Microbial Decontamination and Rapid Cooling of Fresh Food Products by Plasma Integrated Low-Pressure Cooling

by

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Abstract

Fresh food products, including fruits, vegetables, raw meat and poultry have been associated with safety concerns and quality issues, owing to their susceptibility to rapid deterioration and microbial contamination. This research aimed to develop an integrated process to simultaneously cool and decontaminate high moisture food products.

Cold plasma (CP), a novel decontamination technology, was integrated with vacuum cooling to develop a plasma integrated low-pressure cooling (PiLPC) process. To evaluate the rapid cooling and microbial inactivation efficacies of the PiLPC process, fresh cut Granny Smith apples and *Salmonella enterica* serovar Typhimurium ATCC 13311 were used as the model food and microorganism, respectively. The influence of process parameters including treatment time, pressure, and post-treatment storage on inactivation of *Salmonella* on fresh-cut apples was investigated. Inactivation of *Salmonella* increased with treatment time, with a maximum reduction of 3.21 log CFU/g after 5 min of CP treatment at atmospheric pressure. Inactivation of *Salmonella* after CP treatment at 200 mbar were not significantly different from those at atmospheric pressure for the same treatment time. However, there was a significant decrease in the inactivation of *Salmonella* when samples were treated with CP at 50 mbar as compared to atmospheric pressure. Post-treatment storage at ~4 °C for 3 days after 3 min of CP treatment at 200 mbar reduced the total *Salmonella* population by > 6 log CFU/g. Depending on the sample surface to volume ratio, the cut apple temperature was reduced from room temperature to 1 °C in 3 to 7 min, when the pressure was reduced to 7 mbar. However, this PiLPC process resulted in high moisture loss in cut apples.

The second part of this research aimed to achieve higher rates of inactivation of *Salmonella* without an increase in CP treatment time, to reduce the moisture loss in fresh foods during the PiLPC process, and to improve the quality-related issues such as cut-edge enzymatic browning. This study evaluated and compared the effects of citric acid (CA) and CP alone and in combination on the inactivation of *Salmonella*, polyphenol oxidase (PPO) activity and the resulting enzymatic browning, the total phenolic content, and the moisture loss of cut-apples. The highest inactivation of *Salmonella* (5.68 log CFU/g) was observed after treatment of cut apples dipped in 5 % CA for 2 min combined with 3 min CP treatment. The highest PPO inactivation was observed when cut apples were dipped in 5 % CA for 2 min and then treated with 3 min of CP. The color of cut apples remained relatively unchanged with fresh like appearance during 7 days of storage after this combined treatment. Although the cooling time was increased when samples were pre-dipped in CA, it reduced the moisture loss of cut apples by more than 50 % during the PiLPC process. No significant reduction in phenolic content was observed during the PiLPC when the samples were pre-dipped in 5 % CA. These results indicate the potential of the PiLPC process for rapid cooling, microbial, and enzymatic inactivation in fresh food products. Future research using different fresh food products and other important pathogens is required to further develop this technology for industrial applications.

PREFACE

This thesis is an original work done by Abdullahi M. Adam at the Food Safety and Sustainability Engineering Lab at the University of Alberta under the supervision of Dr. Roopesh Mohandas Syamaladevi. The PiLPC apparatus described in chapter 3 and 4 was designed and assembled by me under the supervision of Dr. Roopesh Mohandas Syamaladevi and with the help of Dr. Ying Tsui.

A manuscript based on the study in Chapter 3 was submitted to a peer-reviewed journal for publication. A technical research abstract based on the results in Chapter 3 was peer-reviewed, and an oral presentation was delivered at the Food Innovation and Engineering Conference, held virtually from Nov 4 – 6, 2020. A manuscript based on the study in Chapter 4 is under internal review, to be submitted to a peer-reviewed journal for publication.

The acquisition of the spectra and the data analysis in Chapter 3 was conducted with the help of Barun Yadav. The methodology for estimating the activity of the polyphenol oxidase enzyme and the total phenolics content in Chapter 4 was validated and refined with the help of Brasathe Jeganathan. Several other collaborators contributed to the manuscripts, and their contributions were acknowledged accordingly.

Following manuscripts were prepared and submitted or under internal review.

Adam, A.M., Yadav, B., Prasad, A., Gautam, B., Tsui, Y., & Roopesh. M. S. Cooling of fresh cut apples and plasma assisted inactivation of *Salmonella* at low pressures. (Submitted to Food Research International)

Adam, A.M., Jeganathan, B., Vasanthan, T., & Roopesh. M. S. Improvement in the inactivation of *Salmonella* and polyphenol phenol oxidase by citric acid pre-dipping during plasma integrated low-pressure cooling of fresh cut apples. (Under internal review)

Dedicated

To my beloved

Mom and Dad

Mohamed and Ayaan

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List of Abbreviations

CDC	Centre for Disease Control
CFG	Canada's Food Guide
CFU	Colony forming unit
CP	Cold Plasma
DBD	Dielectric barrier discharge
DNA	Deoxyribose Nucleic Acid
DW	Distilled water
FDA	Food and Drug Administration
GRAS	Generally recognized as safe
PAA	Peracetic acid
PiLPC	Plasma integrated low-pressure cooling
POD	Peroxidase
PPO	Polyphenol oxidase
RH	Relative humidity
RONS	Reactive oxygen and nitrogen species
ROS	Reactive oxygen species
SA:V	Surface area to volume ratio
TPC	Total phenolics content
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UV	Ultraviolet
VC	Vacuum cooling
WHO	World health organization
YE	Yeast extract

Chapter 1: Introduction and Objectives

1.1 Introduction

Microbial foodborne illnesses have been identified as the largest category of emerging infectious diseases in Canada (Arthur, Jones, Fabri, & Odumeru, 2007). Aside from the physical and emotional toll on the individual, foodborne illnesses cost an estimated $1000 per case in health-care costs and lost productivity in Canada (Zhuang, Barth, & Hankinson, 2003). This does not include their costs to the food industry in terms of recall procedures and loss of brand trust. Among food-related outbreaks, 60-80% are of bacterial origin (Bruhn & Schutz, 1999), and among those, *Salmonella* spp. have been the leading cause, accounting for almost half of all bacteria-related outbreaks (Berger et al., 2010; Scallan et al., 2011).

Compared with other food products, the fresh and fresh-cut produce industry faces unique challenges that make it an easy route for human pathogens to reach the consumer plate. These risks include recent changes in farming practices, the globalized nature of the fresh produce supply, changing demographics, and finally, minimal processing of fruits and vegetables (James, 2005). Thermal treatment is often not a viable option for fresh-cut and most whole produce items. Many other treatments that processed foods receive are also not applicable. This means risk reduction is often a critical element for fresh produce. The use of sanitizing solutions in the production chain is the predominant method to reduce spoilage and pathogenic microorganisms (Qadri, Yousuf, & Srivastava, 2015). Novel liquid and gaseous sanitizers with improved lethality, deeper penetration, and less harmful by-products, when compared to traditional sanitizers like chlorine, have also been introduced. These include organic acids, ozone, and hydrogen peroxide (Ukuku, 2004; Whangchai, Saengnil, & Uthaibutra, 2006).

The potential of Cold Plasma (CP) technology has been widely studied lately; it employs multiple active antimicrobial components like reactive oxygen and nitrogen species (RONS), UV radiation, and charged atomic particles to simultaneously attack multiple targets in the bacterial cell. The appeal of CP lies in its potential to replace thermal and chemical processes for microbial inactivation. Its efficiency, lack of toxicity and minimal residues make it a more suitable for a wide range of applications in food decontamination and material sterilization (Patil, Bourke, & Cullen, 2016); where thermal and chemical treatment can damage the product and result in reduced quality. Further, CP can be integrated to existing food processing lines. On the other hand, CP has some disadvantages, such as color changes and elevated levels of moisture loss in some food products (Shashi K. Pankaj, Wan, & Keener, 2018).

Most studies on CP are conducted at atmospheric pressure, which offers several technical advantages by foregoing expensive and complicated setups that need airtight vacuum chambers and expensive vacuum pumps (Sen, Onal-Ulusoy, & Mutlu, 2019). However, low-pressure CP offers multiple advantages like low breakdown-voltages, the ability to utilize larger gaps, and more uniform plasma discharges (Schütze et al., 1998). In many processes that use low-pressure for other purposes, such as cooling or drying, it becomes a viable alternative to atmospheric CP.

Alongside the safety concerns discussed above, fresh and fresh-cut produce items show an accelerated decline in quality, which is initiated right after harvest. The signs of this deterioration include general decay, browning, and softening of cut surfaces. Immediate cooling or modified atmosphere storage are some of the techniques used to slow down and halt the biochemical activity of the products (Mena, Adenso-Diaz, & Yurt, 2011).

Vacuum cooling is one such fast cooling technology that offers much faster cooling times compared to traditional methods by utilizing the evaporative removal of heat, that can take place at lower pressures. However, it can cause elevated levels of moisture loss (Mahajan & Frías, 2012). Dipping in antioxidant and antimicrobial solutions is used to prevent the enzymatic browning of produce, reduce moisture loss, and improve microbial safety. Several organic acids are some of the most commonly used dipping solutions due to their generally recognized as safe (GRAS) status (González-Aguilar, Ruiz-Cruz, Cruz-Valenzuela, Rodríguez-Félix, & Wang, 2004). Recently there has been increased interest in combining different chemical or physical treatments to reduce the chemical doses, temperatures, and treatment times of any given technique (de Oliveira, Cossu, Tikekar, & Nitin, 2017). Combinations of acetic acid, lactic acid, and many other organic acids with ozone or UV have been reported. CP has been combined with multiple organic acids like lactic acid and peracetic acid for increased inactivation and better quality-retention (Liao et al., 2020). Also, CP could be integrated with conventional processes such as cooling, drying, heating in processing lines to improve the efficiency of these processes and reduce microbial load in food products.

1.2 Hypotheses

The overall hypothesis is that an integrated low-pressure plasma and vacuum cooling system will reduce microbial load and achieve the required cold storage temperatures of high moisture fresh food products in a single process. This integrated process can take advantage of existing low-pressure production lines for the simultaneous cooling and decontamination of fresh produce at low pressures (chapter 3).

The process can be divided into few stages depending on the pressure fluctuations in the vacuum chamber (decreasing, stationary, and increasing pressure). The hypothesis was that it is

best to apply CP before any pressure increase takes place in the chamber, since the pressure increase can facilitate bacterial infiltration and thus hinder the inactivation of microorganisms (chapter 3).

Organic acids can damage the cell membrane of microorganisms; the hypothesis was that treatment with citric acid (CA) before CP will facilitate the penetration of RONS into the cell and have a synergistic effect on the inactivation of *Salmonella*.

Pre-dipping the samples in antioxidant/antimicrobial solutions such as CA before CP treatment and vacuum cooling will significantly reduce enzyme activity and increase yield by replacing the moisture lost during CP treatment and cooling at low pressures (chapter 4).

1.3 Objectives

The main objective of this thesis was to develop a plasma integrated low-pressure cooling (PiLPC) process for cooling and microbial decontamination of high moisture fresh food products. The specific objectives were to:

1) Design and build the plasma integrated low-pressure cooling (PiLPC) system (chapter 3),
2) Compare the antimicrobial efficacy of CP at atmospheric pressure and multiple sub-atmospheric pressures, e.g., 200 and 50 mbar (chapter 3) using cut apples and *Salmonella* as model food and microorganism, respectively,
3) Compare the emission spectra of CP at atmospheric pressure with that of CP at 200 and 50 mbar pressures (chapter 3),
4) Evaluate how the CP and cooling sequence affect the level of inactivation of *Salmonella* on cut apples (chapter 3),
5) Monitor the total *Salmonella* population during post-treatment storage at refrigerated

temperatures (Chapter 3),

6) Evaluate how the PiLPC affects the moisture loss and determine the cooling time of the samples (chapter 3),
7) Investigate how the pre-dipping of cut apples in specific concentrations of CA followed by CP treatment affects the inactivation of *Salmonella*, polyphenol oxidase activity, total phenolics content, and color parameters after the PiLPC process, and during the following post-treatment storage in a refrigerated environment (chapter 4), and
8) Evaluate how pre-dipping of cut apples in CA affects moisture loss during the PiLPC process (chapter 4).

Salmonella enterica serovar Typhimurium was selected as the model microorganism in this thesis due to its implication in many recent fresh produce related recalls and outbreaks as described in the next chapter. As a popular snack, cut apples were selected to evaluate the efficacy of the PiLPC process (chapter 3). The sub-atmospheric pressures of 200 and 50 mbar were selected based on preliminary tests involving a wider set of sub-atmospheric pressures. The operating sub-atmospheric pressure (200 mbar) and the process sequence in chapter 4 were selected based on results from chapter 3.

Chapter 2: Literature Review

2.1 Fresh Produce

Fresh produce and fresh-cut fruits are crucial components of any healthy diet and are recommended by global and local health agencies (Denis, Zhang, Leroux, Trudel, & Bietlot, 2016). Canada's Food Guide (CFG) recommends the consumption of 4-10 servings of fruits and vegetables; mostly in the form of edible produce rather than juice (Black & Billette, 2013). The availability of fresh produce increased considerably, with more types of fruits and vegetables being accessible year-round to consumers, which would help them meet these recommended intake levels (James, 2005). However, the consumption of fruits and vegetables decreased by about 13% in Canada from 2004 to 2015 (Tugault-Lafleur & Black, 2019). It also decreased in the 28 European Union (EU) countries by 13.1% in 2013 compared to the average consumption between 2000 and 2006 (Baselice, Colantuoni, Lass, Nardone, & Stasi, 2017).

But, the sub-category of fresh-cut produce, also known as minimally processed fruits and vegetables, experienced tremendous growth since they first appeared on the market in the 1980s. Consumers showed huge interest in them due to their convenience, variety, and flexible serving sizes. Although their share in the fresh produce market is still low, they have been growing at around 16% every year between 2009 and 2014 in the EU (Euromonitor International, 2015). This trend is far more advanced in the USA, where the per capita consumption of fresh-cut fruits and vegetables was 3-10 times higher than what it was in European countries, such as the United Kingdom, France, Germany, Italy, and Spain in 2006 (Abadias, Usall, Anguera, Solsona, & Viñas, 2008). Fresh-cut fruits and vegetables were expected to constitute more than 25% of fresh produce sales in the USA by 2010 (Faye, 2004). Even though they are highly recommended, keeping the

fresh fruit and vegetable supply safe and of high quality poses a considerable challenge (Prado-Silva, Cadavez, Gonzales-Barron, Rezende, & Sant'Ana, 2015).

2.1.1 Foodborne illnesses and outbreaks

Although fresh produce of plant origin was considered relatively safer than foods of animal origin, the number of outbreaks related to fresh produce and unpasteurized fruit juices has been increasing recently. According to Doyle & Erickson (2008), the percentage of foodborne outbreaks associated with fresh produce was around 0.7% in the 1970s. This rose to 6 and 13% of the total identified foodborne cases by 1990 and 2007, respectively. Leafy greens, in particular, were implicated in more illnesses than any other food products constituting 22% of the total reported cases by 2014 (Doona et al., 2015). Table 2.1. shows a number of produce related outbreaks in recent years.

Table 2.1 List of major foodborne outbreaks associated with fresh produce and fresh-cut fruits in the USA between January 2019 and October 2020 (CDC, 2020).

Date	**Product**	**Cases / Hospitalizations**	***Pathogen***
August 2020	Peaches	101 / 28	*Salmonella* Enteritidis
February 2020	Sprouts	51 / 3	*Escherichia coli* O103
December 2019	Fruit mix	165 / 73	*Salmonella* Javiana
December 2019	Fresh Blackberries	20 / 11	Hepatitis A
December 2019	Salad Mix	10 / 4	*E. coli* O157:H7
June 2019	Fresh papaya	81 / 27	*Salmonella* Uganda
Spring 2019	Vegetable trays	5 / 0	*Salmonella* Infantis
April 2019	Pre-cut Melons	137 / 38	*Salmonella* Carrau

In addition to this, it is safe to assume that the number of actual foodborne illnesses is considerably higher than those identified and cataloged by the health authorities. For reference; it is estimated that only 1 in 38 cases of *Salmonellosis* are reported to the responsible authorities (Hanning, Nutt, & Ricke, 2009). This is due to several reasons; firstly, treatment may not be sought or tests not performed in the hospital; secondly, pathogens may be transmitted through other routes like person

to person or through non-food vehicles like water; finally, source attribution of pathogens can complicate identifying food outbreak (Wilcock, Pun, Khanona, & Aung, 2004).

2.1.2 Microbial risks associated with fresh produce

The factors increasing the risk of contamination in fresh and fresh-cut produce include:

Farming practices: The increasing demand for organic products promotes the use of organic fertilizers (manure) rather than chemical fertilizers (Beuchat, 2002). This may be both economically and environmentally beneficial, but it will inevitably lead to increased exposure to fecal contamination in the produce (Jacobsen & Bech, 2012).

Importing: The willingness of consumers to pay for fresh produce year-round and the developments in distribution technologies has created an upward trend towards increased imports of fruits and vegetables (Beuchat, 2002). This is even more true in countries like Canada where the growing season is rather short (Denis et al., 2016). Canada regularly imports far more fruits and vegetables than it exports (AAFC, 2018, 2019). Around 88 and 41% of fruits and vegetables sold in Canada, respectively, were imported from countries with warmer climates, with the primary sources being the USA, Mexico, and Chile (AAFC, 2019). These trends can widen the geographical distribution of outbreaks and make their tracing more difficult.

2.1.3 Microbial risks of minimally processed produce

Pathogenic bacteria do not commonly grow on the surface of produce due to their inability to produce the enzymes necessary to breakdown the epidermis of fruits and vegetables (Artes, Gomez, & Artes-Hernandez, 2007; Qadri et al., 2015). The intact produce surfaces in the farm environment is also an unstable growth medium in terms of available moisture and humidity, the temperature of the surrounding environment, and even the intensity of the UV light from the sun (Harris et al., 2003). However, after cutting or slicing the produce, pathogens have access to the

inner tissue with its nutrient-laden juices and suitable water activity (Rajkowski & Baldwin, 2002; Rolle & Chism, 1987). In addition , the cut surfaces of fruits and vegetables continue metabolizing, especially in the first few hours after cutting or peeling, which will provide more nutrients for spoilage and pathogenic microorganisms (Artes et al., 2007; Rajkowski & Baldwin, 2002).

