3.6 Inoculation of *L. monocytogenes* cocktail on broccoli

Broccoli florets were received in frozen condition from Bonduelle, Inc., Canada. The 25g of broccoli (2 florets) was weighed per sample (Figure 16). All the samples were inoculated by following same procedure as explained above for red pepper.

Figure 16: Weighed 25 g Broccoli sample thawed under BSC

5.3.7 Inoculation of *L. monocytogenes* cocktail on chicken

Chicken thighs were received from Exceldor, Canada. The frozen chicken sample was first thawed and then cut into smaller pieces of about 4x4 cm in length and width using sterile knife. The pieces were boneless and had skin on one side (chicken thighs are sold with skin hence the skin was not removed). 25g of chicken (2 pieces) was weighed per sample. The

chicken samples were inoculated by following same procedure as explained above for red pepper.

5.4 CPC treatment

For each food sample, specific CPC range was selected based on preliminary experiments on each food. Three concentrations were chosen such that, they represented lower, mediate and higher CPC concentrations to inactivate *L. monocytogenes* on each food surface.

5.4.1 CPC treatment on red pepper

The entire experiment was done within the BSC (Class II A/B3 Biological safety cabinet, USA). The red pepper sample (25 g) inoculated with *L. monocytogenes* was transferred into sterile stainless-steel strainer (mesh). The strainer was kept over an empty 2-litre sterile glass beaker (to collect sprayed chemical and water) (Figure 17). The red pepper sample was sprayed with 0.2% CPC solution by using shower head/spraying unit (as shown in Figure 17) for 15 s at 1500 ml/min flowrate by using a pump (Barnant Co., Masterflex®). During spraying the care was taken to avoid splashes (Note: Before using the shower head, it was disinfected with 20 ppm chlorine solution followed by rinse with sterile distilled water). After 0.2% CPC treatment the sample was rinsed immediately with sterile distilled water using another shower for 15 s at 1500 ml/min flowrate (to remove excess CPC and to stop the CPC reaction after 15 s). Similar procedure was followed to give other CPC treatments (0.4% and 0.7%). The control red pepper sample was sprayed with just potable water for total 30 s. First 15 s represented the chemical treatment and second 15 s represented water rinse after chemical use. The control samples were also sprayed with water to know whether the log reduction observed was due to chemical efficiency or the force/flowrate of sprayed waters.

5.4.2 CPC treatment on potato

The same protocol was followed for CPC treatments (0.2%, 0.4%, 0.7%) on potato sample as explained above for red pepper. The control potato sample was sprayed with potable water for a total of 30 s.

5.4.3 CPC treatment on broccoli

The same protocol was followed for CPC treatments (0.4%, 0.7% and 1%) on broccoli sample as explained above for red pepper except with higher CPC concentration levels. The 0.2% CPC was not very effective on broccoli hence the CPC concentration was increased up to 1%. Three CPC concentration (0.4%, 0.7% and 1%) were decided (representing lower, mediate and higher concentration levels) based on preliminary experiments. The control broccoli sample was sprayed with potable water for total of 30 s.

Figure 17: The spraying unit/shower head at left corner (orange in colour) was used for the spraying of CPC solutions onto food samples

5.4.4 CPC treatment on chicken

Same protocol was followed for CPC treatments (0.4%, 0.7% and 1%) on chicken sample as explained above for broccoli. The control chicken sample was sprayed with potable water for total of 30 s.

5.4.5 Microbial Sampling of the Products

Each treated sample and control sample was transferred into stomaching bag (Seaward stomacher lab systems, UK) containing 225 ml sterile saline (0.85%). The bag was stomached for 2 min using a stomacher lab blender-400 (UL laboratory equipment, England) (Figure 18). The stomached content was further analysed for microbial enumeration.

Figure 18: Broccoli sample in a stomaching bag after having been stomached for 2 min

5.4.6 Microbial enumeration

Serial dilutions were prepared in 0.85% saline. *L. monocytogenes* populations were determined quantitatively by plating appropriate dilutions (10^{-1} to 10^{-4}) along with 0.1 ml solution from stomaching bag (without dilution) on Oxford agar (Sigma-Aldrich, USA) with Oxford Listeria selective supplement which contains cycloheximide (Merck KGaA, Germany). Oxford agar is a selective media that contains, Columbia agar base which

provides peptones and other essential nutrients to *L. monocytogenes*. It hydrolyzes esculin to form 6, 7-dihyroxy-coumarin that combines with ferric ammonium citrate to form black precipitate in the media surrounding the colonies. Lithium chloride, acriflavine, cycloheximide, colistin, fosfomycin are selective agents that inhibit the growth of other gram positive and gram-negative organisms.

All plates were incubated at 37°C for 48-72 h and colonies (as shown in Figure 19) were counted. Experiments were done in triplicates and results were reported as $\log_{10}$ CFU/g. (Appendix III).

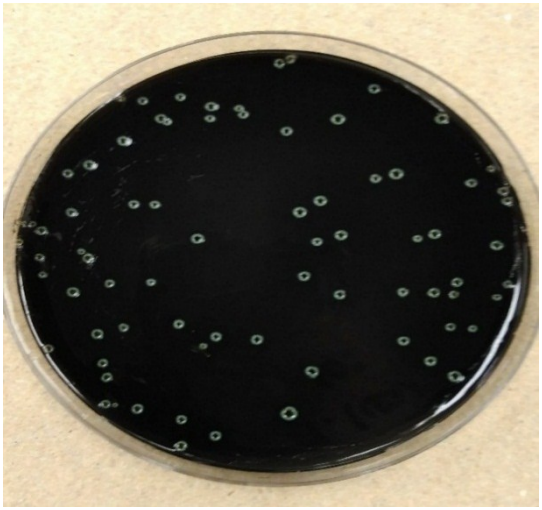


Figure 19: *L. monocytogenes* colonies on Oxford agar after 48 h incubation at 37°C

5.5 Inactivation kinetics

In our study, microbial inactivation was evaluated using inactivation kinetics from the Weibull model. Weibull model is a two-parameter nonlinear model, which was used to analyse the data. The cumulative form of the Weibull distribution is given by (Eq. 1) (van Boekel, 2002).

$$\boldsymbol{log_{10}\left(\frac{N}{N_0}\right) = -\left(\frac{1}{2{\cdot}303}\right)\left(\frac{t}{a}\right)^{\beta}} \quad \text{.....(1)}$$

where, N is the survival population at time t (CFU/g), N_o is the initial population (CFU/g), t is the treatment time, α is the characteristic time, β is the shape factor of the inactivation curve. If β < 1 then it shows concave-upward survival curve and if β > 1, it shows concave-downward survival curve. The Weibull parameters α and β were used to calculate the 90% percentile of the failure time distribution, T_R (Equation 2)

$$\mathbf{T_R = \alpha^*(2.303)^{\beta}} \quad \text{.....(2)}$$

where, T_R is the time required for 90% reduction of the population of the pathogen, α is the characteristic time (sec), and β is the shape parameter.