Another popular practice is, mixing different fruits or vegetables into one prepackaged convenience item after cutting, which can complicate the ability to anticipate the types of organisms and their interactions with each other, and with their new environment (Rolle & Chism, 1987). For example, fruits like cantaloupe and melons that grow directly on the surface have a different microbial composition than ones that grow on trees or those that grow under the ground like carrots. Also, different products have different organic compounds that facilitate or hinder the growth of spoilage and pathogenic microorganisms (Keeratipibul, Phewpan, & Lursinsap, 2011), so the risks that can arise from mixing multiple food items must be considered, as well.

2.2 Pathogens of concern

Typically, the microflora of fruits and vegetables is composed of non-pathogenic spoilage bacteria, yeast, and molds. In fruits, the acidic environment usually prevents the growth of pathogenic bacteria, while in vegetables, the normal flora dominates the local environment, with populations reaching up to $10^4 – 10^7$ CFU/g, the vast majority of which are Gram-negative bacteria such as *Pseudomonas* or *Enterobacter* species (Francis, Thomas, & O'Beirne, 1999), which can make the growth of pathogens unlikely (Rolle & Chism, 1987). Nonetheless, spore-forming bacteria like *Clostridium* spp. and *Bacillus cereus* from the soil (Hanning et al., 2009) or non-spore-forming bacteria like *Campylobacter jejuni* and *Vibrio* spp. can contaminate fresh produce through cross-contamination (James, 2005). The most common microbial hazards associated with fresh produce are gram-negative enteric pathogens, in particular different serovars of *S. enterica*, *E. coli*

O157:H7, and *Shigella* spp., which were identified as the most significant hazards for fresh produce by the Canadian Food Inspection Agency (CFIA, 2008).

2.2.1 *Salmonella* spp.

Majowicz et al. (2010) estimated the global toll of *Salmonella*-caused gastroenteritis to be around 93.8 million cases and around 155,000 deaths. The majority of these were because of foodborne illnesseses in the developed world, while a larger proportion was likely waterborne in the developing world. Since they were first isolated from the intestines of pigs, *Salmonella* spp. were found to live in the intestines of many wild and domesticated animals, but poultry is considered the most common *Salmonella* source, and traditionally poultry and meat were implicated in most cases of salmonellosis. However, increased number of salmonellosis cases are originating in produce (Hanning et al., 2009; Jacobsen & Bech, 2012), table 2.2. shows a list of the latest produce related recalls caused by suspected *Salmonella* contamination.

2.3 Decontamination approaches

Many of the treatments that processed foods receive, such as thermal treatment, are not applicable to fresh produce. This means risk reduction is often a critical element for fresh produce. Essential factors that reduce the risk of contamination are water quality control, protection from fecal contamination, and cold chain temperature management (Lynch, Tauxe, & Hedberg, 2009).

The use of sanitizers is the predominant method used to reduce spoilage and pathogenic microorganisms (Qadri et al., 2015). The efficacy of the sanitization step depends on product-related factors such as surface characteristics, microbial factors including species of the bacteria, surface attachment, and biofilm formation, and sanitizer related factors including concentration and pH (Allende, Selma, López-Gálvez, Villaescusa, & Gil, 2008).

Table 2.2 Recent *Salmonella* related recalls associated with fresh produce between January 2019 and October 2020 in the USA and Canada* (CFIA, 2020; FDA, 2020).

Date	Brand name	Recalled product	Company
10/07/2020	Kandy Brand, Meijer Brand	Whole Cantaloupe, Select Cut Cantaloupe Fruit Trays & Bowls	Meijer
08/22/2020	Wawona, Prima, Kroger, Organic Marketside, and Wegmans	Peaches	Prima Wawona
08/22/2020*		Fresh peaches	Multiple
08/21/2020	Wawona, Prima, Kroger, Organic Marketside, and Wegmans	Bagged Peaches	Prima Wawona
08/19/2020	Hello Fresh	Onions	HelloFresh
08/10/2020	Progressive Produce LLC	Red and yellow onions	Progressive Produce LLC
07/30/2020*		Red onions	Sysco
04/09/2020	Organic Go Smile	Raw Coconut	International Harvest, Inc
03/11/2020*	Sobeys and Foodland	Asian vegetable mix	Sobeys Inc.
03/10/2020*	Fresh Sprouts brand	Fresh bean sprouts	Fresh Sprout International
12/08/2019	Tailor Cut Produce	Fruit Luau	Tailor Cut Produce
12/03/2020*	President choice	Coleslaw	Loblaw Companies Limited
12/03/2019	President's Choice, Marketside	Colorful Coleslaw	Dole Fresh Vegetables, Inc.
08/09/2019	Dole	Baby Spinach	Dole Fresh Vegetables, Inc.
05/02/2019	Caribeña	Papaya Maradol	Grande Produce
04/18/2019	Caito Foods, Renaissance Food Group, Open Acres, Garden Highway, and others	Fresh cut watermelon, honeydew, cantaloupe, and mixed fruit melons	Caito Foods
04/29/2019	ChloroFields	Micro-Greens Asian Mix	ChloroFields
02/01/2019	Satur Farms	Baby Spinach and Mesclun	Satur Farms
01/23/2019*	Veg-Pak	Thai chili peppers	Veg-Pak
01/21/2019*	Hello Fresh, Chefs Plate	Red chili	HelloFresh Canada Inc
01/20/2019*	Canada Herb	Red chili	Canada Herb

However, adhesion and internalization of pathogens into the product limits the efficacy of washing the surface with chemical sanitizers. According to Niemira, (2012), conventional washing can often result in less than a 2 log reduction of pathogens.

Natural preservatives like bacteriocins, essential oils, and edible coatings (Qadri et al., 2015) can also be used to improve the safety of fresh fruits and produce. Several bacteriocins are recognized as safe through a long history of human exposure, and have been approved as GRAS (Schulz et al., 2015). Also, lactic acid bacteria showed promising antimicrobial action by deploying several mechanisms, mainly in the form of producing antimicrobials including organic acids, and bacteriocins (Siroli, Patrignani, Serrazanetti, Gardini, & Lanciotti, 2015).

In a meta-analysis of the effect of alternative sanitizers (e.g., ozone and organic acids) on the three main bacterial pathogens *Salmonella*, *E. coli* O157:H7, and *Listeria monocytogenes,* Prado-Silva et al., 2015*,* analyzed results from 44 studies and found that both treatment time and temperature significantly affected the mean log reduction of pathogens. They also reported that *E. coli* had more resistance to ozone, while *Salmonella* and *L. monocytogenes* were more resistant to organic acids. In terms of product characteristics, bacteria on leafy produce exhibited less reduction compared to that on non-leafy products.

In conclusion, a perfect solution, which is extremely effective for microbial decontamination in fresh produce while being cost effective and sustainable, does not exist. In this context, novel sustainable while effective antimicrobial approaches will be useful for the fresh produce industry. Also, hurdle approaches that combine multiple treatments are mostly necessary to deliver safe and high quality fresh and fresh-cut produce items (Jacobsen & Bech, 2012).

2.4 Cold Plasma

Plasma the forth state of matter, is a partially ionized gas generated by the progressive application of electric energy to a wide range of gases that include air, nitrogen, noble gases like helium and argon, and mixtures of two or more gases (Niemira, 2012). Plasma, which typically consist of free electrons and fully ionized atoms (ions) or partially ionized atoms/molecules (ions) can be divided into hot thermal plasma and cold non-thermal plasma (CP). When the nucleus (protons and neutrons) are in thermal equilibrium with the electrons, and the plasma temperature in such conditions can be in the order of 10^6 to 10^8 K, the plasma is characterized as thermal. On the other hand, in cold non-thermal plasma, the partially ionized atoms/molecules are at nearly ambient temperature, although the free electrons are at much higher temperatures, the heavier partially ionized atoms/molecules are at nearly room temperature in such cases (Zainal, Redzuan, & Misnal, 2015).

CP is generated using several sources and mechanisms, but currently, dielectric barrier discharge (DBD) and plasma jet are the two most commonly studied in food-related applications (Deng et al., 2020). DBD utilizes a high voltage electrode and a ground electrode with a dielectric barrier covering one or both electrodes to limit current conduction and charge transfer (Roth, Rahel, Dai, & Sherman, 2005). It is a simple, safe, and flexible design. With the recent advances in their design as thin-layers of flexible electrodes that form a plasma container for in-package plasma treatments, they promise to achieve a more uniform and standardized surface treatment (Lu, Cullen, & Ostrikov, 2016; Yong et al., 2015).

2.4.1 Reactive species

Depending on the working gas and process parameters such as voltage and excitation frequency, a wide variety and concentrations of reactive species, UV photons, and atomic particles are

generated in CP. The reactive species and UV light are formed by the primary collisions of electrons with heavy particles (atoms and molecules) and the secondary collisions of ions and radicals – formed from the primary collisions – with each other and with molecules in their environment. These processes generate numerous reactive oxygen species (ROS) and reactive nitrogen species (RNS).

Table 2.3 shows a number of these primary and secondary collisions that take place during the generation of CP (Björn Surowsky, Schlüter, & Knorr, 2015; Whitehead, 2016). Many of these reactions are one step in a chain of reactions, and their by-products, like hydroxyl radical, have extremely short half-lives measured in nanoseconds (Hrycay & Bandiera, 2015). The very high reactivity of such species enables them to interact and oxidize all the components of living cells, making them crucial components of CP.

Table 2.3 Primary and secondary plasma collisions that generate the reactive species of CP.

Primary Collision processes			
Process name	***Formula***	***Example***	***Radical***
Excitation	$A + e^- \rightarrow A^* + e^-$	$O_2 + e^- \rightarrow O^{\cdot} + O^{\cdot} + e^-$	Atomic Oxygen
Dissociation	$AB + e^- \rightarrow A + B + e^-$	$H_2O + e^- \rightarrow H^{\cdot} + OH^{\cdot} + e^-$	Hydroxyl
Ionization	$A + e^- \rightarrow A^+ + 2e^-$	$H_2O + e^- \rightarrow H_2O^+ + 2e^-$	Ionized Water
Secondary collision processes			
Oligomerization	$A + B \rightarrow AB$	$NO_2 + N \rightarrow N_2O$	Nitrous oxide.
Recombination	$A + B + C \rightarrow AB + C$	$O + O_2 + O_2 \rightarrow O_3 + O_2$	Ozone

A, B and C represent heavy particles like atoms and molecules, and e^- represents electrons. The (*) indicates an atom or a molecule in an excited state.

2.4.2 Working mechanisms

The different components of CP act simultaneously on multiple targets in the bacterial cell as shown in fig 2.1. There are multiple microbial inactivation mechanisms during CP treatment, possibly due to the presence of several reactive species that could effectively reduce the number of highly resistant bacterial pathogens in food (Bourke, Ziuzina, Han, Cullen, & Gilmore, 2017).

The high variability in the contribution of different reactive species also makes it difficult to identify their relative contribution to microbial inactivation, which can change widely based on process conditions and target microorganisms (Liao et al., 2018; Patange, 2019).

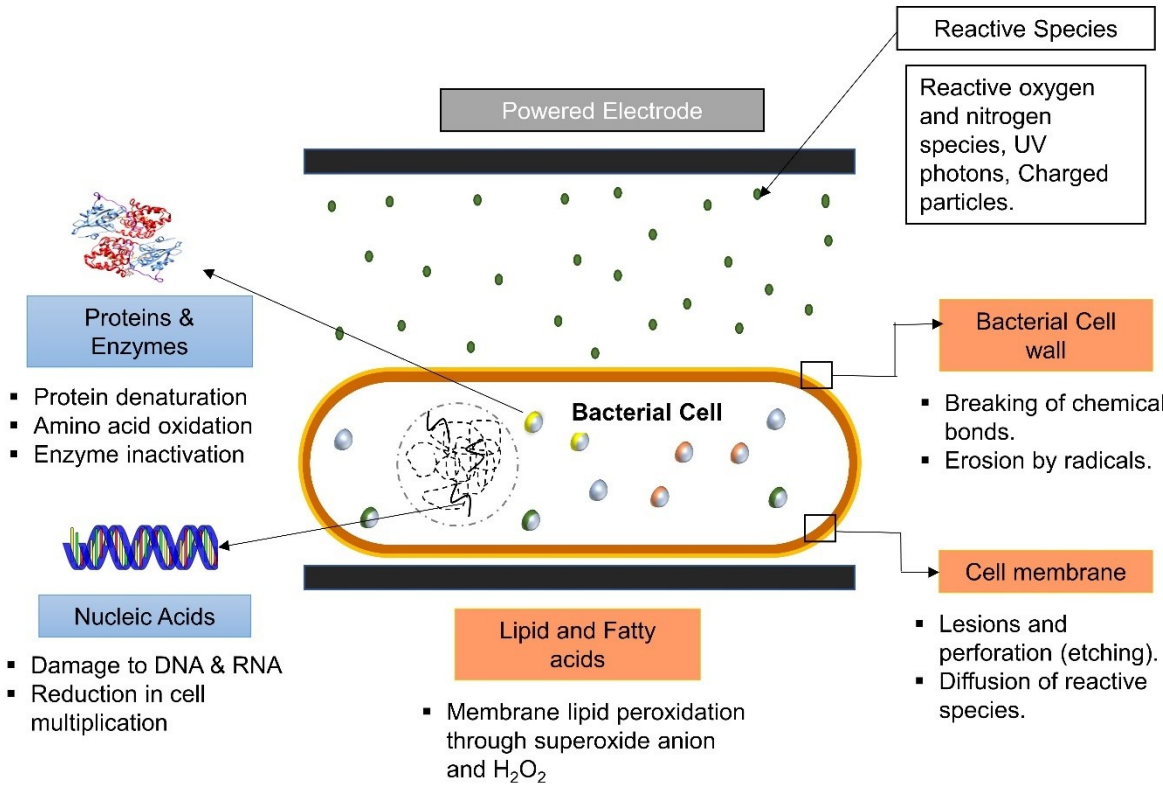

Figure 2.1 diagram of the mechanisms of cold plasma inactivation of bacteria (adapted from Misra and Jo 2017).

The components of CP can be divided into three main categories.

Reactive oxygen and nitrogen species (RONS) are considered the primary contributors to bacterial inactivation (Liao et al., 2020; Patange, 2019; Sohbatzadeh, Hosseinzadeh Colagar, Mirzanejhad, & Mahmodi, 2010). They include species such as ozone (O_3), atomic oxygen ($O^{\cdot}$), hydroxyl radical (OH), nitrite (NO_2^-), nitrate (NO_3^-), and hydrogen peroxide, that put all macromolecules of the cell under high oxidative and nitrosative stress (Mai-Prochnow, Clauson,

Hong, & Murphy, 2016). Their first target is the lipids in the cell membrane; for example, the OH radical causes lipid peroxidation by subtracting a hydrogen atom from unsaturated fatty acids leading to the formation of methylenedianiline, which is used as an indicator for lipid peroxidation (Patange, 2019). They also significantly reduce membrane-bound proteins, decomposing them into amino acids (Hou et al., 2008). Finally, their list of targets includes DNA, which at atmospheric pressure is degraded through oxidation rather than UV radiation (Korachi & Aslan, 2011).

Charged particles are responsible for the etching and erosion effects of CP. The charge accumulation on cell membranes may exceed their tensile strength and cause electrostatic disruption (Feizollahi, Misra, & Roopesh, 2020). The formed lesions open the gateways for RONS into the cell causing total disintegration of the cell and leakage of its components if the lesions are big enough (Mai-Prochnow et al., 2016). The abundance and relative importance of charged particles may increase in the absence of oxygen and nitrogen, such as times when pure; noble gases are used for CP generation (Deng et al., 2020).

UV radiation damages the microbial cells by directly destroying the genetic material, inducing a reaction between two thymine bases. UV radiation is believed to be of minor importance in atmospheric plasma treatments since air molecules absorb most UV photons before they reach the sample. However, UV is of major significance in low-pressure plasma sterilization, where less of the photons are absorbed by the air molecules (Moisan et al., 2002).

2.4.3 Factors influencing cold plasma efficacy

The rate of inactivation depends on factors such as the plasma generation setup, the characteristics of the bacterial species, growth phase, biofilm formation, characteristics of the product or surface, (Feizollahi et al., 2020). For example, Gram-negative bacteria are seemingly more sensitive to CP than Gram-positive bacteria. A study that compared three Gram-positive and three Gram-negative

bacteria in planktonic and biofilm forms found that the Gram-positive bacteria had higher resistance to CP (Mai-Prochnow et al., 2016). They attributed this mainly to the thickness of the cell wall, which was around 20-80 nm in Gram-positive bacteria and less than 10 nm in Gram-negative bacteria. Several studies also reported that Gram-positive bacteria showed signs of intracellular damage more often than Gram-negative bacteria, which were more likely to show signs of disruption and disintegration (Patil, Bourke, & Cullen, 2016; Stoffels, Sakiyama, & Graves, 2008); this indicates that different inactivation mechanisms are at play.

2.4.4 Studies on low-pressure CP

Although earlier research in CP utilized low-pressures, research on the plasma applications for sterilization shifted towards atmospheric pressure applications (Fiebrandt, Lackmann, & Stapelmann, 2018). Atmospheric CP offers a number of engineering advantages since it does not use expensive and complicated setups that need airtight vacuum chambers and vacuum pumps (Sen et al., 2019), and it can also offer a continuous operation with conveyor belts; which promises a high throughput process. On the other hand, the size of the vacuum chamber limits low-pressure CP applications.

Nonetheless, low-pressure CP offers several advantages such as a low breakdown voltage (often at higher voltages, arcing occurs between the electrodes), a uniform discharge over a large volume of gas, and a relative abundance of ions and many radicals like atomic oxygen even compared to atmospheric CP (Schütze et al., 1998). Considering that many fresh food processes already utilize vacuum for packaging, cooling, drying, and other applications (An, Park, & Lee, 2009; Sablani, 2006; Thompson & Chen, 1988), the use of low-pressure CP in these processes becomes a viable alternative.

A number of recent studies used low-pressure CP for the inactivation of *E. coli* and *Listeria innocua* on fresh-cut apples (Segura-Ponce, Reyes, Troncoso-Contreras, & Valenzuela-Tapia, 2018), and *S.* Typhimurium on the surface of spinach, tomato, and potato (M. Zhang, Oh, Cisneros-Zevallos, & Akbulut, 2013); however, no direct comparison between the antimicrobial efficacy of plasma at open-air and lower pressures, in the range used in the food industry has been reported in the literature.

2.4.5 CP-based hurdle technologies

Often a combination of treatments is necessary to maintain quality and keep the microbial population under control in foods (Rajkowski & Baldwin, 2002). Recently, CP has been combined with a range of other treatments such as organic acids (Chaplot, Yadav, Jeon, & Roopesh, 2019), ultrasonication (Liao et al., 2018), and thermal treatment (Mehta & Yadav, 2020) to improve the rate of microbial inactivation and quality retention, while keeping the treatment temperature or chemical doses to a minimum.

The order of the different treatments can have a significant effect on the rate of inactivation. For example, (Chaplot et al., 2019) reported that treating the samples with peracetic acid first, followed by CP, resulted in 2 times more inactivation than when they were treated with CP first. On the other hand, (Liao et al., 2018) attained maximum inactivation when they started with CP and followed it by ultrasonication. The microbial inactivation mechanisms of different treatments can widely vary, but it is necessary to consider how different treatments can interact in ways that improve their efficacy or provide cross-protection; where the first treatment evokes resistance in the treated microbial population, making the subsequent treatment less effective (Liao et al., 2020).

2.5 Post-harvest cooling

Refrigeration is one of the most crucial technologies for the modern food supply (Floros et al., 2010). The dependence of the food supply on refrigeration is mainly due to the seasonality of many products and, more importantly, the increasing distance between farms and population centers. This is only projected to increase in the future, with 68% of the global population projected to live in urban areas by 2050 (United Nations, 2018).

The term used to describe this is the cold chain, which is the interconnected steps and techniques necessary to deliver the products – especially fresh ones – to the final consumer. It starts with the initial cooling after harvest using techniques like hydro cooling, vacuum cooling, or cooling using crushed ice, followed by storage, utilizing other techniques like cold rooms down to cold transportation and finally the home refrigerators (Mahajan & Frías, 2012). Proper management of the cold chain is crucial to avoid spoilage and ultimately reduce waste (Mena et al., 2011). In addition to maintaining quality, proper temperature control also determines the potential for pathogen growth (James, 2005).

Vacuum cooling: Vacuum cooling is one such common technique for the initial cooling of produce and fruits. It is an evaporative cooling method that has been in use since 1948 (Thompson & Rumsey, 1984). Its advantage over other traditional cooling methods like air blast, water immersion, and cold room lies in its faster cooling times, which can reach up to 400-800% faster (Zheng & Sun, 2004). This made it preferable for cooling of foods like vegetables and some fruits that require fast cooling after harvest for quality preservation and shelf life prolongment (McDonald & Sun, 2000). Vacuum cooling is also being successfully applied to liquid and semi-liquid foods such as soups and baked products (Kratochvil & Holas, 1984). The main

disadvantages of vacuum cooling are moisture loss, which is more than other traditional cooling methods, and the high equipment cost (Mahajan & Frías, 2012).

2.6 Quality issues associated with fresh and minimally processed produce and intervention techniques

The short shelf life of fresh-cut products significantly impairs their value. While the expected shelf life of raw produce is likely to be weeks or even months, fresh-cut produce items have a short shelf life of around 4 to 10 days (Siroli et al., 2015). The most serious quality issues besides the safety concerns discussed earlier include: softening, flavor deterioration, and decay by spoilage microorganisms (Rajkowski & Baldwin, 2002). In particular, browning is considered as a leading contributor to product waste during postharvest processing (Mathew & Parpia, 1971), since appearance and color are the initial factors that determine consumer acceptance. Several browning causes have been identified, but cut-edge browning, in particular, is catalyzed by endogenous food enzymes like polyphenol oxidase (PPO) (Han, Cheng, & Sun, 2019).

2.6.1 Polyphenol oxidase enzyme

Most of the enzymatic browning reactions in fresh produce are caused by PPO (Gorny, Hess-Pierce, Cifuentes, & Kader, 2002), also known as tyrosinase. PPO is composed of a polypeptide chain and two copper atoms. Usually, it is stored in the chloroplast, separate from the phenolic compounds in the vacuole, but after cutting, this compartmentalization ends, and the two compounds mix and interact with each other (Otwell & Iyengar, 1992).

PPO catalyzes a hydroxylation and an oxidation reaction; in the first one, the mono-phenols are slowly converted to the colorless di-phenols, which are in turn oxidized into the highly reactive o-quinones (Suttirak & Manurakchinakorn, 2010). These compounds can form brown pigmentation (melanin) by polymerizing or interacting with other food components, like amino

acids (Mayer & Harel, 1978). Aside from the color changes, enzymatic browning can also oxidize important nutrients like ascorbic acid, reducing the nutritional value of the product (Pizzocaro, Torreggiani, & Gilardi, 1993). Ordinary refrigeration is not adequate to stop or reverse these changes (Kolk, 1975), so, up to six different classes of anti-browning agents are currently in use. Their action mechanisms include inhibiting the enzyme by binding its copper atoms and unfolding its polypeptide chain by reducing the pH of its environment.

2.6.2 Anti-browning additives and mechanisms

Heat treatments like blanching are the most effective ways to inactivate endogenous food enzymes and are still used for frozen and canned fruits or vegetables but are not practical for fresh-cut products (Otwell & Iyengar, 1992). Here, several surface treatments, typically dipping are used, the most common of which are sulfites. But, sulfites have some health risks, especially for certain vulnerable groups, which lead to several regulatory agencies banning their use in salads and some fresh produce items (FDA, 1986).

Organic acids can be good anti-browning agents; they are non-toxic and have a GRAS status (Generally Recognized As Safe). Many of the most common organic acids like citric acid (CA), oxalic acid, and acetic acid are weak acids that are naturally present in plant foods (González-Aguilar et al., 2004). Their efficacy depends on their concentration, and it can vary based on the product type and cultivar (Suttirak & Manurakchinakorn, 2010) or the temperature of the treatment solution.

2.6.3 Citric acid

CA is the most abundant organic acid in plants (Del Campo, Berregi, Caracena, & Santos, 2006), and it is the most widely used for browning inhibition. It uses two different mechanisms to inhibit enzymatic browning; it reduces the superficial pH of the cut fruits to less than the optimal level

for PPO activity (Otwell & Iyengar, 1992), and it acts as a chelating agent, binding the Cu atoms of the PPO enzyme (Son, Moon, & Lee, 2001).

CA application in the cut-fruits industry usually involves dipping in a diluted solution containing CA, and dipping times in the literature range from 1-5 min (Soliva-Fortuny & Martín-Belloso, 2003). At very low concentrations, CA can act as a prooxidant, promoting PPO activity, but its anti-browning efficacy increases with increased concentrations (Jiang, Pen, & Li, 2004). CA is considered safe, and no toxicity level has been reported.

2.6.4 CP for enzyme inactivation

CP showed an interesting ability to reduce the activity of browning enzymes like PPO and peroxidase in fresh produce (Surowsky, Fischer, Schlueter, & Knorr, 2013). It induces conformational changes in the secondary structure of the enzymes leading to inactivation, and the extent of this inactivation depends on treatment time and voltage (Tappi et al., 2014). The ROS are believed to be the leading factors in these conformational changes. These highly reactive species can cleave peptide bonds and oxidize chemically reactive amino acid side chains (Bjoern Surowsky et al., 2013).

CP treatment is also reported to increase the firmness of the samples; this is contrary to the tissue softening common in other anti-browning treatments that involve dipping and usually require the use of anti-softening compounds (Tappi et al., 2014).

2.7 Summary

Significant research is needed to understand the bacterial inactivation efficacy of CP at the sub-atmospheric pressure ranges at which low-pressure food processing operations like vacuum cooling and vacuum drying take place. This gap in the literature is addressed in Chapter 3, where a plasma integrated low pressure cooling (PiLPC) system was developed with the intention of

using low pressures not only for produce decontamination with CP, but also for evaporative cooling. Also, a range of sub-atmospheric pressures were compared to atmospheric pressure in terms of bacterial inactivation efficacy during CP treatment. Chapter 3 also identified other relevant parameters for bacterial inactivation during CP treatment at sub-atmospheric pressures such as the timing of CP treatment in the process sequence. In Chapter 4 the PiLPC process was further optimized to address quality issues related to the the cut-produce such as enzymatic browning and to achieve microbial inactivation levels in the range recommended by regulatory agencies without increasing the CP treatment time.

Chapter 3: Cooling of Fresh Cut Apples and Plasma Assisted Inactivation of *Salmonella* at Low Pressures

3.1 Introduction

Although the demand for minimally processed fruits and vegetables keeps growing, the need to preserve their fresh like properties for extended periods remains a challenge (Soliva-Fortuny & Martín-Belloso, 2003). Fresh fruits, vegetables, and produce can get contaminated at different stages of the farm-fork continuum, from irrigation water and wild birds before harvest, equipment and workers during harvest, and transportation and retail operations after harvest (James, 2005). The most common processes to produce ready-to-eat fruits and vegetables include cutting and peeling, which may result in physiological deterioration and microbial contamination (Suttirak & Manurakchinakorn, 2010). Also, cutting and peeling may reduce the natural defenses of fruits and vegetables, resulting in increased outbreak risks of ready-to-eat products (Doyle & Erickson, 2008). Hence, the disinfection of fresh fruits, vegetables, and produce is extremely important. A wide variety of liquid and gaseous disinfectants are used by the food industry to improve the microbial safety of fresh fruits, vegetables, and produce (Joshi, Mahendran, Alagusundaram, Norton, & Tiwari, 2013).

CP has received increased attention as a novel decontamination technology recently. CP contains a mixture of reactive species, UV photons, and positively and negatively charged atomic particles (Laroussi & Leipold, 2004) that have germicidal effects. These CP reactive species can oxidize the outer cellular structures of bacteria, interfere with cellular respiration systems, and rupture the cell membrane of gram-negative bacteria (De Geyter & Morent, 2012). Several studies tested the inactivation efficacy of CP against pathogens on fresh fruits, including *Salmonella* on apples (Niemira & Sites, 2008) and cantaloupe (Critzer, Kelly-Wintenberg, South, & Golden,

2007), and *Escherichia coli* O157:H7 on strawberries (Schwabedissen, ŁAcińSki, Chen, & Engemann, 2007), and cut mango (Perni, Shama, & Kong, 2008).

Fresh food products have a short shelf life, and they start deteriorating immediately after harvest. Rapid cooling is necessary to preserve the quality of these products and extend their shelf life (Ambaw et al., 2013). Vacuum cooling, based on the concept of evaporative cooling, is a rapid industrial cooling method used for fresh food products. This method can be used to reduce the temperature of porous, high moisture products, multiple times faster than the conventional cooling methods such as air blast and water immersion (Wang & Sun, 2001). Conventional cooling methods rely on convection and conduction, where heat transfer occurs from the surface of the product to the center or vice versa. However, in vacuum cooling, water is simultaneously evaporated from the surface and the inside matrix of the product through its cavities and pores, generating a fast and uniform cooling effect (Mc Donald & Sun, 2001; Zheng & Sun, 2004). Vacuum cooling has long been established as a cooling method for leafy vegetables such as lettuce, but it also shows promising results for the fast cooling of fruits like strawberries, carrots, and melons (McDonald & Sun, 2000).

The PiLPC process takes advantage of existing production lines for the generation of CP at sub-atmospheric pressure environments, i.e., generating CP with less power (Niemira, 2012) and maintaining larger gaps between the two plasma generating electrodes. This is possible due to the smaller gas density at lower pressures. CP treatment at sub-atmospheric pressures allows the use of a wider variety of product shapes and sizes. Cut apples were selected as the model food product to understand the cooling and microbial inactivation efficacy of the PiLPC process since apples were associated with a number of microbial outbreaks in the past. To the best of our knowledge, this study is the first to integrate CP with vacuum cooling in a single process for

microbial inactivation and rapid cooling of high moisture products. This study aims to develop a new Plasma Integrated Low-Pressure Cooling (PiLPC) process that can simultaneously cool and decontaminate ready-to-eat fresh products by integrating CP treatment with vacuum cooling in a single process.

3.2 Materials and Methodology

3.2.1 System design and development

The main components of the PiLPC system (Fig. 3.1) included a 40 L hermetically sealed steel vacuum chamber, a cold trap with a 250 mL condensate capacity, and 1000 mL coolant capacity, in which dry ice (solid CO_2) was used as the coolant (Vacuubrand Inc., Wertheim, Germany), and a rotary vane pump with a 2.3 m^3/h speed and a minimum final pressure of 0.3 mbar (Vacuubrand Inc.). To generate CP inside the chamber at different pressures, an adjustable steel platform covered with a layer of dielectric material was used as the ground electrode, with a powered electrode on the top. Both electrodes were connected to the plasma power supply (PG 100-D, Advanced Plasma Solutions, Malvern, PA, USA) through feedthroughs on the walls of the vacuum chamber. A convection vacuum gauge with a bar/mbar output, and 0.1 mbar resolution was used to monitor the pressure (CVM211, InstruTech, Longmont, CO, USA). For temperature measurement, a type K thermocouple connected to a high accuracy handheld thermometer with a 0.1 °C resolution and a range of -200 to 260 °C was used (Omega Engineering, Saint-Eustache, QC, Canada). For measuring relative humidity (RH) inside the chamber, a wireless RH logger was used (HOBO MX1100, Onset Computer Corp., Bourne, MA, USA) which had a relative humidity range of 1 – 90 %. To assess changes in RH at lower pressures, the RH logger was placed in the region between the two electrodes, and changes were monitored with or without a sample inside the chamber and the RH% was recorded at 10 s intervals. The decrease in pressure inside the

chamber was monitored when the vacuum pump was operated to test the PiLPC system. The vacuum pump was able to reduce the pressure from atmospheric to 6 mbar within 4 min of operation. A drastic decrease in pressure from atmospheric to 130 mbar was obtained in the first 1 min of vacuum pump operation; however, further reduction in pressure required more time (Fig. 3.2A).

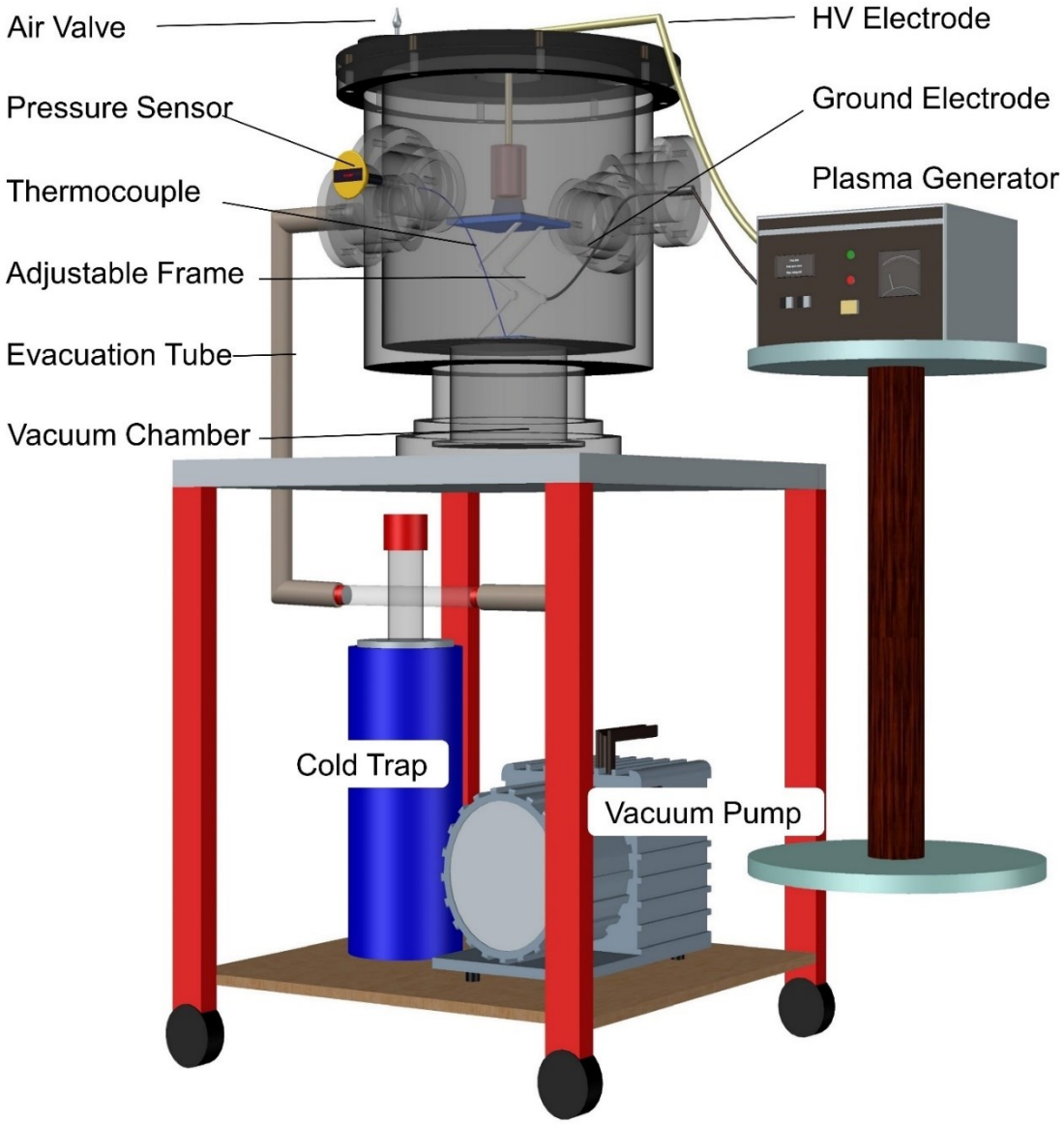

Figure 3.1 Schematic diagram of the Plasma Integrated Low-Pressure Cooling PiLPC system, featuring the different components of the PiLPC system.