5.5.1 Statistical analysis

Effect of CPC concentrations and treatment times on the inactivation of *L. monocytogenes* on each food sample were tested by two-way ANOVA, by using GraphPad Prism 7 software. Also, differences between sample means which analysed by using Student's *t*-test, were considered significant when *P*-value <0.05. The survival curves of *L. monocytogenes* were drawn by using GraphPad Prism 7 software (Appendix IV).

The goodness of fit for the Weibull model was determined in terms of a parameter R^2, defined as the ratio of the difference between the corrected sum of squares and the model sum of squares. MATLAB R2018a (MATLAB R2018a, McGill, CA) was used to perform the nonlinear regression and to calculate Weibull parameters.

5.6 Results and Discussion

Most of the previous CPC work has been done on poultry, beef, shrimps and other meat products. Its effect on vegetables has not been studied extensively. Hence, our study on CPC treatment on vegetables along with chicken makes a special contribution. In this study, the effect of CPC and treatment time on *L. monocytogenes* adhered on the surface of cut red pepper, potato strip, broccoli floret and chicken were studied through the use of the Weibull

model, which is a more expanded version of the log-linear first order model. Recovery of *L. monocytogenes* from food surfaces treated with combination of different CPC concentrations at different treatment times was evaluated.

5.4.1 Effect of CPC and treatment time on potato inoculated with *L. monocytogenes*

The results of inactivation of *L. monocytogenes* cocktail by CPC on the surface of potato is shown in Figure 20. This figure summarizes the survival level of *L. monocytogenes* cocktail when treated with 0.2%, 0.4% and 0.7% CPC at room temperature (23ºC) for up to 60 s exposure time. Weibull model was used to fit the curves obtained from log reduction versus treatment time. Weibull parameters α and β values for CPC treatments calculated from the model are shown in Table 12.

Two-way Analysis of Variance (ANOVA) of mean $\log_{10}$ CFU/ g of *L. monocytogenes* recovered from the potato samples treatment with CPC (0.2%, 0.4% and 0.7%) followed by water rinse showed significant differences ($P<0.05$) between treatment times (15 s and 60 s) when selective media (Oxford agar) was used. The statistical analysis indicated no significant differences ($P>0.05$) in the recovery of *L. monocytogenes* between non-treated (control) samples.

Treatment of CPC on *L. monocytogenes* cocktail inoculated on potato surface with 0.2%, 0.4% and 0.7% concentration for 60 s resulted in log reductions of 0.4, 2 and 4.3, respectively. It shows that, increased concentration of CPC has an effect on the destruction of *L. monocytogenes* cocktail inoculated on potato surface.

As shown in Figures 20, the bacterial reductions achieved by 0.2% CPC treatment did not significantly increase when the treatment time was increased from 0 to 60 s. However, a significant difference ($P<0.05$) was observed when the treatment time was increased from 30 s to 60 s when the potato samples were treated with 0.4% and 0.7% CPC. In addition, 0.4% CPC (60 s) and 0.7% CPC (15, 30 and 60 s) treatment showed significant ($P<0.05$)

bactericidal effect against *L. monocytogenes* cocktail as compared to control and 0.2% CPC treatments (Figure 20).

The two-way ANOVA results indicated that a combination of different treatment times and 0.7% CPC concentration had a significant effect ($P<0.05$) on the destruction of *L. monocytogenes* on the potato sample. After 15 s treatment time, 2.4 log reduction was observed while after 60 s it increased to 4.3 log with 0.7% CPC. Enumeration of *L. monocytogenes* from CPC treated samples on oxford agar plates has a detection limit of ≤ 4.5 log in our protocol, depending on number of cells attached to the surface of food. These results suggest that, the most effective CPC concentration for destruction of *L. monocytogenes* was 0.7% as compared to other concentrations of CPC with non-treated (control) on potato sample. Also, the increase in log reduction was negligible (<1 log cycle) between 15 s to 30 s among all CPC treatments carried out on potato. It indicates that, the CPC treatment time should be increased to certain threshold time to observe effectiveness of CPC.

The results of CPC treatments and R^2 in Table 12 indicate that the Weibull model provided the best fit for inactivation curves. For each treatment, the Weibull parameter β is less than 1, representing the upward concavity of the inactivation curves (Table 20).

T_R values of *L. monocytogenes* in different CPC treatments were obtained by using the Weibull model equation are shown in Table 12. The T_R is defined as the treatment time required for the one log reduction of the pathogen. The mean values of RMSE were 0.0056, 0.0129 and 0.2224 for *L. monocytogenes* after 15 s, 30 s and 60 s treatment time respectively. Regression coefficients (R^2) in all treatments were 0.99.