3.2.2 Theoretical considerations

The temperature at which a liquid starts evaporating is dependent on the surrounding pressure. Since the generated vapor needs energy to maintain a higher energy level (the gaseous phase), it absorbs heat from the product and its surrounding environment, cooling them both. Any product with free water can be vacuum cooled, but the efficiency of the process depends more on the product's surface area and its porosity (McDonald & Sun, 2000). The flash point of evaporation where cooling starts can be calculated using the Clausius–Clapeyron equation (Masterton & Hurley, 2015).

$$ln(\frac{P_2}{P_1}) = -\frac{L}{R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right) \quad (3.1)$$

Where L in equation 1 is the specific latent heat of the substance, and R is the universal gas constant (8.314 J/mol K), P and T stand for the pressure and temperature (K).

In the case of plasma generation, the breakdown voltage ($V_{breakdown}$) necessary to ionize a gas between two electrodes of a dielectric barrier discharge (DBD) system at different gas densities and electrode distances can be calculated using Paschen's law. The minimum voltage (Equation 3.2) required for ionization is a function of the gas pressure (p) and the distance between the electrodes (d).

$$V = f(pd) \quad (3.2)$$

For determining the working gap between the two electrodes at varying pressure levels at constant voltage output, equation 3.3 was used (Burm, 2007).

$$V_{breakdown} = \frac{B\,p\,d}{ln(A\,p\,d) - ln\left(ln\left(1+\frac{1}{\gamma}\right)\right)} \quad (3.3)$$

Where A is the saturation ionization in the gas, and B is related to the excitation and ionization energies, both are constants that stay roughly the same over different voltages and pressures. For

air, the value of A, B, and the second Townsend coefficient (γ) are 10.95 $Pa^{-1}\,m^{-1}$, 273.78 V $Pa^{-1}m^{-1}$, and 0.025, respectively (Burm, 2007). Since the breakdown voltage is positively correlated to pressure and gap between the electrodes (Equation 3.3), with decrease in pressure, a higher gap between the electrodes can be maintained for a specific breakdown voltage value. Fig. 3.2B shows that at fixed output discharge voltage (30 kV), with the decrease in pressure level, plasma can be generated using larger electrode gaps. Below 300 mbar, the values of the gap between the electrodes increased drastically with a small change in pressure (Fig. 3.2B).

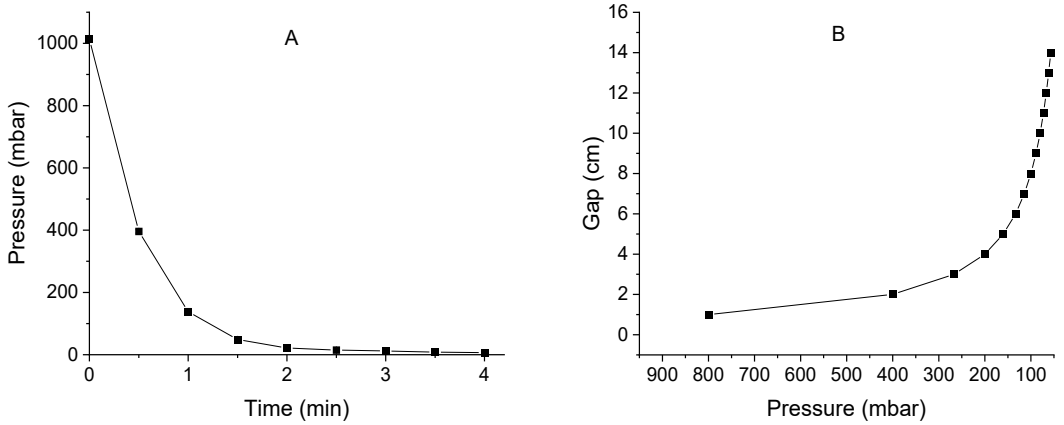

Figure 3.2 (A) The measured chamber pressure as a function of pumping time; (B) The maximum gap (cm) between the powered electrode and the ground electrode that allows for plasma generation at different pressures when the electrical output is fixed at 30 kV.

3.2.3 Bacterial strain and inoculum preparation

Salmonella enterica subsp. *enterica* serovar Typhimurium ATCC 13311 was used as the model pathogenic bacteria in this study as it was associated with a number of recalls and outbreaks (CDC, 2020). The strain was streaked from a frozen stock culture (-80 °C) onto tryptic soy agar with 0.6% yeast extract TSA-YE and incubated for 24 h at 37 °C. One isolated colony was transferred into 5 mL tryptic soy broth (TSB) and kept in the shaking incubator for 24 h at 37 °C, followed by another subculture into a fresh 5 mL TSB that was incubated 18 h. To prepare the inoculum, 100 µL of the

18 h subculture was spread on TSAYE plate and incubated for 24 h at 37 °C. The resulting lawn was scraped off and suspended in 1 mL of 0.1% w/v peptone water (Difco, Becton-Dickinson, MD, USA). The suspension was centrifuged at 10,000 rpm for 3 min (Sorvall Micro 21R microcentrifuge, Thermo Fisher Scientific, Ottawa, ON, Canada) and resuspended in 1 mL of 0.1% w/v peptone water. Finally, the concentration of the inoculum was determined using spectrophotometric analysis at OD_{600} nm absorbance and was plated on TSA plates for confirmation.

3.2.4 Sample selection, inoculation, and bacterial enumeration

Granny smith apples were obtained from a local grocery store (Edmonton, AB, Canada) and stored at 4 °C in a refrigerator for not more than three days before use. The apples were kept at room temperature until they reached room temperature (~23 °C) on the day of experiments. After washing, apple pieces (with height, length, and width of 4, 12, and 12 mm, respectively, and weight of approximately 0.6 g) were aseptically sliced. They were inoculated and uniformly distributed with 20 µL of the *S.* Typhimurium ATCC 13311 on the surface and were not allowed to flow over the edge of the samples. A final concentration of 8 log CFU/g of *Salmonella* population on the cut apple was obtained. The inoculated samples were kept in a biosafety cabinet for 15 min for the inoculum to get fully absorbed on the surface of the cut apples. For plasma treatment, samples were placed on sterile glass slides in Petri dishes to move to and from the treatment chamber. After treatments, samples were mixed with 5 mL of peptone water in a polyethylene stomacher bags (Fisher Scientific Co. Ottawa, ON, Canada). Then they were crushed, pureed, and vortexed for 30 s and finally, the suspended cells were serially diluted, and 100 µL aliquot of appropriate dilution was plated on TSA-YE plates. After 24 h incubation at 37 °C, the microbial colonies were counted, and the results were expressed as log CFU/g.

3.2.5 CP treatment

The plasma generator was set to 3500 Hz frequency, 70% duty cycle, and a voltage output of 28-32 kV. Preliminary optimization experiments were performed to determine the appropriate gap between the two electrodes at different pressures. Sub-atmospheric pressures of 200 and 50 mbar were selected based on preliminary tests and were determined to be the most representative of the sub-atmospheric pressures for our study. To obtain a uniform and continuous plasma discharge, the gap between the powered and ground electrodes were set to 5, 12, and 22 mm at 1013 mbar (atmospheric), 200, and 50 mbar, respectively. To determine the influence of process parameters such as pressure level and treatment time on the inactivation of *Salmonella*, samples were treated for 30 s, 1, 3, and 5 min at three different pressure levels, including 1013 (atmospheric), 200 ± 10, and 50 ± 10 mbar, respectively. The ground electrode was covered with one layer (1 mm) of dielectric material (glass slide) at atmospheric pressure and two layers (2 mm) of material at all the selected sub-atmospheric pressures to reduce arcing and improve the uniformity of the plasma discharge.

3.2.6 Effect of process sequence on inactivation of *Salmonella*

There are several possible PiLPC process sequences, and different levels of bacterial inactivation can be expected depending on the sequence used. The pressure profile of the PiLPC process can be divided into three main stages: an initial evacuation stage where pressure reduction takes place, a stationary stage where pressure is kept at 7 mbar, where cooling takes place, and a final re-pressurization stage where the pressure is brought back to atmospheric pressure. At this final stage, bacterial infiltration can take place, where cells on the surface can be pushed into the tissue of the food product due to the sudden increase in ambient pressure (Ranjbaran & Datta, 2019). To identify the optimal window for CP application, two protocols were developed (Fig. 3.3A). These

two protocols evaluated the effect of CP treatments for 3 and 5 min at 200 mbar. In protocol 1: after placing the sample in the vacuum chamber and reducing the pressure, the samples were first treated with CP at 200 mbar and then the pressure was further reduced to 7 mbar to allow cooling to take place. In protocol 2: after placing the samples in the chamber, pressure was decreased from 1013 mbar (atmospheric) to 7 mbar for cooling to take place first, and then pressure was increased to 200 mbar to treat the samples with CP. Fig. 3.3B represents the starting and endpoints of the PiLPC process mapped onto the phase diagram of water and shows where CP treatment takes place in protocols 1 and 2.

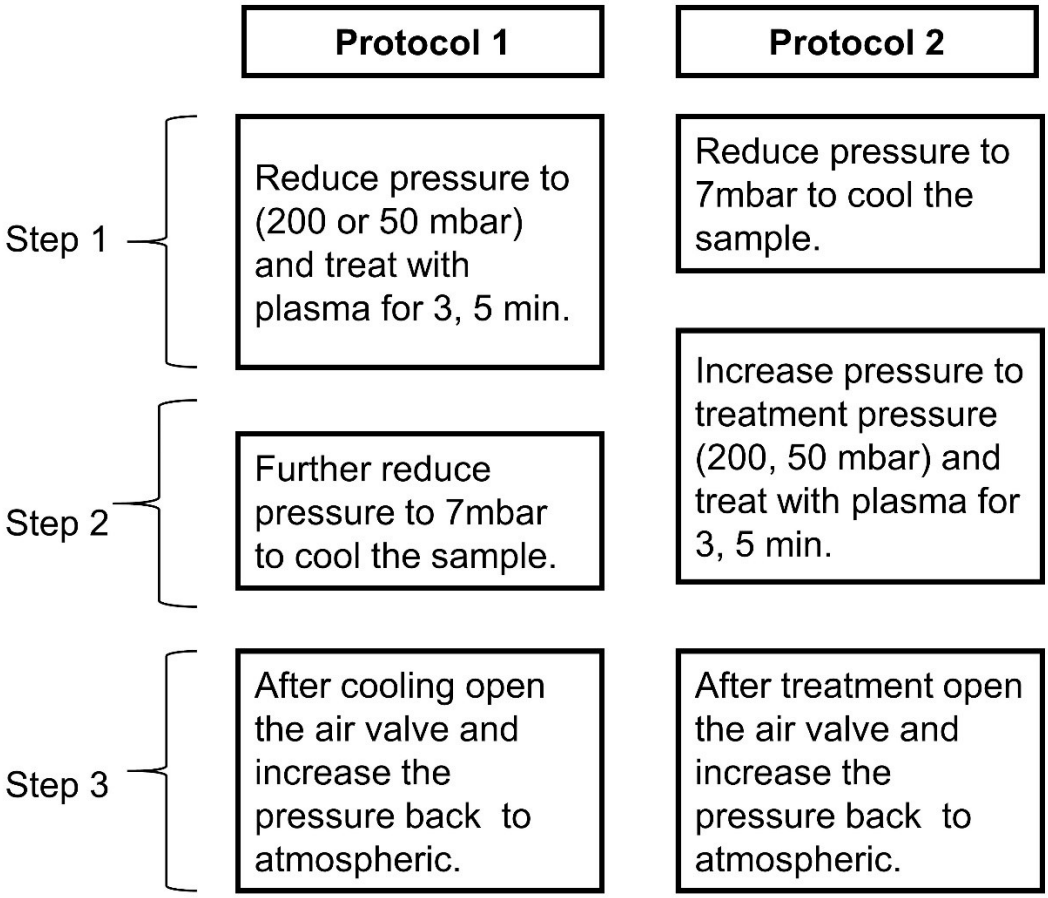

Figure 3.3. (A) Details of the two protocols (process sequences) used in this study.

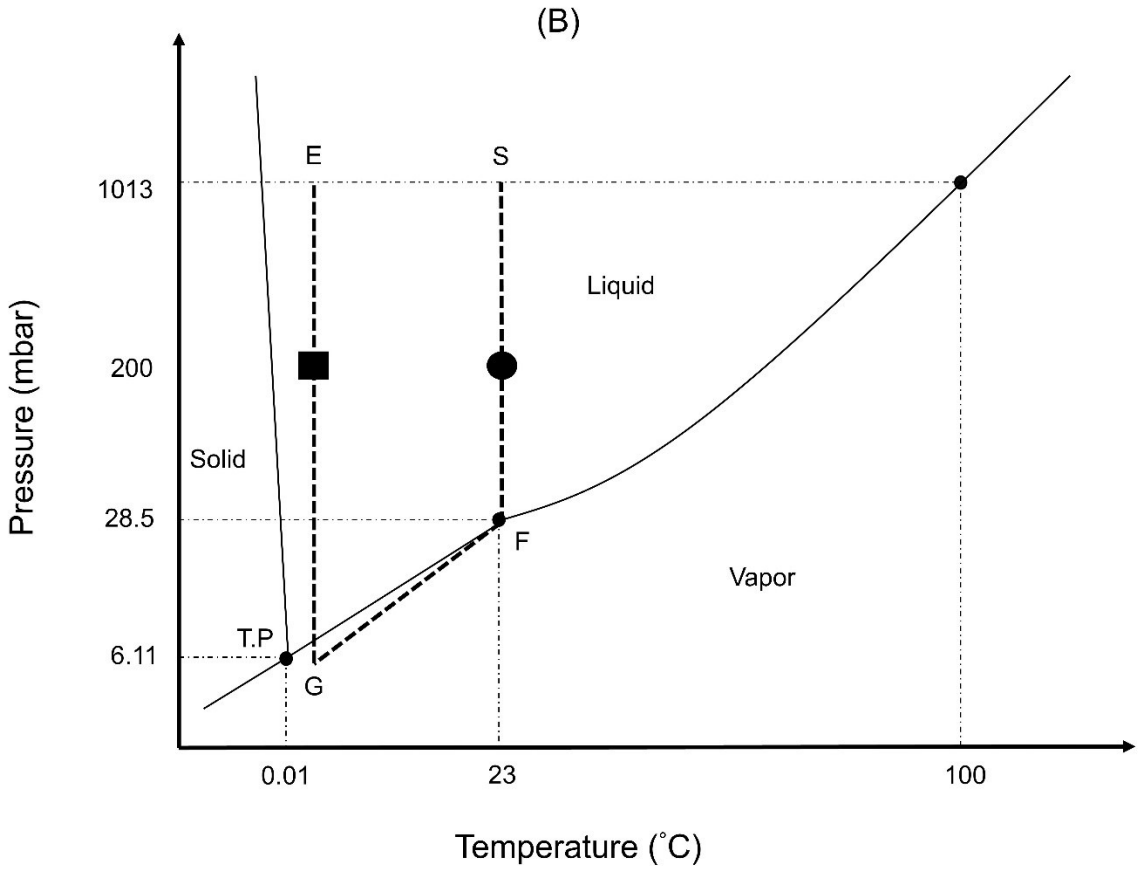

Figure 3.3 (B) PiLPC process mapped on the phase diagram of water. "S" represents the starting point of the PiLPC process at atmospheric pressure and room temperature, while "F" represents the flash point of evaporation and "G" the final targeted temperature, and "E" is the endpoint. Based on protocol 1, plasma treatment was performed at 200 mbar pressure, shown in a circle, and based on protocol 2, plasma treatment was performed at 200 mbar pressure, shown in a solid square – [Figure is representative; not size to scale].

3.2.7 Effect of post-treatment storage on the inactivation of *Salmonella*

To assess the effect of post-treatment storage, samples were inoculated with *S.* Typhimurium ATCC 13311 and separated into a treatment group (3 min CP treatment at 200 mbar) and a control group. *Salmonella* was enumerated immediately after treatment, and after 1, 3, and 5 days of storage at ~4 °C. During storage, the samples were kept in pre-sterilized containers sealed with parafilm. At the end, samples were aseptically opened in the biosafety cabinet. The samples were crushed and vortexed in peptone water, and after the appropriate serial dilutions, 0.1 mL was plated

on TSAYE. Plates were incubated at 37 °C, counted after 24 h, placed at room temperature and checked for any new growth after 48 h and the results were expressed as log CFU/g.

3.2.8 Quantification of cooling time and moisture loss

Apple cubes with 5 different sizes i.e., 1, 1.5, 2, 2.5, and 3 cm^3, corresponding to Surface Area to Volume (SA:V) ratios of 6:1, 4:1, 3:1, 2.4:1 and 2:1 were prepared. The core temperature of the apple cubes was 20 ± 237 °C. The dimensions of the cubes were measured using a digital caliper with a 0.1 mm resolution. For cooling time determination, samples were placed in the PiLPC system with a type K thermocouple inserted near the geometric center of each sample, and the temperature was recorded in 30 s intervals.

The moisture loss in the samples during vacuum cooling was recorded and expressed as percentage weight loss per 10 °C reduction in product temperature (% weight loss/10 °C) or L_{10}. The L_{10} was calculated using equation 3.4.

$$L_{10} = \frac{\left(\frac{\Delta m}{m_i} \times 100\right)}{\frac{\Delta T}{10}} \tag{3.4}$$

where Δm and ΔT are the changes in sample weight and temperature. The measured moisture loss was compared to the predicted moisture loss values (L_p) using equation 3.5 (McDonald & Sun, 2001).

$$L_p = \frac{mC_p\Delta T}{h_{fg}} \tag{3.5}$$

where m is the weight of the sample before vacuum cooling, and h_{fg} is the latent heat of vaporization of water (kJ/kg) at room temperature. The specific heat C_p was calculated using equation 3.6.

$$C_p = 0.03349M_c + 0.8374 \tag{3.6}$$

whereas the moisture content of the sample was determined by oven drying at 105 °C for 8 h (Heratherm OGS60, Thermo Scientific, Waltham, MA, USA) and calculated using equation 37.

$$M_c = \frac{W_i - W_f}{W_i} \times 100 \quad (3.7)$$

where W_i is the initial weight of the sample, and W_f is the weight of the sample after drying.