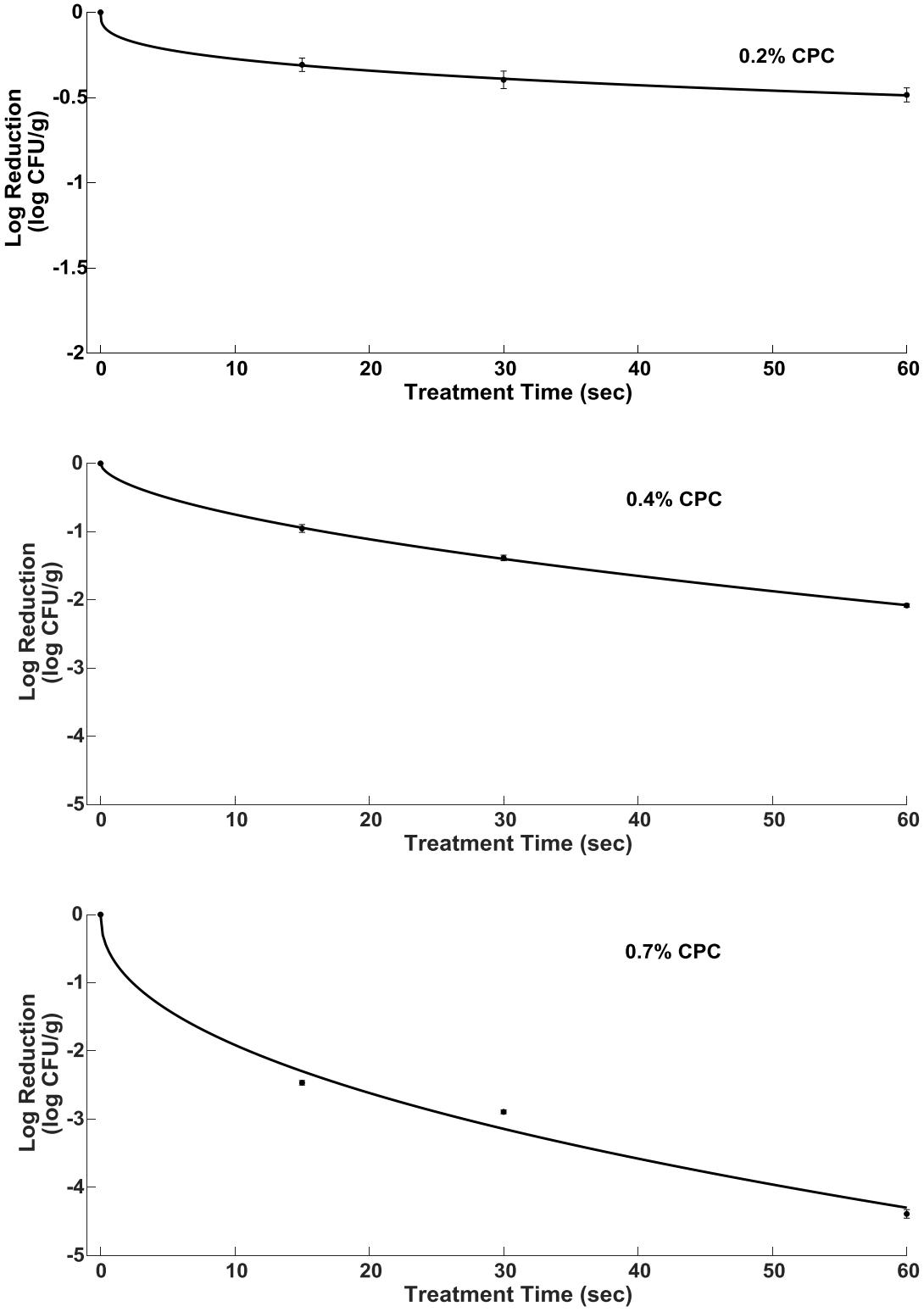


Figure 20: Survival curves of *L. monocytogenes* cocktail inoculated on the surface of potato ($3x10^7$ CFU/g and treated with different concentrations of CPC solutions (0.2%, 0.4% and 0.7%) for up to 60 s treatment time. Points represent the mean±std. error of log CFU/g. Curves are fitted using Weibull model

Table 12: Weibull model parameters (mean± SD), R^2 values, RMSE, SSE and T_R for three CPC treatments on each food sample

Food sample	CPC (%)	α		β		R^2	RMSE	SSE	T_R (min)
Potato	0.2	42.05	±4.73	0.32	±0.07	0.9995	0.0057	0.0001	54.92
	0.4	3.84	±0.69	0.57	±0.04	0.9900	0.0129	0.0003	6.18
	0.7	0.38	±1.24	0.45	±0.32	0.9901	0.2224	0.0989	0.55
Red pepper	0.2	0.12	±0.05	0.22	±0.01	1.0000	0.0055	0.0001	0.14
	0.4	0.00	±0.02	0.19	±0.10	0.9984	0.0584	0.0068	0.01
	0.7	0.02	±0.33	0.29	±0.60	0.9582	0.4753	0.4518	0.03
Broccoli	0.4	15.85	±12.43	0.59	±0.42	0.9859	0.0582	0.0068	25.95
	0.7	3.01	±5.48	0.42	±0.29	0.9914	0.4753	0.0110	4.27
	1	4.43	±7.02	0.84	±0.55	0.9843	0.2534	0.1284	8.96
Chicken	0.4	11.80	±6.70	0.39	±0.17	0.9967	0.0101	0.0002	16.34
	0.7	6.60	±19.15	0.51	±0.78	0.9490	0.1602	0.0513	10.07
	1	0.32	±1.45	0.33	±0.31	0.9890	0.1362	0.0371	0.43

5.4.2 Effect of CPC and treatment time on red pepper inoculated with *L. monocytogenes*

The results of inactivation of *L. monocytogenes* cocktail by CPC on the surface of red pepper are shown in Figure 21. This summarizes the survival level of *L. monocytogenes* cocktail when treated with 0.2%, 0.4% and 0.7% at room temperature (23ºC) for up to 60 s exposure time. Weibull model was used to fit the curves obtained from log reduction versus treatment time. Weibull parameters are shown in Table 12.

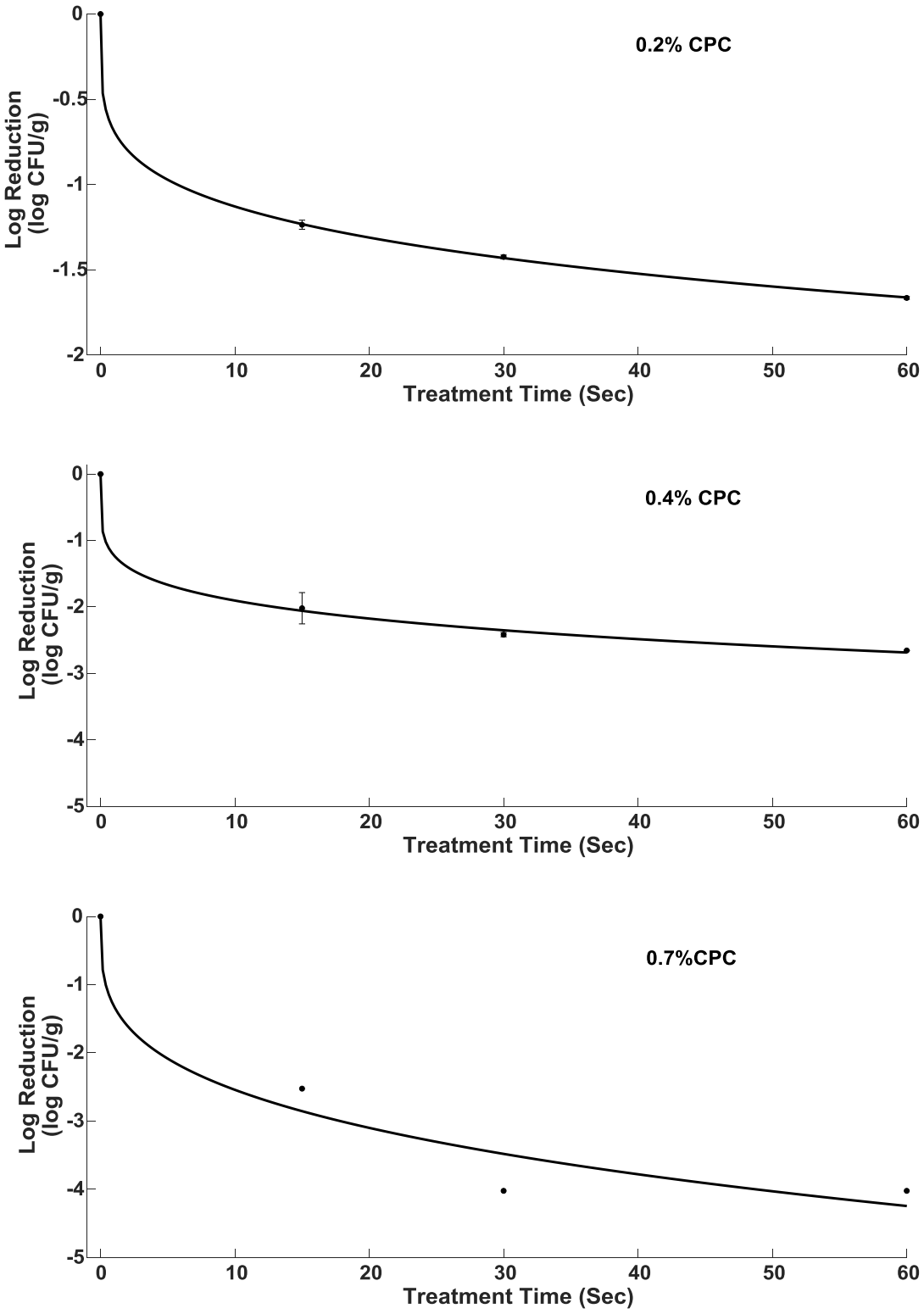


Figure 21: Survival curves of *L. monocytogenes* cocktail inoculated on the surface of red pepper ($3x10^7$ CFU/g and treated with different concentrations of CPC solutions (0.2%, 0.4% and 0.7% for up to 60 s treatment time. Points represent the mean±std. error of log reduction. Curves are fitted using Weibull model

Two-way Analysis of Variance (ANOVA) of mean $\log_{10}$ CFU/ g of *L. monocytogenes* recovered from the red pepper samples treatment with CPC (0.2%, 0.4% and 0.7%) followed by water rinse showed significant differences (P<0.05) between treated and non-treated (control) samples when selective media (Oxford agar) was used.

It can be observed in the Figure 21 that, 0.4 % CPC treatment for 15, 30 and 60 s treatment time caused 2.0, 2.4 and 2.6 log reductions in *L. monocytogenes* cocktail on red pepper, respectively. There was no significant difference (P<0.05) between 30 s and 60 s.

The results of CPC treatments and R^2 in Table 12 indicate that the Weibull model provided the acceptable estimation for describing the inactivation curves. The T_R is defined as the treatment time required for the one log reduction of the pathogen. Results showed the decrease in T_R value with increasing CPC concentration as well as treatment time. It reflects that these two factors are capable to enhance the efficiency of CPC in the *L. monocytogenes* cocktail reduction. In addition, the minimum T_R value for the *L. monocytogenes* cocktail was obtained by 0.4% CPC treatment for 30 s on red pepper, indicating that the pathogen was most sensitive to this combined treatment on red pepper.

For each treatment, the parameter β is less than 1, representing the upward concavity of the inactivation curves. The T_R values of *L. monocytogenes* in different CPC treatments were obtained by using the Weibull parameters α and β as shown in Table 12. The mean values of RMSE were 0.0054 and 0.0584 for *L. monocytogenes* after 15 s, 30 s and 60 s treatment time respectively. Regression coefficients (R^2) in 0.2%, 0.4% and 0.7% treatments were 1.0, 0.99 and 0.95 respectively.

5.4.3 Effect of CPC and treatment time on Broccoli inoculated with *L. monocytogenes*

The results of inactivation of *L. monocytogenes* cocktail by CPC on the surface of broccoli are shown in Figure 22. These summarizes the survival level of *L. monocytogenes* cocktail when treated with 0.4%, 0.7% and 1% at room temperature (23ºC) for up to 60 s

exposure time. Weibull model was used to fit the curves obtained from log reduction versus treatment time. Weibull parameters are shown in Table 12.

The two-way ANOVA indicated that CPC treatment and treatment time had a significant effect (P<0.05) on the destruction of *L. monocytogenes* on the broccoli as compared to non-treated (control). Treatment of CPC with 0.4%, 0.7% and 1% concentration for 60 s on broccoli surface inoculated with *L. monocytogenes* cocktail resulted in log reductions of 0.9 $\log_{10}$ CFU/g, 1.4 $\log_{10}$ CFU/g and 3.9 $\log_{10}$ CFU/g respectively. Also, as shown in Figures 22. 0.7% CPC showed significant (P<0.05) bactericidal effect against *L. monocytogenes* cocktail between 30 s as well as 60 s and between 15 s and control. Further, 1% CPC spray for 30 and 60 s were significantly (P<0.05) different to inactivate *L. monocytogenes* cocktail as compared to control and 0.4% CPC spray treatments.