To quantify the moisture loss during CP treatment, samples were treated with CP without cooling for 1 and 2 min at 1013 (atmospheric) and 200 mbar. The weight of the samples was recorded before and after CP treatment and compared with untreated control.

3.2.9 Plasma characterization

The emission spectrum of the plasma discharge in the vacuum chamber was acquired using optical fiber (F600-UVVIS-SR, StallerNet, Inc., Tampa, USA), connected to an enhanced UV sensitive concave grating spectrophotometer (Black comet, C-25, S/N 17060712, StallerNet Inc., Tampa, USA). The emitted light of CP discharge at different pressure levels was captured using the coupled optical fiber, at a wavelength range of 180 to 850 nm. One end of the optical fiber was connected to a collimating lens, and the other end to the spectrophotometer (F600-UVVIS-SR, StallerNet, Inc., Tampa, USA). The optical fiber with the attached collimating lens was placed perpendicular to the plasma discharge by a transparent glass on the side of the vacuum chamber, pointing at the middle of the plasma discharge. The distance between the lens and the central region of plasma discharge was approximately 22 cm. The spectrum was acquired at 3 different pressures (atmospheric (1013), 200 ± 10, and 50 ± 5 mbar) to compare their peaks and intensity values. A uniform set integration time of 500 ms and scan to an average of 10 was used. The spectra were collected after 1 min of plasma discharge and analyzed using the SpectraWiz software (StellerNet Inc.).

3.2.10 Statistical analysis

All experiments were performed three independent times. The data was subjected to analysis of variance using the generalized linear mixed-effects procedure of SAS® software which uses Rodger method to calculate variance (Proc Glmmix; SAS Institute, 2011 student version, Cary, NC, USA). The interaction effects between pressure and treatment time on *Salmonella* inactivation were analyzed using two-way ANOVA. Whereas the effect of storage time on *Salmonella* inactivation at a single pressure was analyzed using one-way ANOVA. A significance level of $(P < 0.05)$ was used in the tests.

3.3 Results and Discussion

3.3.1 Effect of CP treatment time and pressure on inactivation of *Salmonella*

The mixed model ANOVA analysis revealed a significant two-way interaction between CP treatment time and pressure ($F(6, 24) = 2.76$; $P < 0.05$). Maximum log reductions of 3.21, 2.82, and 1.76 log CFU/g were observed after 5 min of CP treatments at 1013 (atmospheric), 200, and 50 mbar, respectively (Fig. 3.4). CP efficacy increased with treatment time at all pressures; however, the relationship between inactivation and treatment time was not linear. Several studies reported non-linear microbial inactivation rates during CP treatment, where increased treatment time did not translate to an equivalent increase in bacterial inactivation (Critzer, Kelly-Wintenberg, South, & Golden, 2007). The top layer of bacteria inactivated during the initial stage of CP exposure could act as a physical barrier to the diffusion of plasma reactive species and reduce the effect of CP treatments for longer durations (Fernández, Shearer, Wilson, & Thompson, 2012). The initial action of CP can lead to the rupture of the outer membranes of bacterial cells, which leads to the release of the highly oxidizable membrane lipids and free fatty acids, which in turn might end up quenching the ROS and RNS in CP (Critzer et al., 2007). Wang et al. (2012) reported higher inactivation of *Salmonella* sp. CGMCC 1.1552 for products with less surface moisture,

such as carrots, compared to more moist products like pears and cucumbers after CP treatment. They reasoned that the moisture on the surface of a cut product can facilitate the migration of bacteria deeper into the product (Perni, Shama, & Kong, 2008), CP treatment might result in lower inactivation rates on cut fruits, compared to that of whole fruits with smooth and waxy surfaces.

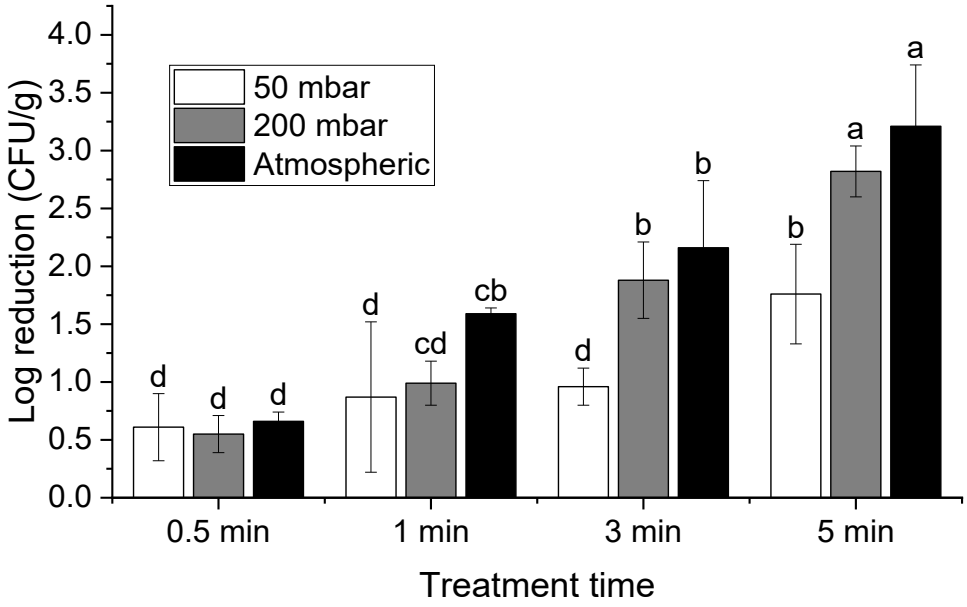

Figure 3.4 Effect of gas pressure during CP treatment and treatment time on the inactivation of *Salmonella* during the PiLPC process. Data are the mean of 3 independent experimental results ± standard deviations. Means with different letters are significantly different *($P < 0.05$)*.

Samples treated by CP at atmospheric pressure and at 200 mbar exhibited a similar level of inactivation of *Salmonella*, while those treated at 50 mbar exhibited significantly lower inactivation levels after 3 and 5 min of treatment (Fig. 3.4). The CP discharge at sub-atmospheric pressure can lead to reduced electron density, electromagnetic field, and less ionization, and excitation collisions (Samir, Liu, & Zhao, 2018). A lower inactivation of *Salmonella* by CP at sub-atmospheric pressures could be due to a reduced concentration of reactive species and gas-phase collision reactions (Fridman, 2008). However, since we did not observe any significant difference

in the *Salmonella* reduction efficacy of CP at 200 mbar in comparison to that at atmospheric pressure, CP treatment would be useful at those sub-atmospheric pressures for practical applications along with the additional advantage of product cooling. Moisan et al. (2002) reported that UV radiation was the main plasma component responsible for the microbial inactivation at low pressures since more UV photons would reach the sample instead of being absorbed by the gas molecules. However, Rossi, Kylián, & Hasiwa (2006) stated that etching was the primary mechanism of inactivation at low pressures. According to their study, UV mainly acts only on isolated cells and the first layer of complex microbial colonies. A direct comparison of the low-pressure plasma treatment efficacy obtained in this study with the studies reported in the literature is not possible due to the different plasma sources, and pressure levels used.

Relative humidity (RH) is another important variable that can affect the nature and concentration of the reactive species in plasma. Environmental RH can widely vary depending on the geographical location and season of the year, from 30 to 80% (Hähnel, Von Woedtke, & Weltmann, 2010). Fig. 3.5 shows the RH between the two electrodes during the PiLPC process with and without a sample. As the vacuum pump removes water vapor from the chamber, RH decreased from an average of 55% at atmospheric pressure to 35% and 25% at 200 and 50 mbar pressures, respectively. The concentration of water vapor, and hence the RH of the surrounding environment, can influence the generation of specific reactive species such as hydroxyl radical ($^{\bullet}$OH) during plasma discharge. The $^{\bullet}$OH radical is a very important reactive species that can be produced by the primary collision process, that involves the dissociation of water (Whitehead, 2016). Further, $^{\bullet}$OH radicals can be a precursor for the production of other important reactive species such as peroxides and hydroperoxides (Hähnel et al., 2010). The lower inactivation of

Salmonella observed during CP treatment at 50 mbar could be attributed to the lower RH in the environment and hence possibly the lower •OH produced during CP treatment.

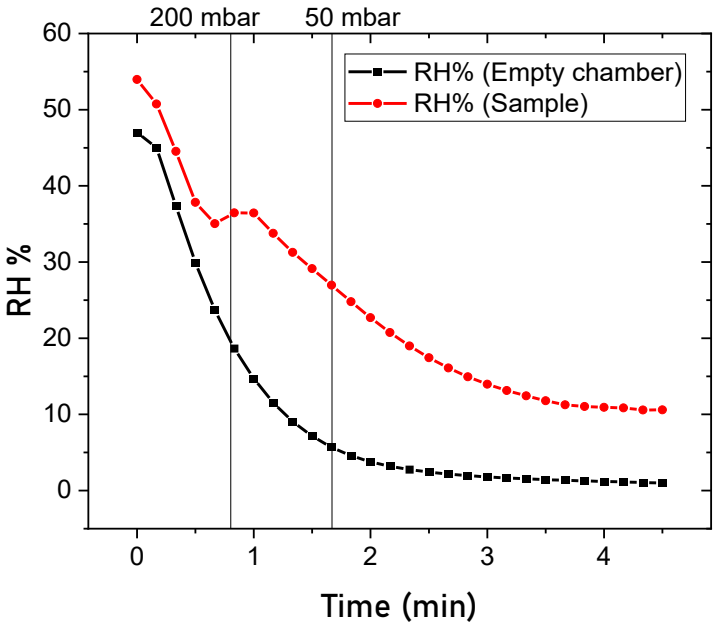

Figure 3.5 The relative humidity (RH) between the two electrodes inside the treatment chamber with and without a sample during the PiLPC process. The presented RH values are the mean of 3 independent experimental results.

3.3.2 Evaluation of CP reactive species at different pressure levels

The emission spectrum of the plasma at different pressure levels was captured in the wavelength region from 180 to 850 nm (Fig. 3.6A). For all pressure levels, the majority of the distinct peaks were in the near UV region, with strong emission from N_2 and N_2^+ (Machala et al., 2007). A weak atomic oxygen emission at around 770 nm was also identified for the 200 and 50 mbar pressures (Wang et al., 2012). The majority of the active oxygen species could not be identified, possibly due to the long half-life of oxygen, which tends to lose energy from particle collisions, quenching its energy before it emits detectable light (Ziuzina, Patil, Cullen, Keener, & Bourke, 2013). The similarity in the emission peaks indicates that the major reactive species interacting on the bacterial

cells were identical at all pressures. Hence, similar mechanisms of inactivation of *Salmonella* by the reactive species at all the tested pressure levels could be possible. However, the relative and absolute concentration of these reactive species may vary at different pressures, and a quantitative study on the changes in their concentrations at lower pressures and at different relative humidity levels is required.

Fig. 3.6B shows images of the plasma discharge at atmospheric, 200, and 50 mbar pressures. The plasma discharge at atmospheric pressure is characterized by filament-like channels, with each channel extending between the two electrodes. Each one of these channels can be considered as a micro-discharge (Kogelschatz, 2003), but at sub-atmospheric pressures, plasma was more diffuse with glow-like characteristics. Glow DBD discharges could be easily obtained at low gas pressures but are more difficult to generate at atmospheric pressure (Patil, Bourke, & Cullen, 2016). This difference in the nature of the discharge is probably the reason for the huge difference in the intensity between the plasma generated at atmospheric and sub-atmospheric pressures.

However, not all diffuse (glow-like) discharges are actual glow discharges (Kogelschatz, 2002). To confirm this, experiments using intensified charge coupled device cameras will need to be performed (Patil et al., 2016). Although the peaks were similar at all pressures, the intensity of the plasma discharge increased up to 10 folds at lower pressures; this could be attributed to the difference in the nature of the plasma discharge at different pressures. Several discharge modes such as filamentary (channels), diffuse, and glow discharges could exist, when using DBD, depending on the gas pressure and mixture, the dielectric surface properties, and the operating conditions (Kogelschatz, 2002).

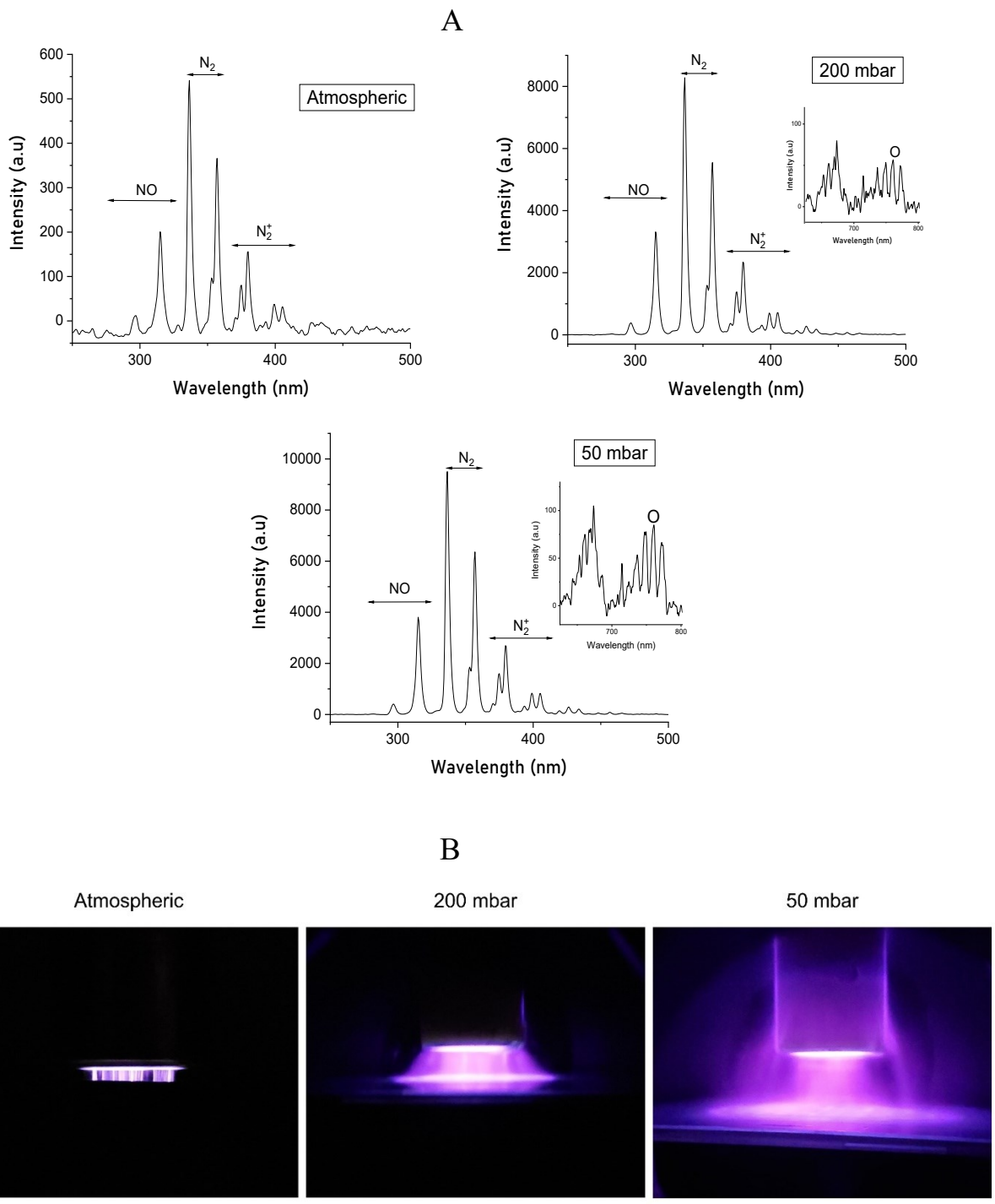

Figure 3.6 Effect of pressure on plasma discharge. (A) Optical emission spectra of plasma discharge generated with 30 kV output voltage at 1013 (atmospheric), 200 mbar, and 50 mbar pressures. (B) Images of plasma discharge at atmospheric pressure, 200 mbar, and 50 mbar.

3.3.3 Effect of PiLPC process sequence on *Salmonella* inactivation

To identify the optimal PiLPC process sequence for inactivation of *Salmonella*, two protocols, each with a different process sequence were used. Protocol 1 (CP treatment at 200 mbar before cooling and then increasing the pressure) resulted in a significantly greater reduction in the *Salmonella* population ($P < 0.01$) compared to protocol 2 (cooling at 7 mbar first and increasing the pressure to 200 mbar for CP treatment) (Fig. 3.7). This means the cells were already exposed to oxidative stress, and they were further inactivated by cold stress when protocol 1 was used. In protocol 2, the cells of *Salmonella* were exposed to both oxidative stress by CP treatment and cold stress by cooling, but the order of exposure to these stresses could contribute to the difference in the inactivation of *Salmonella*. For the rest of the inactivation experiments in this study, protocol 1 was used. No significant influence of treatment time (3 and 5 min) on the inactivation of *Salmonella* was observed when either of the protocols was used. Vacuum cooling alone reduced the *Salmonella* population only by 0.12 log CFU/g. Li, Tajkarimi, & Osburn (2008) reported that vacuum cooling modified the microstructure of lettuce. The evaporated water forcibly migrated through the openings on the lettuce surface and increased the number and size of these openings. At the end, when transitioning from vacuum to atmospheric pressure, conditions that favor infiltration are created.