The combination of 1% CPC concentration and 60 s treatment time gave highest log reduction (3.9 $\log_{10}$ CFU/g) in broccoli sample followed by log reduction observed with 1% CPC spray for 30 s gave 1.9 $\log_{10}$ CFU/g reduction in *L. monocytogenes* population. It indicates that, as the CPC concentration and treatment time was increased, the inactivation level of *L. monocytogenes* cocktail on broccoli surface also increased. However, the inactivation kinetics of *L. monocytogenes* cocktail on broccoli was not similar to inactivation kinetics observed in potato and red pepper. It indicates that, the type of food plays a vital role in the effectiveness of CPC.

ANOVA results also indicated no significant difference between log reduction observed at 15 s with 1% CPC and at 60 s with 0.7% CPC, as both treatments gave 1.4 log reduction of *L. monocytogenes* cocktail inoculated on broccoli surface. This result suggests that, higher level of inactivation can be observed by keeping CPC concentration constant and increasing treatment time. In other words, the combination of low concentration-high

treatment time and high concentration-low treatment time give rise to similar amount of bacterial inactivation in case of CPC treatments.

The T_R values of *L. monocytogenes* in different CPC treatments were obtained by using the Weibull model equation are shown in Table 12. The mean values of RMSE were 0.0582, 0.4753 and 0.2534 for *L. monocytogenes* after 15 s, 30 s and 60 s treatment time respectively. Regression coefficients (R^2) in 0.4% and 1% treatments were 0.98 respectively and 0.7% treatments were 0.99.

5.4.4 Effect of CPC and treatment time on chicken inoculated with *L. monocytogenes*

The results of inactivation of *L. monocytogenes* cocktail by CPC on the surface of chicken are shown in Figures 23. These summarizes the $\log_{10}$ CFU/ g of *L. monocytogenes* recovered from the chicken when 0.4%, 0.7% and 1% CPC was used. Initial bacterial loads of the product were determined from control sample. Weibull model was used to fit the curve obtained from log reduction versus treatment time. Weibull parameters such as α and β values for CPC treatments were calculated from the model are shown in Table 12.

Treatment of CPC with 0.4%, 0.7% and 1% concentration for 60 s on chicken sample inoculated with *L. monocytogenes* cocktail resulted in log reductions of 0.3 $\log_{10}$ CFU/g, 1.2 $\log_{10}$ CFU/g and 2.5 $\log_{10}$ CFU/g respectively. In other words, a significant (P<0.05) bactericidal effect against *L. monocytogenes* cocktail was observed between all CPC treatments (0.4%, 0.7% and 1%) at 60 s treatment time. It indicates increased level of inactivation level of pathogen with increased CPC concentration.

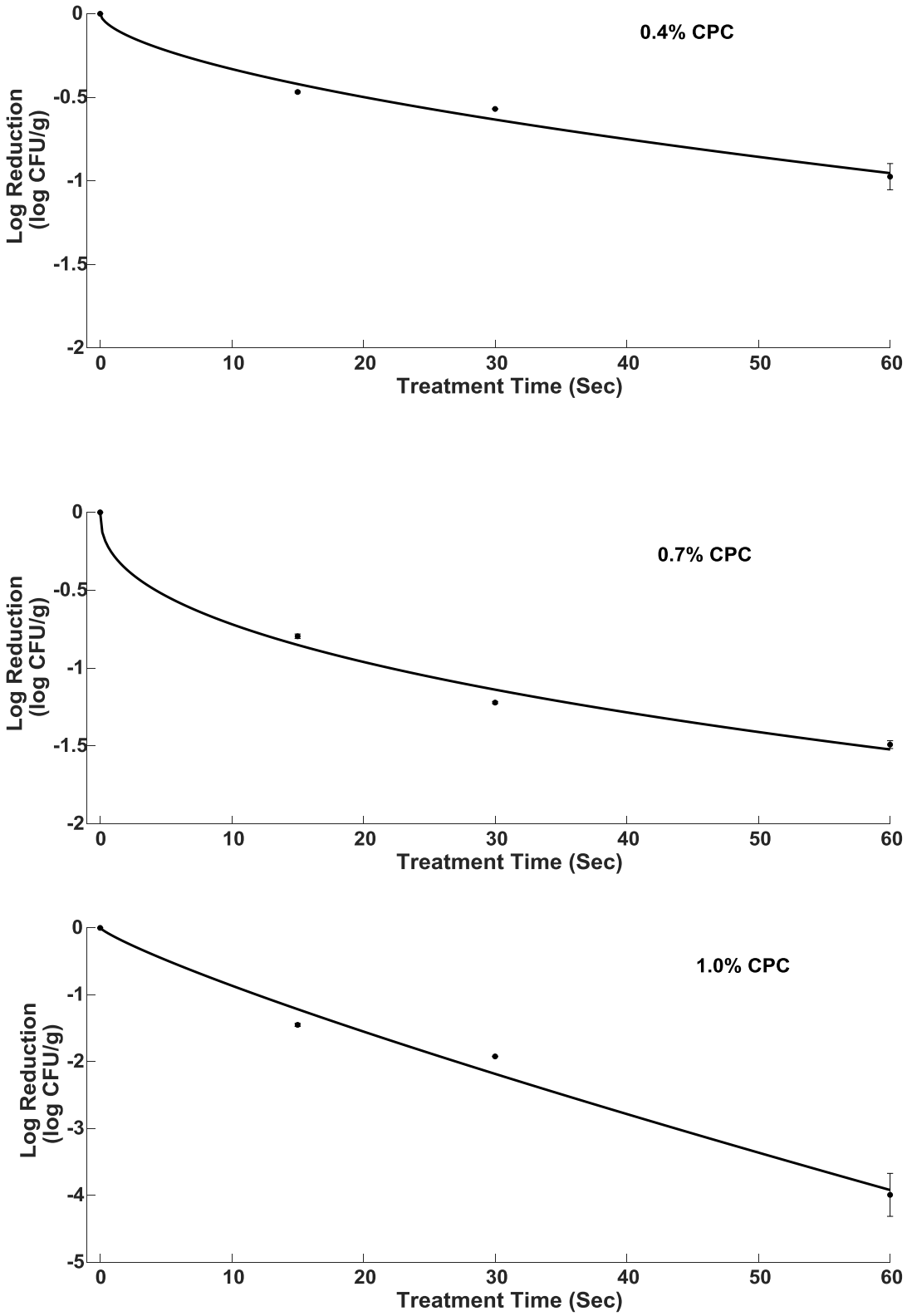


Figure 22: Survival curves of *L. monocytogenes* cocktail inoculated on the surface of Broccoli ($3x10^7$ CFU/g and treated with different concentrations of CPC solutions (0.4%, 0.7% and 1% for up to 60 s treatment time. Points represent the mean±std. error of log CFU/g. Curves are fitted using Weibull model

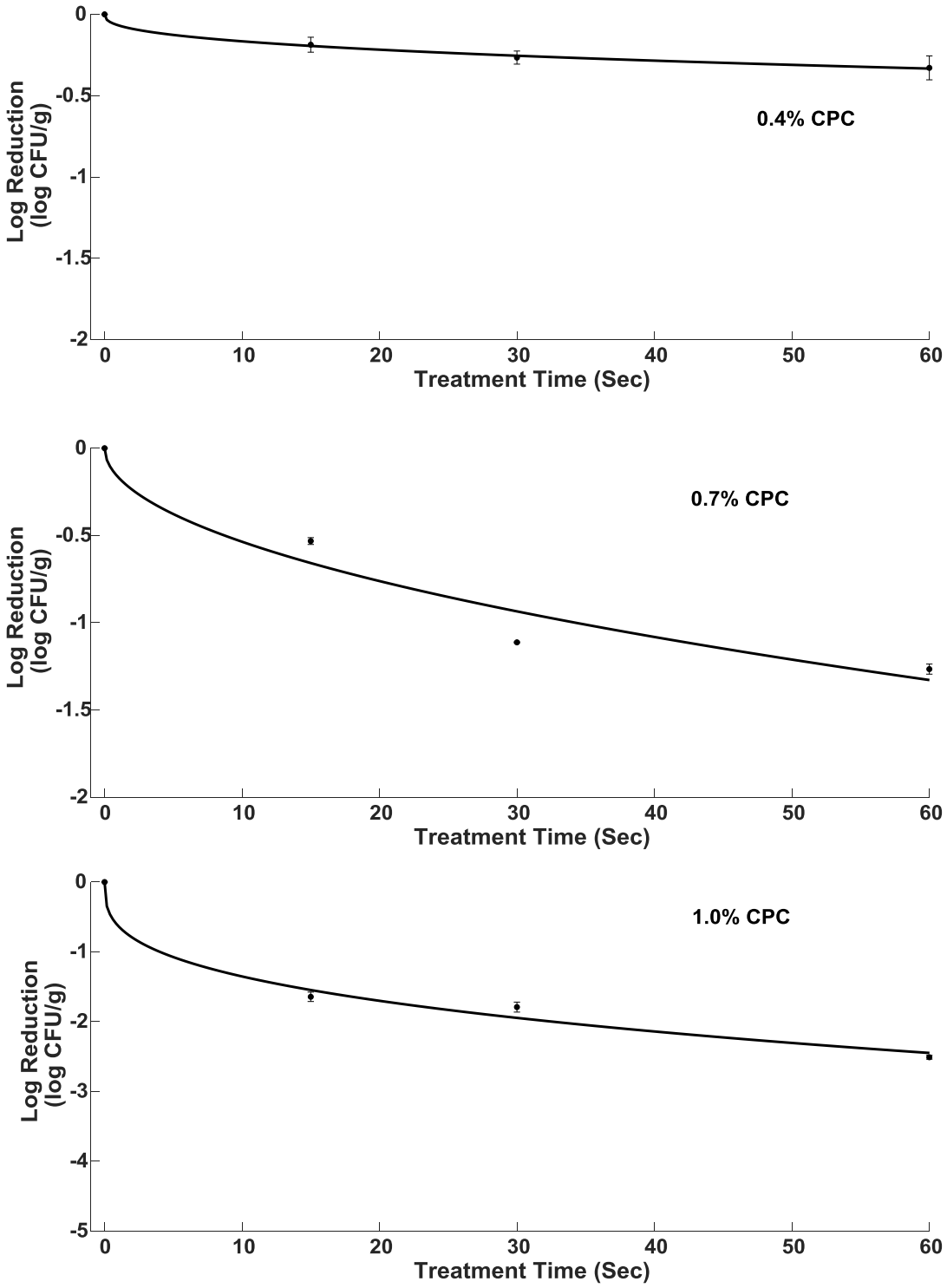


Figure 23: Survival curves of *L. monocytogenes* cocktail inoculated on the surface of chicken ($3x10^7$ CFU/g and treated with different concentrations of CPC solutions (0.4%, 0.7% and 1% for up to 60 s treatment time. Points represent the mean±std. error of log CFU/g. Curves are fitted using Weibull model

As evidenced from Figure 23, there was no significant difference (P>0.05) in log reductions irrespective of the time of exposure of CPC on the inoculated chicken sample treated with 0.4% CPC. Here the increase in log reduction from 15 s to 60 s observed was negligible. However, the significant difference was observed with 0.4% CPC spray solution for 15 s and 60 s treatment time that showed >1 CFU/g and 2 $\log_{10}$ CFU/g reduction of *L. monocytogenes* cocktail on potato respectively.

The two-way ANOVA results obtained from 0.4% CPC treatment, indicate no significant difference (P>0.05) in log reduction of *L. monocytogenes* (0.3 $\log_{10}$ CFU/ g) over 60 sec treatment time. In addition, there is no significant effect on recovery of *L. monocytogenes* after 0.7% CPC treatment at 30 s and 60 s treatment time. It was observed that treatment time (15 s and 60 s) of 1% CPC treated samples and non-treated samples had a significant effect (P<0.05) on the destruction of *L. monocytogenes* cocktail present on chicken surface.

The T_R values of *L. monocytogenes* in different CPC treatments were obtained by using the Weibull model equation are shown in Table 12. The mean values of RMSE were 0.0054 and 0.0584 for *L. monocytogenes* after 15 s, 30 s and 60 s treatment time respectively. Regression coefficients (R^2) in 0.4%, 0.7% and 1% treatments were 0.99, 0.94 and 0.98 respectively.