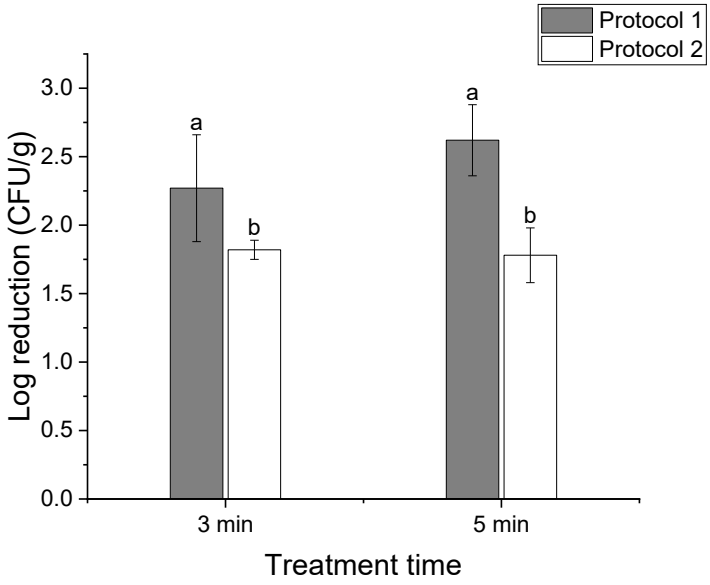

Figure 3.7 Inactivation of *Salmonella* with CP treatment for 3 or 5 min at 200 mbar pressure with protocols 1 and 2. Data are the means of 3 independent experimental results ± standard deviations. Means with different letters are significantly different *(P < 0.05)*.

3.3.4 Effect of post-treatment storage on inactivation of *Salmonella*

Samples were inoculated with an average initial population of 7.5 log CFU/g of *Salmonella* and exposed to CP treatment for 3 min at 200 mbar. Survivability of *Salmonella* was evaluated immediately after treatment (0 days), and 1, 3, and 5 days of post-treatment storage at refrigeration temperature (~ 4 °C) and compared with untreated control samples. Untreated control samples were also stored at the same condition as treated samples after vacuum cooling. During the storage period, the *Salmonella* population on untreated control samples were reduced by less than 0.5 log CFU/g after 5 days of storage (Fig. 3.8). After 5 days of post-treatment storage, *Salmonella* on CP treated samples was reduced to below the detection limit, which was 1 log CFU/g, meaning that there was > 6 log reduction in *Salmonella* during post-treatment storage.

Prasad, Mehta, Bansal, & Sangwan, (2017) reported increased inactivation of *Escherichia coli* inoculated on the surface of tomatoes after 24 and 48 h of post-treatment storage after CP

treatment. Ziuzina, Patil, Cullen, Keener, & Bourke, (2013) reported a similar trend with the total inactivation of *E.coli* after 24 h of storage. Yadav et al. (2020) obtained >6 log reduction *Listeria monocytogenes* after 7 days of storage after in-package CP treatment. This increased inactivation during the post-treatment storage period may be due to the diffusion of reactive species in the microbial cells, and the residual reactive species remaining in contact with the stressed bacterial cells during storage (Ziuzina et al., 2013). However, other factors could affect the extent of bacterial inactivation during the post-treatment storage, including factors intrinsic to the food such as pH, food microstructure, and salt concentration; or extrinsic factors such as storage conditions, including temperature (Smet et al., 2019; Yadav et al., 2019).

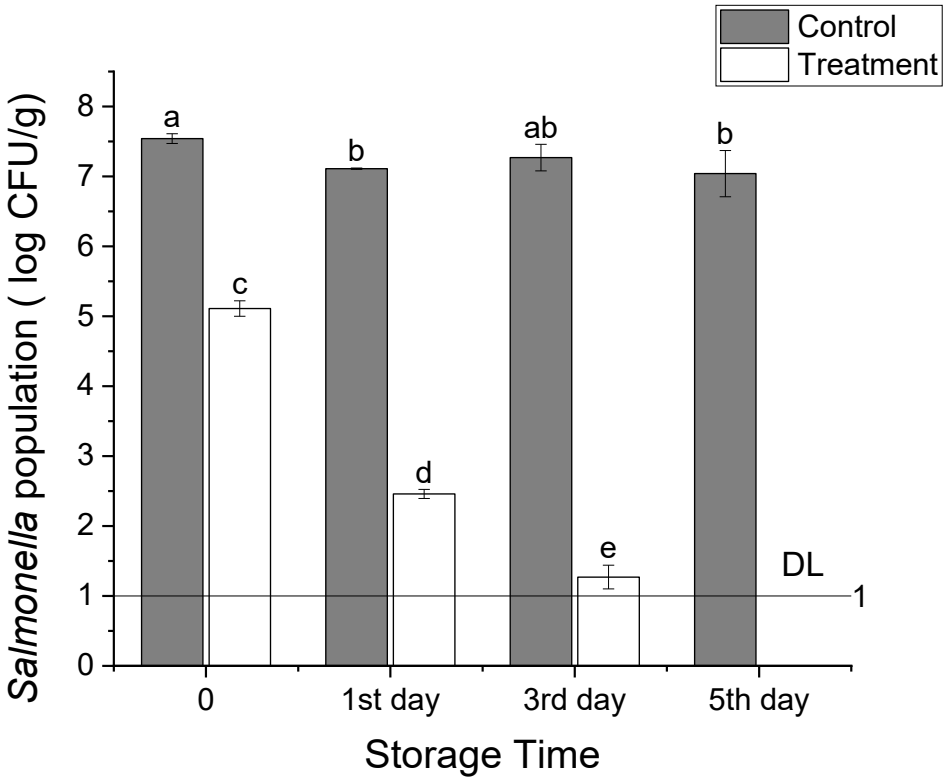

Figure 3.8 Effect of post-treatment storage (4 °C) on total *Salmonella* population of samples treated with CP for 3 min at 200 mbar and untreated control samples using protocol 1. BDL means below detection limit. Data is the mean of 3 independent experimental results ± standard deviations. Means with different letters are significantly different *(P < 0.05)*.

3.3.5 Cooling time, cooling loss, and percent weight loss per unit temperature reduction

Rapid cooling of the apple cubes started when the pressure reached the flash point of evaporation (25-30 mbar). The cooling time (the time taken to cool the samples from 20 °C to 2 °C) increased from 3 min for the smallest samples (1 cm^3) to 7 min for the largest one (2.5 cm^3) (Fig. 3.9). The surface area to its volume (SA:V) of samples was the most important factor affecting the cooling rate. The cooling time of the samples increased as their SA:V ratio decreased, indicating that the surface area available for water evaporation is one of the most significant variables determining the cooling rate of apple slices during vacuum cooling.

The measured moisture loss of the apple cubes per 10 °C reduction in temperature was determined and compared with the moisture loss predicted using equation 5 (Fig. 3.10). For the smallest sample, the measured moisture loss was three times higher than the predicted value, but with the increase in the apple cube size, the measured moisture loss decreased. However, for the largest sample (3 cm^3), measure moisture loss was statistically similar to the predicted moisture loss. This discrepancy between the predicted and the measured moisture loss of vacuum cooled products could be because equation 5 does not account for all the variables that can contribute to moisture loss, but only the amount of evaporated water necessary to obtain a unit reduction in the temperature of a unit mass, during vacuum cooling. McDonald & Sun (2001) reported the difference between the predicted and measured moisture losses and concluded that the extra weight loss might be due to the factors not accounted in the equation, such as water drip. Browning of the apple slice surfaces was visually observed immediately after cutting and during the storage.

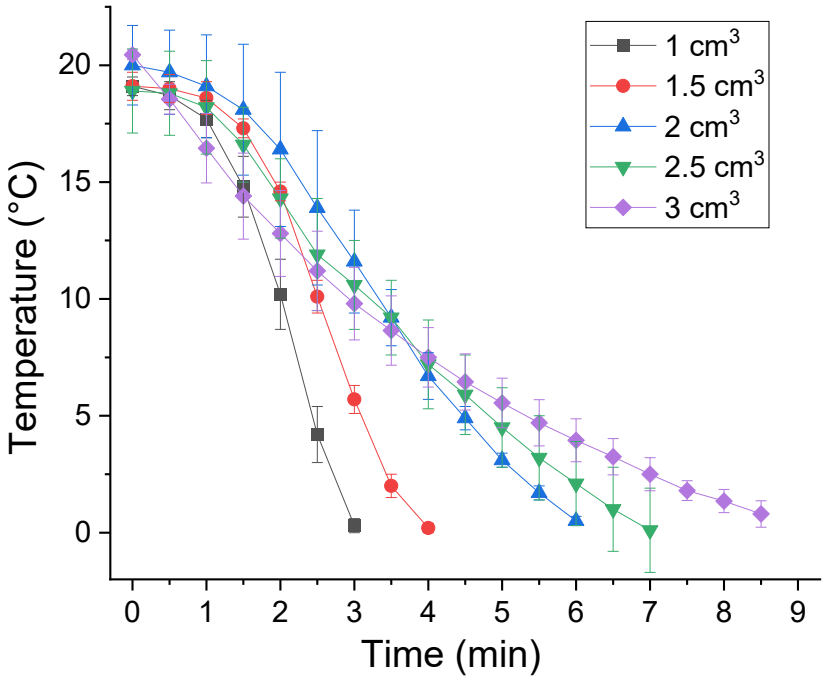

Figure 3.9 Temperature changes in apple cubes with different surface area to volume ratios. Data are the means of 3 independent experimental results ± standard deviations.

The excess weight loss in this study (a loss that is not from evaporation) could be due to surface drying from increased air velocity during the evacuation and re-pressurization of the chamber. Increased air velocity can accelerate the rate of evaporation and moisture loss (Putra & Ajiwiguna, 2017; Vega-Gálvez et al., 2012). To test this, control samples were placed in the chamber and pressure was reduced to 200 mbar without reaching the flash point of evaporation, where evaporative cooling starts. These samples lost 2.32% of their weight during this experiment. This also explains the difference between the measured and predicted moisture losses, which was the highest for small samples with large SA:V ratios, since the rate of air drying directly correlates with the SA:V ratio, even when the air temperature is not high (Mercer, 2014). To the best of our knowledge, this is the first study to link the SA:V ratio of vacuum cooled products to their moisture

loss. To predict the moisture loss during vacuum cooling more accurately, the effect of other variables such as evacuation rate, surface area, re-pressurization rate, the temperature and RH of the air during re-pressurization should be considered.

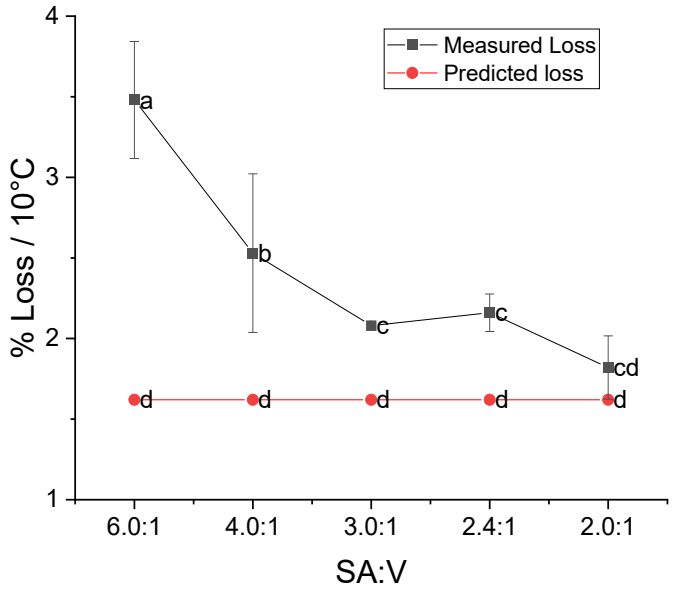

Figure 3.10 Comparison between the measured moisture loss in apple cubes with 5 different surface area to volume (SA: V) ratios and predicted loss from evaporation. Data is the mean of 3 independent experimental results ± standard deviations. Means with different letters are significantly different *(P < 0.05)*.

Table 3.1 shows plasma-induced moisture loss of apple slices after 1 and 2 min of treatment at atmospheric and 200 mbar pressures. A significantly higher moisture loss was observed in cut apple cubes after CP treatment at 200 mbar compared to atmospheric pressure, irrespective of CP treatment time. The higher drying rates at 200 mbar could be attributed to higher vapor pressure difference between the apple cubes and the surrounding environment. Irrespective of the applied pressure level, the moisture loss increases with the increase in CP treatment time. The increased moisture loss during a longer duration of CP treatment could be due to the modification of the surface microstructure of cut apple surfaces, that supported the moisture migration. Additionally,

the drying of cut fruits during CP treatment could be partially due to the slightly elevated temperature of air plasma, which could reach 30-45 °C (Wang et al., 2012).

Table 3.1 Average moisture loss of samples treated with CP at atmospheric (1013 mbar) and 200 mbar pressures for 1 and 2 min.

CP treatment time (min)	Pressure (mbar)	Average moisture loss (%)
0	1013	0.5 ± 0.09^{d}
1	1013	4.25 ± 0.8^{c}
2	1013	7.07 ± 1.35^{b}
0	200	2.32 ± 0.15^{d}
1	200	7.3 ± 0.67^{b}
2	200	11.31 ± 1.1^{a}

Values are the mean ± SD of three independent measurements

3.4 Conclusions

This study developed a Plasma Integrated Low-Pressure Cooling (PiLPC) process by integrating the CP treatment with vacuum cooling and identified the critical parameters determining the *Salmonella* inactivation efficacy of this process, using cut apples as a model food. The CP treatment time and post-treatment storage influenced the inactivation of *Salmonella* on cut apples. The inactivation levels of *Salmonella* by CP at 200 mbar and atmospheric pressure were not significantly different. These low pressures enabled us to maintain large gaps between the electrodes, which would allow for a wider variety of product sizes and shapes for CP treatment. Different PiLPC process sequences were tested to optimize the process for higher inactivation of *Salmonella*. The cooling time and moisture loss during the PiLPC process directly correlated with the surface area to volume ratio of the samples. Future investigations will help explore the antimicrobial and cooling efficacy of the PiLPC for a variety of products and optimize this process to reduce product moisture loss and cut-surface browning. Studies to compare the cost and antimicrobial efficacy of PiLPC process with other antimicrobial treatments in the future are required to understand its commercial viability.

Chapter 4: Improvement in the inactivation of *Salmonella* and polyphenol phenol oxidase by dipping in citric acid before plasma integrated low-pressure cooling of fresh cut apples

4.1 Introduction

An increasing consumer demand recently fueled the expansion of the fresh-cut fruit and vegetable industry. They first appeared on the market in the 1980s and are increasingly becoming a popular consumer product in many developed countries due to their convenience, variety, and flexible serving sizes (Abadias et al., 2008; Euromonitor International, 2015). The processing steps necessary to produce these convenient fresh-cut fruit and vegetable products include cutting, peeling, and poring, all of which could trigger biochemical modifications such as the browning and softening of cut edges and flavor deterioration (Tappi et al., 2014). In addition, the processing steps can amplify microbial contamination by opening the inner tissues, with their favorable conditions, to colonization (Artes et al., 2007). These challenges made the maintenance of microbial safety and organoleptic properties – by the inactivation of the endogenous enzymes of fruits and vegetables – a main priority for the food processing industry (Cocci, Rocculi, Romani, & Dalla Rosa, 2006). Among endogenous food enzymes, polyphenol oxidase (PPO) is the critical enzyme involved in enzymatic browning. In normal circumstances, PPO is isolated in the plastids while the phenolic compounds are located in the vacuoles of the plant cell (Queiroz, da Silva, Lopes, Fialho, & Valente-Mesquita, 2011), but when the PPO enzyme comes into contact with the phenolic substrates due to cutting, it oxidizes them to quinones that react with each other or with other food components to form colored pigments.

Thermal treatments like blanching have been conventionally used to stop negative biochemical processes and reduce the microbial load (Chutia, Kalita, Mahanta, Ojah, & Choudhury, 2019). But thermal treatments negatively affects the sensory and nutritional quality of

fresh produce with significant reductions in bioactive compounds like ascorbic acid and phenolic substrates (Rawson et al., 2011). Some of the most common alternatives for thermal treatment include edible coatings and dipping in solutions containing synthetic or natural additives (Tinello & Lante, 2018). Several novel treatments like UV, ozone, high-pressure processing, and cold plasma (CP) have also been studied for microbial reduction or quality enhancement purposes.

Cold plasma in particular, has been extensively studied for its antibacterial action in cut fruits and vegetables (Li et al., 2019). Recently there has been an increased interest in its effect on the quality of fresh-cut products, including; cut-apples, cut-melons, and sliced kiwifruits (Ramazzina et al., 2015; Tappi et al., 2016, 2019). The potential of CP to reduce the activity of browning enzymes like PPO and peroxidase (POD) depends on its ability to induce conformational changes, initiated by the reactive species and the UV radiation. Most of these conformational changes take the form of a reduction in the proportion of α-helixes and an increase in that of β-sheets in the secondary structure of the enzyme. Structural changes like these make the enzyme unable to bind its substrate owing to the key-lock principle (Surowsky et al., 2013).

An increasingly popular practice is the combination of multiple treatments to take advantage of their unique mechanisms for increased microbial reduction rates, improved quality characteristics and the ability to use lower temperatures, chemical doses, and shorter treatment times. These include combining a physical treatment like blanching with a chemical agent at reduced temperatures (Almeida & Nogueira, 1995), or combining a novel technology like ozone, UV, or CP with an organic acid such as peracetic acid (PAA) or citric acid (CA) for optimal results (Chen, Hu, He, Jiang, & Zhang, 2016; Yuk et al., 2006).

In the previous study when cut apples were treated with CP followed by low-pressure cooling, it resulted in inactivation of *S.* Typhimurium by 3.21 log CFU/g, while browning and high

moisture loss in cut apples were observed (Chapter 3). The enzymatic browning of cut apples by PPO – which is a main issue with fresh cut produce – could be reduced by applying antioxidants such as CA with specific concentrations while achieving microbial inactivation, since CA's antimicrobial properties were previously reported (In, Kim, Kim, & Oh, 2013). The hypothesis is that a combination of CA and CP before low-pressure cooling will have multiple benefits i.e., this combined treatment will improve the microbial safety of cut apples with their synergistic antimicrobial actions, while the application of CA will reduce the moisture loss and PPO activity, hence the browning of cut apples during CP treatment followed by low-pressure cooling.