5.4.5 Inactivation kinetics of *L. monocytogenes* on different foods

Inactivation kinetic models are mainly used to determine the mechanism of microbial inactivation and survival and to develop an adequate food preservation process (Parish et al., 2003). Xiong et al. (1999) combined the first order kinetic model, the Buchanan model and Cerf's model and proposed a new model that could fit the four commonly observed types of survival curves such as linear curves, curves with a shoulder, biphasic curves and sigmoidal curves.

However, in our study (Table 12) R^2 values of 0.94 and above were obtained for all the CPC treatments on all food surfaces studied. It suggests that the Weibull model was a good fit for the experimental data being analysed. The T_R is another parameter that can be calculated by using Weibull parameters. T_R is defined as the treatment time required for the one log reduction of the pathogen. Results (Table 12) showed the decrease in T_R value with increasing CPC concentration as well as treatment time. It reflects that these two factors are capable to enhance the efficiency of CPC in the *L. monocytogenes* cocktail reduction.

In our study among all CPC treatments, 0.7% CPC spray for 60 s gave 4.3 $\log_{10}$ CFU/g reduction of *L. monocytogenes* on potato surface, followed by 4 $\log_{10}$ CFU/g reduction of *L. monocytogenes* on red pepper surface. These are the highest log reductions observed in this study. Other studies have been reported the effective CPC concentrations on poultry carcasses as 0.1% and 0.5% (Kim & Slavik, 1996; Xiong et al., 1998). Study on ready to eat (RTE) sausages showed that treatment of *L. monocytogenes* inoculated RTE polish sausages with a 1% CPC spray for 30 s exposure resulted in immediate reductions of around 1-3 log CFU/g. After 42 days of 4^{o}C storage, *L. monocytogenes* populations of treated samples were shown reduction up to 4 log CFU/g (Singh et al., 2005).

We carried all CPC treatments on food at room temperature (23^{o}C). However study on shrimps showed the reduction *L. monocytogenes* counts on the surface of raw and cooked shrimp stored at 4°C and -20°C (Dupard, 2005). Also Wang et al., (2016) evaluated 0.1% CPC spray for 30 s exposure time against *Salmonella* on chicken skin at three different temperatures (10, 35, and 60^{o}C) and five spray pressures (from 30 to 150 psi). Spray pressure had no significant effect on treatment effectiveness at low temperatures but ineffective at high temperatures.

The treatment with 1% CPC did not totally kill all population of *L. monocytogenes* on the surface of broccoli and chicken but significantly ($P<0.05$) reduced *L. monocytogenes*

number and kept them significantly (P>0.05) lower than the untreated control. Study done by Bosilevac et al., (2004) also revealed that treatment with 1% CPC significantly reduced hide to carcass transfer of contaminants and enhances the overall beef carcass.

Treatment time was main factor to enhance antimicrobial activity of CPC in our study. But some studies have noted no increase in efficacy with increased in pressure or exposure time. Researchers have used CPC spray treatments to reduce *Salmonella* populations on chicken skin, and at 2 mg/ml CPC concentrations the log reductions observed were similar for 1 and 3 min exposure times (Breen et al., 1997).

In study, the CPC treatment on food surfaces at room temperature for up to 60 s was found to be the most effective in reduction of *L. monocytogenes* present on the red pepper and potato strips. Figures 21 and 20 showed that the most effective reduction was achieved by 60 s with 0.7% CPC treatment, which promoted 4 log CFU/g and 4.3 log CFU/g reductions of *L. monocytogenes* in red pepper and potato respectively. Results shown in Figures 22 and 23, indicate that log reduction of *L. monocytogenes* achieved in case of broccoli and chicken are not high as compared to potato and red pepper. These results indicate that, the bactericidal effect is probably dependent on the type of vegetable or food and its texture. Luo & Oh, (2016) studied inactivation kinetics of *L. monocytogenes* and *S. enterica* on bell pepper by applying acidic electrolyzed water in combination with ultrasound and mild heat. They also suggested that, bactericidal efficacy might be dependent on the type of fruits and vegetables.

This was further supported by study on beef, demonstrated that surface type (sliced versus exterior) clearly affect the effectiveness of the CPC treatment for reducing *L. monocytogenes* on cooked roast beef, with the pathogen more easily reduced on exterior than on sliced surfaces (Singh et al., 2005). Hence, surface type is also an influencing factor.

Wang et al., 2001 investigated the effect of 0.1% and 0.5% CPC against *L. monocytogenes*, *E. coli O157:H7*, and *S. typhimurium*, on fresh-cut vegetables (broccoli, cauliflower, and radishes). Vegetables samples were dip inoculated into 10^5 CFU/ml bacterial solutions for one h at room temperature. After inoculation, all samples were rinsed with tap water for one min. Then, the vegetables samples were treated with CPC by dipping vegetable samples into 0.1% CPC or 0.5% CPC solutions for 1 min at room temperature. In this experiment it was observed that CPC-treated vegetables showed greater reduction of *L. monocytogenes* and *S. typhimurium* than *E. coli O157:H7*. Furthermore, the results obtained were according to analysis done by (Talaro & Talaro, 1993) who concluded that QACs are less effective against gram-negative bacteria than gram-positive bacteria; because the outer membrane of gram-negative bacterium provides an extra barrier that slows down the activity or inhibits the entry of few antimicrobial agents. Due to this reason, the gram-negative bacteria are more difficult to kill than gram-positive bacteria.

The effect of CPC treatments on the reductions of attached bacteria (*L. monocytogenes*, *E.coli O157:H7*, and *S. typhimurium*) to the vegetables surfaces (broccoli, cauliflower, and radishes) varied depending on the type of vegetables and microbial strains (H. Wang et al., 2001). Even in our study, the effect of CPC was largely dependent on type of vegetable (potato, red pepper, broccoli).

5.7 Conclusions

A CPC treatment (0.7%) at room temperature for 60 s was found to be the most effective method for reduction of *L. monocytogenes* present on the red pepper and potato strips with approximately 4 and 4.3 $\log_{10}$ CFU/g reduction respectively. Whereas for broccoli and chicken, 1% CPC for 60 s treatment time showed significant effect with approximately 4 and 2.5 $\log_{10}$ CFU/g reduction of *L. monocytogenes* respectively.