This study aims to evaluate and compare the effect of CA and CP alone and in different combinations, applied before low-pressure cooling on the inactivation of *Salmonella* and the main quality characteristics of fresh-cut Granny Smith apples, such as PPO activity, total phenolics content, color, and moisture loss, after treatment and during storage.

4.2 Materials and Methodology

4.2.1 Plasma Integrated Low-Pressure Cooling (PiLPC) system

The Plasma Integrated Low-Pressure Cooling (PiLPC) system developed in chapter 3 was used in this study. The system was built to cool and sanitize fresh produce simultaneously by treating them with plasma at low pressures, where vacuum cooling can occur. The optimum operating pressure for the inactivation of *Salmonella* in that study was identified as 200 mbar, and the samples were cooled after the plasma treatment for maximum inactivation. In this study, similar operating conditions (200 mbar, 30 kV, and a 12 mm distance between the electrodes) were used for the CP treatment.

4.2.2 Optical emission spectroscopy

The optical emission spectra of the CP at 200 mbar were collected to identify the main reactive species. The spectrophotometer (Black Comet, C-25, S/N 17060712, StellarNet Inc., Tampa, FL, USA) was used in the wavelength range from 180 to 850 nm with a set integration time of 500 ms and scan to an average of 10. The optical fiber was placed perpendicularly and pointed at the discharge through a transparent glass window on the vacuum chamber, and the distance between the center of the chamber where the discharge was occurring, and the lens was 22 cm. The spectra were collected after 1 min CP treatment, and the results were normalized to one.

4.2.3 Preparation of CA solutions

CA solutions were prepared by diluting lab-grade CA (Fisher Scientific, Ottawa, ON, Canada) with sterilized distilled water to final concentrations of 0, 1, 2.5, 5, and 10 % w/v. The solutions were stored at refrigeration temperatures (<4 °C) until the experiment day and were kept at room temperature (20-23 °C) before final use.

4.2.4 Inoculum preparation

Salmonella enterica serovar Typhimurium ATCC 13311 was used as the model microorganism in this study. A loop from a frozen stock culture stored at (-80 °C) was streaked on a Tryptic Soy Agar (TSA) plate and incubated for 24 h at 37 °C. One isolated colony was transferred to 5 mL of Tryptic Soy Broth (TSB), and incubated for 24 h at 37 °C, after which 100 µL of this were transferred to another tube containing 5 mL of TSB and incubated at similar conditions for 18 h, after which 100 µL were plated on a TSA plate, and after overnight incubation, the lawn was collected and suspended in 0.1 % peptone water (Difco, Becton-Dickinson, MD, USA). The suspension was centrifuged for 3 min at 10,000 rpm (Sorvall Micro 21R microcentrifuge, Thermo Fisher Scientific, Ottawa, ON, Canada), washed, and resuspended twice. Finally, the cell density

of the inoculum was determined using spectrophotometric analysis at OD_{600}, and the inoculum was diluted to obtain a final concentration corresponding to 8 log CFU/g.

4.2.5 Sample preparation and inoculation

Granny Smith apples were obtained from a local store (Edmonton, AB, Canada) and selected for uniformity in appearance and size; any diseased or bruised apples were discarded. Rectangular samples (H × L × W: 5 × 12 × 12 mm), weighing around 0.7 g, were prepared with sterilized equipment and were aseptically transferred to the biosafety cabinet. Before inoculation, samples were checked for background microflora. The apple slices were inoculated with 20 µL of *S.* Typhimurium ATCC 13311, evenly distributed on the top surface and not allowed to flow over the edges. A final concentration of 10^8 CFU/g was obtained, and the samples were allowed to dry in the biosafety cabinet for 15 min until the inoculum was fully absorbed. Samples were then dipped in different solutions (multiple CA concentrations or distilled water) for 2 min. The submersion times in the literature ranged from 1 to 5 min (Soliva-Fortuny & Martín-Belloso, 2003), and 2 min was selected in this study due to the relatively smaller size of the samples. Finally, the samples were allowed to dry for another 15 min before CP treatment or plating.

4.2.6 Effect of CP and CA treatments on inactivation of *Salmonella*

After inoculation and submersion in one of three solutions with different CA concentrations (1, 2.5, and 5 %) for 2 min, the samples were treated with CP for 0, 1, 2, or 3 min. The results were compared with control samples that were submerged in distilled water (DW) and treated with CP for 0, 1, 2, and 3 min. After the treatments, 5 mL of peptone water was added, and the samples were crushed, pureed, and vortexed for 30 s in polyethylene stomacher bags (Fisher Scientific Co., Ottawa, ON, Canada); finally, the suspended cells were serially diluted, and 100 µL aliquot of the

appropriate dilution was plated on TSA plates. After 24 h of incubation at 37 °C, the microbial colonies were counted, and the results were expressed as log CFU/g.

To assess the effect post-treatment storage, samples were inoculated with *S.* Typhimurium ATCC 13311 and separated into 3 treatment groups (only CP for 2 min, only 2.5 % CA, and sequential treatment of 2.5 % CA followed by 2 min of CP) and a control group. The population of *Salmonella* immediately after treatment were compared to the population after 12 h, 1 day, and 2 days of storage at ~4 °C. During storage, the samples were kept in pre-sterilized containers and sealed with parafilm, and at the end, samples were aseptically opened in the biosafety cabinet and were plated and enumerated.

4.2.7 Effect of CP and CA treatment on selected quality parameters

To determine and compare the effect of CP and CA treatments on important quality parameters, such as polyphenol oxidase activity (PPO), total phenolics content (TPC), and the maintenance of natural color, samples were dipped in 2.5, 5, or 10 % CA solutions, treated with CP for 0, 1 or 3 min, or exposed to a sequential treatment of CA followed by CP. Finally, the results were compared to control samples. Also, quality changes during post-treatment storage were monitored, and the PPO activity, TPC, and color were determined immediately after treatment and after 24 h, and 7 days of storage at < 4 °C.

4.2.8 Polyphenol oxidase (PPO) activity

The PPO enzyme was extracted based on methods described in published research (Du, Fu, & Wang, 2009; Rico, Martín-Diana, Frías, Henehan, & Barry-Ryan, 2006) with small modifications. To extract the enzyme, the samples were placed in 0.2 M sodium phosphate buffer (pH 7) containing 2 % polyvinylpyrrolidone. The ratio of the sample to the homogenization solution was 1 g: 2 mL (apple: buffer solution). The samples were homogenized using a polytron homogenizer

set to 12,000 rpm in an external ice bath for two 1.5 min periods separated by 30 s intervals to avoid heating. The homogenate was then centrifuged at 12,000 *g* for 10 min in a refrigerated centrifuge (4 °C), and the final supernatant was used as the crude enzyme.

To determine the enzyme activity, absorbance was measured spectrophotometrically at 406 nm, which was identified as the lambda max where the samples had the highest absorption. The reaction mixture contained 0.4 mL of the enzyme extract and 1.2 mL of the substrate solution (0.02M catechol dissolved in a 0.05M sodium phosphate buffer, pH 7). The reference cuvette contained only the substrate solution. The rate of catechol oxidation was monitored for 2 min at room temperature, and one unit of enzyme activity was defined as the amount that can cause a 0.001 change in absorbance per minute.

Enzyme activity was measured using the flowing equation.

$$\text{Enzyme activity (U/mL)} = [(AF - AI) / (0.001 \times t) \quad (4.1)$$

where U is the unit of enzyme activity, and AF and AI are the final and initial absorption of the sample, while t is the reaction time. The results were expressed as relative activity compared to the untreated samples of the same cultivar, which was considered as 100 %.

4.2.9 Total phenolics content (TPC)

The TPC was extracted using the method described by Li & Cheng (2010) with small modifications. One gram of the apple flesh was extracted in 4 mL of 70 % methanol solution containing 2 % formic acid using a polytron homogenizer set to 12,000 rpm. The homogenate was shaken at 30 °C for 30 min at 200 rpm and was vortexed at 6 min intervals. After which, it was centrifuged for 10 min at 10,000 *g*. The total phenolics content was estimated using the Folin-

Coicalteu (FC) method described by Singleton & Rossi (1965) with minor modifications. 0.2 mL of the sample extract (diluted with distilled water at a ratio of 1:3) was mixed with 1 mL of FC reagent, and within 3-5 min, 0.8 mL of sodium carbonate (7.5% w/v) was added, the mixture was then allowed to interact at room temperature for 1 h. At the end, the absorbance was measured at 760 nm, gallic acid was used as the standard. To plot the calibration curve, 10 concentrations ranging from 1 to 92 μg/mL were used. Finally, the results were expressed as mg of gallic acid equivalent per 100 g of fresh weight.

4.2.10 Color measurement

The color changes of samples were measured using a chromameter (CR-410, Konica Minolta Sensing Inc., Osaka, Japan). The L* (lightness), a* (red-green), and b* (yellow-blue) values were taken at three different locations on the surface of each sample surface, and averaged to give a final reading.

4.2.11 Statistical analysis

All experiments were conducted in three independent trials and were repeated three times. Data was subjected to a one-way analysis of variance. Two-way ANOVA was used to estimate the interaction effects between CA and CP treatment on *Salmonella* inactivation. The SAS software was used for data analysis (Proc Glmmix; SAS Institute, 2011 student version, Cary, NC, USA), and a significance level of ($P < 0.05$) was used in the tests.

4.3 Results and Discussion

4.3.1 The emission spectra of the plasma

Fig 4.1 shows the emission spectra of CP at a 200 mbar pressure. The spectra were acquired at the range between 180 to 850 nm. No significant emissions were observed above 500 mbar, which

means the reactive oxygen species which emit light at the 700 nm range (Wang et al., 2012) could not be identified. This might be due to the long half-life of oxygen, which tends to lose energy before emitting any detectable light (Ziuzina et al., 2013). The main identifiable peaks were in the near UV region, mainly from N_2 and N_2^+ (Machala et al., 2007).

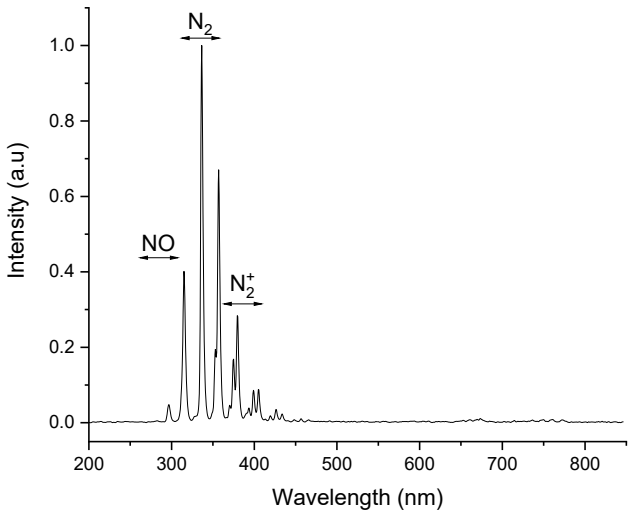

Figure 4.1 Optical emission spectra of plasma discharge generated with 30 kV output voltage at 200 mbar.

4.3.2 Effect of sequential CP and CA treatment on inactivation of *Salmonella*

The inactivation of *Salmonella* increased with CA concentration and with CP treatment time (Fig. 4.3). The greatest inactivation of 5.68 log CFU/g was obtained after a sequential treatment using 5 % CA followed by 3 min of CP treatment. For samples dipped in distilled water instead of CA (control sample), the inactivation of *Salmonella* was significantly lower *($P < 0.05$)* than those dipped in CA before CP treatment of 3 min (Fig. 4.3). CP treatment time and CA concentration had a significant effect *($P < 0.001$)* on the inactivation of *Salmonella* for cut apples, and there was a significant but less pronounced two-way interaction (*($P < 0.05$*) between CA concentration and CP treatment time on *Salmonella* inactivation.

Although not fully understood, the bactericidal properties of organic acids are linked to their easy crossing of the lipid membrane of bacterial cells in undissociated form. They, then dissociate to protons and anions in the neutral pH environment of the cytoplasm (In, Kim, Kim, & Oh, 2013; Ricke, 2003). This increased concentration of charged ions reduces the pH of the cell, which requires a near-neutral environment to maintain its biological functions (Fig. 4.2). In another proposed mechanism, organic acids increase cell permeability by up to 3 times its normal level (de Oliveira, Cossu, Tikekar, & Nitin, 2017). Our hypothesis was that pre-dipping in CA before CP treatment would increase cell permeability and make it easier for reactive species to get into the bacterial cell, thus creating a synergistic effect. A previous study reported the synergistic microbial inactivation effect of a "hurdle" treatment using CP and peracetic acid. The individual treatment of CP and peracetic acid resulted in 1.3 and 2.3 log reductions of *Salmonella*, respectively, while in combination they resulted only a 5.3 log reduction (Chaplot et al., 2019). Other examples include a combined CP and clove oil or CP and ultrasound treatments, wherein in both cases, the hurdle treatment resulted in nearly twice the inactivation levels of the sum of the separate treatments (Liao et al., 2020). However, sequential treatment of CA followed by CP had a mainly additive effect in this study. For instance, CP treatment for 3 min resulted in 2.39 log CFU/g reduction, while 2.5 % CA treatment resulted in 1.05 log CFU/g reduction, while a sequential treatment of 2.5 % CA followed by 3 min of CP resulted in 3.51 log CFU/g, which is nearly the sum of the two (Fig. 4.3). In conclusion, despite the lack of synergy between the two treatments, they complement each other by enabling an increase in inactivation levels without increasing CP treatment time. This approach avoids all the quality-related problems that long CP treatment times could cause.

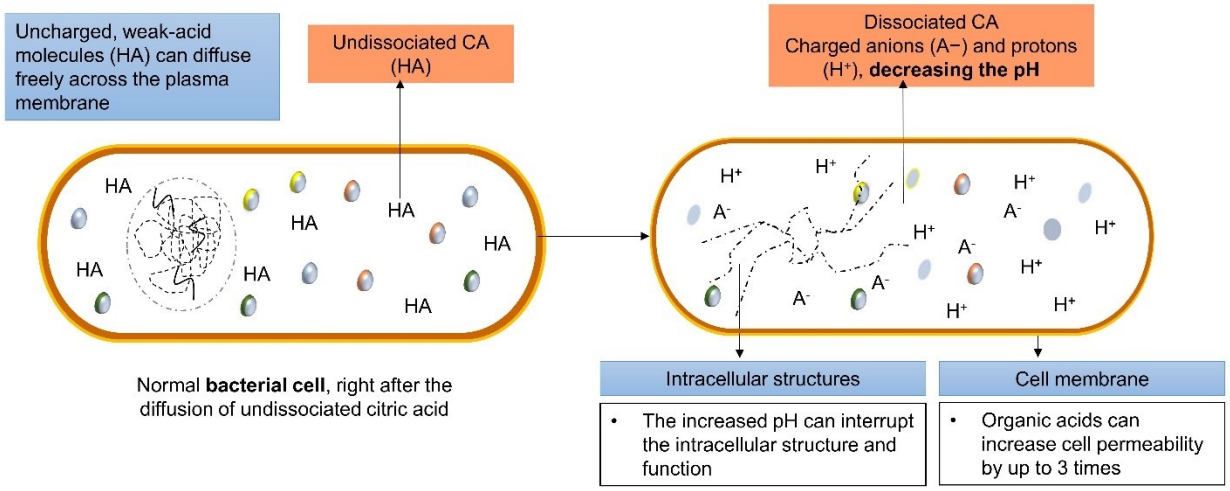

Figure 4.2 The bacterial inactivation mechanisms of citric acid.

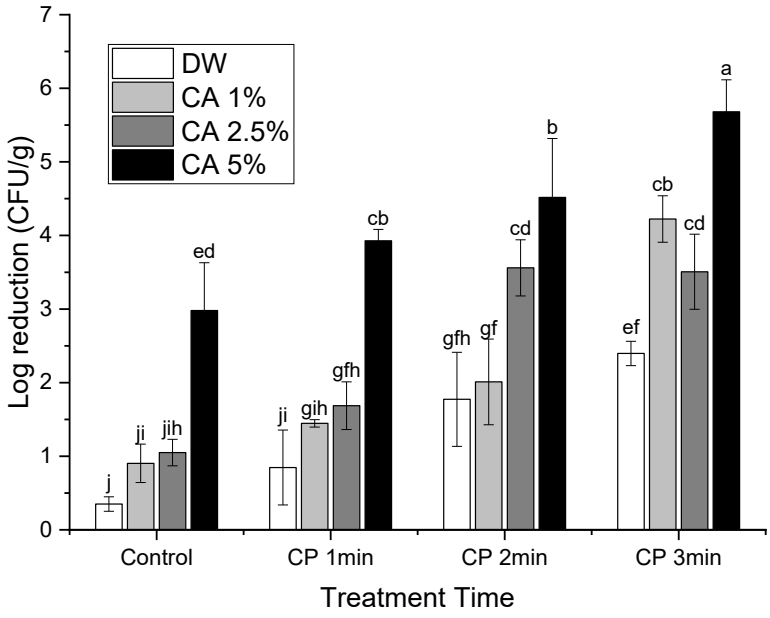

Figure 4.3 Effect of dipping in different concentrations of CA or in DW prior to CP treatment on the inactivation of *Salmonella* after CP treatment of 0, 1, 2 or 3 min. Data are the means of 3 independent experimental results ± standard deviations. Means with different letters are significantly different *(P < 0.05)*.