The minimum T_R value for the *L. monocytogenes* cocktail was obtained by 0.4% CPC treatment for 30 s on red pepper, indicating that the pathogen was most sensitive to this combined treatment on red pepper as compared to other CPC treatments and food surfaces. Differences in T_R values were observed among all food surfaces, that indicates the type of food surface also plays an important role in inactivation of pathogen present on its surface.

The results obtained from these experiments were further supported by many studies that suggested, various factors such as concentration of chemical, treatment time, temperature during the treatment (Wang et al., 2016), type of food (Luo & Oh, 2016), surface of food (Singh et al., 2005) can have huge impact on destruction of bacterial contamination.

CHAPTER 6

GENERAL CONCLUSION

In this study the effect of cetyl pyridinium chloride on the destruction of *L. monocytogenes* in an aqueous suspension (liquid) was first evaluated at two different temperatures (23^{o}C and 1^{o}C) as function of CPC concentration and contact/treatment time. All three factors studied - CPC concentration, treatment time and temperature were significant factors influencing the destruction of the pathogen. As can be expected higher concentration, temperature and treatment time had an enhancing effect on pathogen destruction. Combination of these three factors could be employed to reduce the population of *L. monocytogenes* by more than 6 logarithmic cycles.

Destruction kinetics of *L. monocytogenes* was also studied on several food surfaces - chicken breast, red pepper, par fried potato and broccoli - with surface inoculated *L. monocytogenes*. Results had showed that, CPC was more effective against *L. monocytogenes*, when the pathogen is present in suspension form where CPC contact with the pathogen is efficient. The study revealed that the microbial counts on vegetable and chicken tissue could be significantly reduced by CPC at concentration levels higher than 0.4% with contact times of up to one minute at room temperature. The effectiveness of destruction however was dependent on the product type and surface. Each food matrix gave different inactivation kinetic results, which indicates that the inactivation procedure needs to be individually optimized for each food matrix. If higher concentrations are permitted for application, the inactivation becomes more efficient and complete.

Weibull model demonstrated a good fit for the destruction kinetics with R^2 values of 0.94 and above for all the food samples and CPC concentrations studied. It also suggested that the destruction pattern deviated from the traditional log-linear first order model demonstration some convexity (shoulder) with the inactivation kinetic profile.

Overall, results indicated that, many factors such as concentration of CPC, treatment time, temperature during treatment, type of food surface have vital role in destruction of *L. monocytogenes.* Therefore, low concentration-high treatment time or high concentration-low treatment time can result in same inactivation level of *L. monocytogenes* at certain time-concentration combinations. Hence, it is essential to find such combinations for various food surfaces to achieve highest level of *L. monocytogenes* destruction.

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APPENDICES

Appendix I:

Table: Antimicrobial effect of CPC solution on *Listeria monocytogenes* cocktail (suspension) at RT. All analysis was based on three replicates with each mean log reduction and std. dev values are shown below

CPC conc. (ppm)	**Mean (log CFU/ml)**	**SD**
0	7.4866	±0.0082
2	7.2350	±0.0417
4	6.3926	±0.4252
6	5.9166	±0.1128
8	4.1882	±0.1145
10	1.0000	±0.0000
15	1.0000	±0.0000
20	1.0000	±0.0000
25	1.0000	±0.0000

Appendix II:

Table: Antimicrobial effect of CPC solution on *Listeria monocytogenes* cocktail (suspension) at CT. All analysis was based on three replicates with each mean log reduction and std. dev values are shown below

CPC concentration (ppm)	average	SD
0	7.9555	±0.7189
10	6.0966	±0.1245
15	5.2733	±0.3279
20	3.1599	±0.1349
25	1.5652	±0.1245

Appendix III:

Table: Mean log CFU/g of *Listeria monocytogenes* cells on potato strips surfaces after different CPC treatments.

Time	treatment with CPC solution (0.2%)	std dev	treatment with CPC solution (0.4%)	std dev	treatment with CPC solution (0.7%)	std dev
0	6.3891	±0.125	6.389076	±0.125	6.389076	±0.125
15	6.0816	±0.046	5.435307	±0.010	3.922301	±0.059
30	5.9932	±0.022	5.003276	±0.043	3.495613	±0.069
60	5.9048	±0.042	4.306604	±0.073	2	±0.000

Table: Mean log CFU/g of *Listeria monocytogenes* cells on red pepper surfaces after different CPC treatments.

Time	treatment with CPC solution (0.2%)	std dev	treatment with CPC solution (0.4%)	std dev	treatment with CPC solution (0.7%)	std dev
0	6.022	±0.153	6.022	±0.153	6.022	±0.153
15	4.716	±0.000	4.003	±0.082	3.498	±0.010
30	4.598	±0.131	3.604	±0.121	2.000	±0.000
60	4.358	±0.169	3.366	±0.157	2.000	±0.000

Table: Mean log CFU/g of *Listeria monocytogenes* cells on broccoli surfaces after different CPC treatments.

Time	treatment with CPC solution (0.4%)	std dev	treatment with CPC solution (0.7%)	std dev	treatment with CPC solution (1%)	std dev
0	6.513	±0.094	6.513	±0.094	6.513	±0.094
15	6.045	±0.083	5.718	±0.065	5.063	±0.048
30	5.944	±0.097	5.292	±0.080	4.591	±0.101
60	5.539	±0.251	5.022	±0.044	2.521	±0.736

Table: Mean log CFU/g of *Listeria monocytogenes* cells on chicken surfaces after different CPC treatments

Time	treatment with CPC solution (0.4%)	std dev	treatment with CPC solution (0.7%)	std dev	treatment with CPC solution (1%)	std dev
0	6.235	±0.166	6.235	±0.166	6.235	±0.166
15	6.048	±0.074	5.703	±0.127	4.590	±0.032
30	5.969	±0.085	5.122	±0.160	4.443	±0.023
60	5.906	±0.019	4.969	±0.108	3.725	±0.221

Appendix IV:

Figure: Survival curves of *L. monocytogenes* cocktail inoculated on potato, red pepper, broccoli and chicken samples.