Post-treatment storage of cut apples at < 4 °C significantly influenced *(P < 0.05)* the *Salmonella* population in cut apples at different rates. The greatest *Salmonella* inactivation during storage was

observed on samples treated with 2.5 % CA followed by 2 min of CP (Fig. 4.4). In these samples, the total population was beyond the detection limit after 48 h of post-treatment storage. Previous studies reported the continued inactivation of bacterial pathogens in food products after CP treatment (Yadav et al., 2019, 2020). This continued inactivation during post-treatment storage may be due to the residual activity of the reactive species of CP, which remain in contact with the stressed bacteria for a longer period of time (Ziuzina et al., 2013). However, in this study, the samples treated with only CP for 2 min had a less inactivation during storage compared to those treated with a sequential CA and CP treatment or CA alone. For instance, after 48 h, the total population on these samples was only reduced to 4.5 log CFU/g after 2 min of CP treatment only, compared to 1.5 log CFU/g for ones treated with only 2.5% CA. This could be due to the abundance of CA after dipping, which remained active during storage. While the most reactive species of CP have a very short half-life, except for a few, like hydrogen peroxide, which can remain stable and active even after the treatment (Mahnot, Mahanta, Keener, & Misra, 2019).

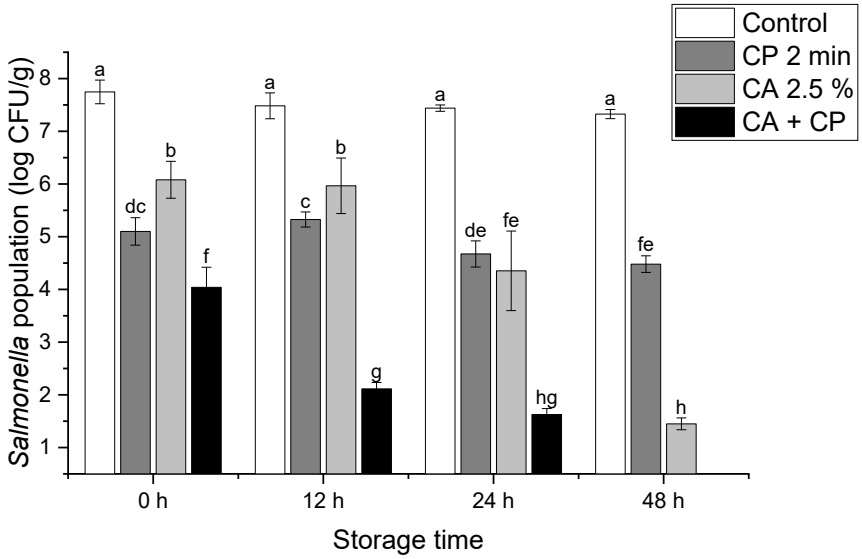

Figure 4.4 Effect of post-treatment storage on the inactivation of *Salmonella* after treatment with 2 min of CP, 2.5% CA alone, and in combination. Data is the mean of 3 independent experimental results ± standard deviations. Means with different letters are significantly different *(P < 0.05)*.

4.3.3 Inactivation of PPO immediately after treatments and during post-treatment storage

Fig. 4.5 shows the residual activity of polyphenol oxidase (PPO) expressed as a percentage of the control sample after CA and CP treatments. The PPO inhibition increased with longer CP treatment times and CA concentrations, and generally, CP was a better PPO inhibitor than CA. For instance, no significant PPO inhibition took place in samples treated with 2.5% CA, but it increased at larger concentrations of 5 and 10 %. The sequential treatment of 5 % CA followed by 3 min of CP resulted in the greatest inactivation, with only 22 % PPO residual activity remaining.

A 90 % inhibition of PPO was reported by Surowsky et al., (2013) after 3 min of CP treatment of a model food system. Bußler, Ehlbeck, & Schlüter (2017) reported a 62 % reduction of PPO activity in fresh-cut apples after 10 min of CP treatment. In this study, 60 % of PPO inhibition was obtained after only 3 min of CP treatment. In addition to the effect of different operating conditions, the improved inhibition in this study could be due to the effect of sample size. Bußler et al., 2017 used apple cubes with a size of 12 mm, which was considerably larger than the rectangular sample ($5 \times 12 \times 12$ mm) used in this study. The greater PPO inactivation might be due to the greater surface area to volume ratio, that could lead to a better diffusion of reactive species during CP treatment.

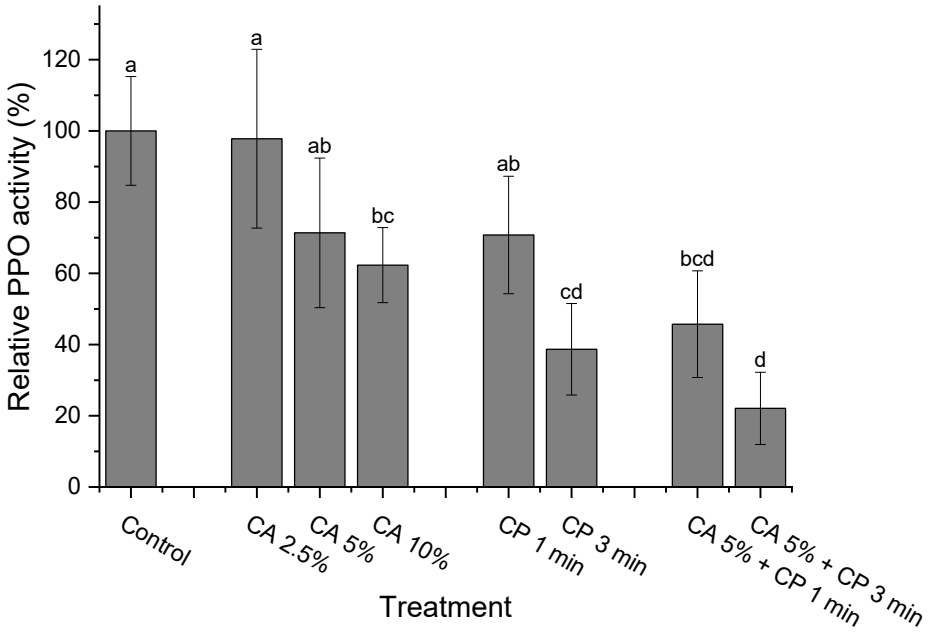

Figure 4.5 Comparison between the PPO inactivation efficacy of CA and CP immediately after treatment. Data are the means of 3 independent experimental results ± standard deviations. Means with different letters are significantly different *(P < 0.05)*.

Different mechanisms for PPO inhibition by CA and CP have been reported. CA inhibits the enzyme by reducing the surrounding pH to less than optimal for its activity, which leads to the loss of enzyme functionality. CA can also act as a chelating agent, binding the copper atoms (Cu), which are crucial parts of the enzyme structure (Son, Moon, & Lee, 2001). On the other hand, the inactivation of the enzyme by CP is mainly attributed to the several plasma radicals that oxidize the reactive side chain of its amino acids (Ramazzina et al., 2015). Also, CP may reduce the sample's pH which in turn can contribute to the inactivation of enzymes. However, factors including the structural properties of the enzyme, such as its molecular weight, cross-linking, disulfide bonding, and enzyme concentration, could influence their changes during CP treatment. Further, process-related factors, such as voltage level, treatment length, pressure level, and humidity, can also affect CP's efficacy as an enzyme inhibitor (Misra, Pankaj, Segat, & Ishikawa, 2016). Although all samples showed further reductions in PPO activity after 24 h of post-treatment

storage, the activity was significantly restored *(P < 0.05)* in CP-treated and control samples after 7 days of storage except for the sample treated with 5 % CA (Fig. 4.6). This indicated that the inhibition of PPO by CP can sometimes be partially reversible, and that it can regain its original structure and thus function after prolonged storage. Gui et al., (2006) reported a significant reactivation of PPO in apple juice after two weeks of inactivation by supercritical carbon dioxide.

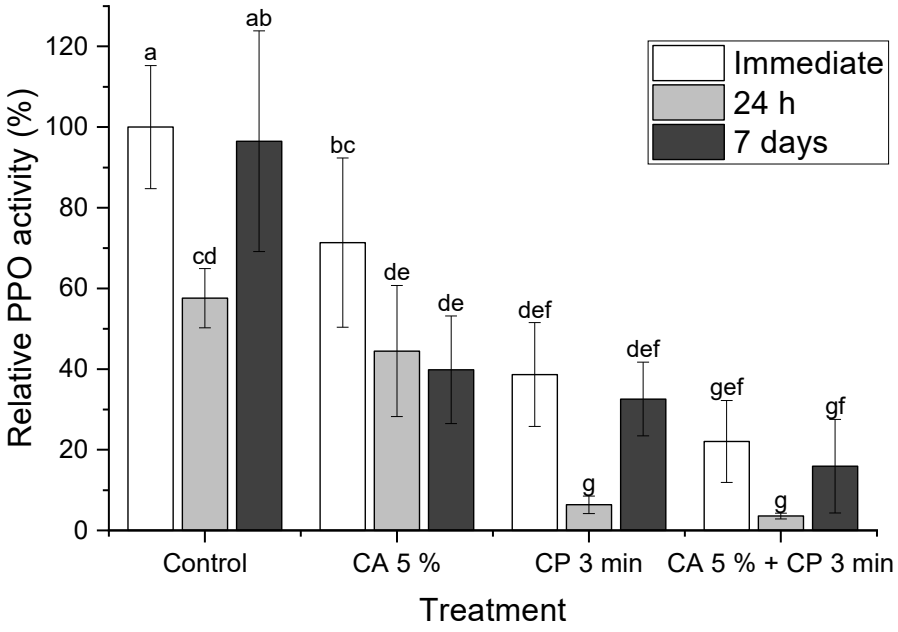

Figure 4.6 Comparison between the PPO inactivation efficacy of CA and CP during post-treatment storage. Data are the means of 3 independent experimental results ± standard deviations. Means with different letters are significantly different *(P < 0.05)*.

4.3.4 Effect of the CP and CA treatments on total phenolics content

The total phenolics content (TPC) in the control samples was around 74 mg gallic acid equivalent (GAE) per 100 g of fresh weight (Fig. 4.7). This value was lower than some of the values reported in the literature for Granny Smiths apples, which widely varied from 118 mg/100 g (Landl, Abadias, Sárraga, Viñas, & Picouet, 2010) up to 200 mg/100 g fresh weight (Henríquez et al., 2010). However, the results were comparable to other apple cultivars such as fresh-cut Fuji (66

mg/100 g) and Braeburn (63 mg/100 g) (Chen et al., 2016). The TPC did not change after CA treatment but had slightly increased but not significantly with CP treatment. This may be due to the biosynthesis of many secondary metabolites like phenols, accelerated in plant cells under stress conditions (e.g., during UV and ROS exposure). CP species probably act as abiotic elicitors of these stress responses, but it is important to note that this process can affect distinct phenolic compounds differently, with some increasing and others remaining unchanged (Li et al., 2019).

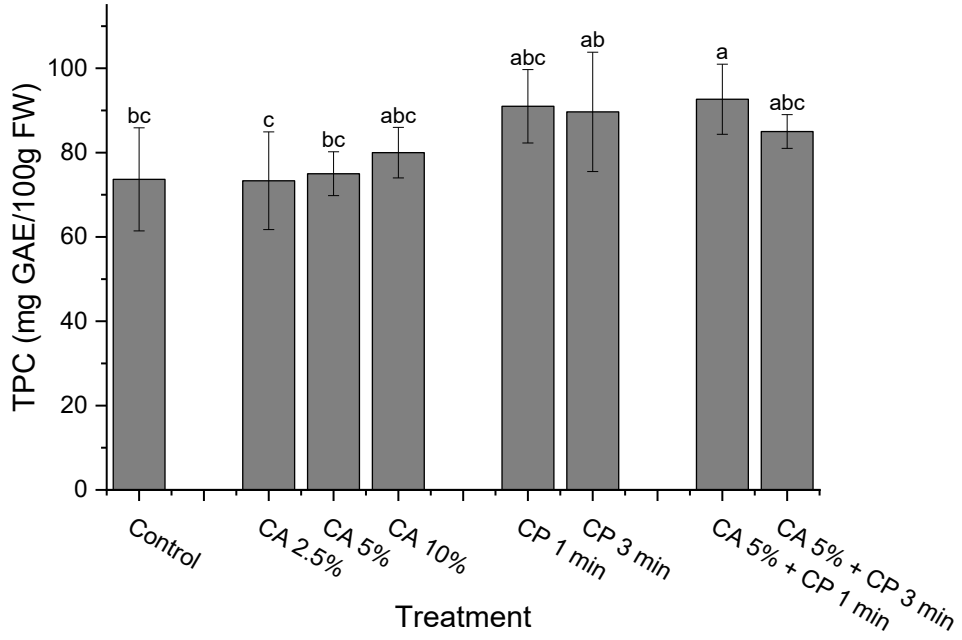

Figure 4.7 The effect of CP and CA treatment on TPC of cut-apples. Data is the mean of 3 independent experimental results ± standard deviations. Means with different letters are significantly different *(P < 0.05)*.

TPC significantly increased *(P < 0.05)* in all the samples after 24 h of storage, even in control samples that were not exposed to CP (Fig. 4.8). This indicates that the biosynthesis of TPC is elicited by cutting the samples. However, the TPC decreased to their original levels by the 7th day. A similar trend was reported by Rocha & Morais (2002), after the cutting of Jonagored apples, the TPC significantly increased *(P < 0.05)* in the first 0 to 3 days but decreased back to their original

levels by the 7th day. The results of our study indicate that the same trend (i.e., initial increase within the first few days of storage followed by a decrease to initial levels) applies here as well. The only difference observed was that the CP treated samples had significantly greater TPC *(P < 0.05)* after 24 h, but by the 7th day, they were similar to the control samples. No other study has reported the changes in the TPC of CP treated apples during storage.

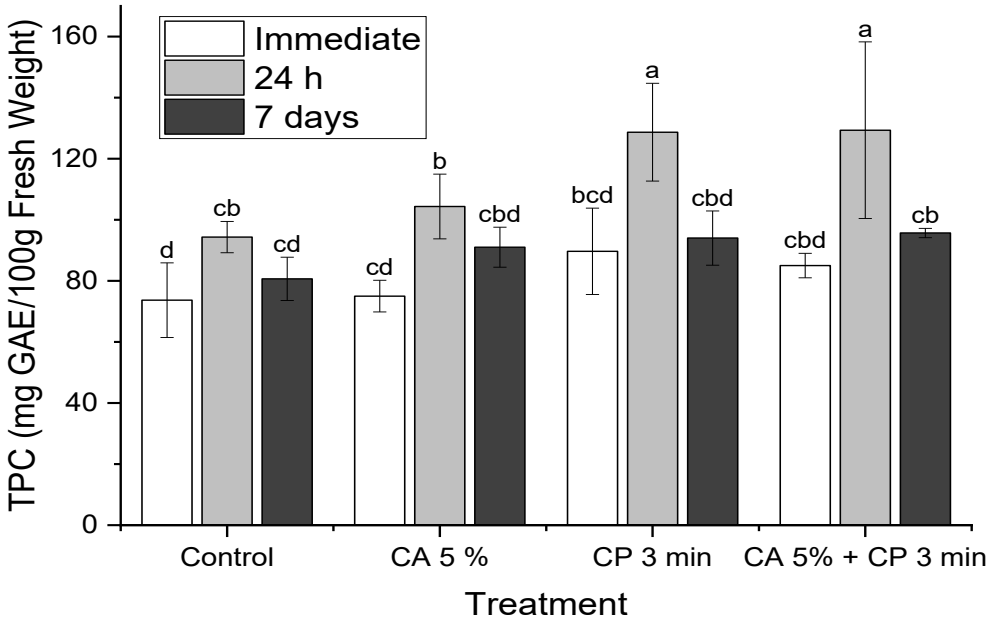

Figure 4.8 The effect of CP and CA treatment on TPC of cut-apples during a post-treatment storage. Data are the means of 3 independent experimental results ± standard deviations. Means with different letters are significantly different *(P < 0.05)*.

4.3.5 Effect CP and CA treatments on color parameters

Table 4.1 shows the impact of separate or sequential treatment of CA and CP on the color parameters of cut apples. The color parameters of two controls were selected; the first control (the cut samples which remained without any treatment for 1 h before measurement) and a negative control (the color parameters of samples immediately after cutting). The main changes were in the

L* (brightness) value, which was at its least for the control samples and greatest for fresh-cut samples (negative control), making them the brightest. The b* (blue-yellow) was statistically similar for both the negative and positive controls, which indicates that it was weakly correlated with surface browning. These results concur with Rocha & Morais (2002), in which the strongest correlation of PPO activity with color parameters was with L* and a* values while the correlation with the b* value was minor. However, b* significantly decreased *($P < 0.05$)* for samples treated by CA ($P < 0.05$) , which showed the development of a blue-shifted hue. The length of the CP treatment time and the concentration of the CA did not have a statistically significant effect on the color parameters ($P > 0.05$).

Table 4.1 Color parameters (L*, a*, and b*) of samples after treatments

Sample	*L**	*a**	*b**
Control	90.46 ± 0.55 ^{a}	-3.25 ± 0.28 ^{d}	8.68 ± 0.68 ^{a}
Control (1 h)	85.58 ± 1.4 ^{d}	0.31 ± 0.61 ^{a}	10.02 ± 1.74 ^{a}
CA 2.5%	89.7 ± 0.72 ^{a}	-2.1 ± 0.32 ^{c}	5.75 ± 1.1 ^{b}
CA 5%	89.79 ± 0.44 ^{a}	-1.83 ± 0.6 ^{c}	5.36 ± 1.23 ^{b}
CA 10%	90.22 ± 0.31 ^{a}	-1.63 ± 0.49 ^{bc}	4.14 ± 1.27 ^{b}
CP 1 min	86.68 ± 0.98 ^{cd}	-0.77 ± 0.55 ^{b}	8.87 ± 0.98 ^{a}
CP 3 min	87.95 ±0.71 ^{cb}	-1.52 ± 0.8 ^{bc}	8.38 ± 1.56 ^{a}
CA 5 + CP 1	88.15 ± 0.87 ^{b}	-3.37 ± 0.32 ^{d}	9.13 ± 0.48 ^{a}
CA 5 + CP 3	88.24 ± 0.35 ^{b}	-3.45 ± 0.36 ^{d}	9.61 ± 0.56 ^{a}

Values are the mean ± SD of three independent measurements

Immediately after treatment, and after 24 h and 7 days of storage (Table 4.2), samples treated with a sequential treatment of CA and CP were the closest to the fresh samples, not only in terms of lightness (L*), but also other color values like a* and b*, which indicates pre-dipping in CA significantly improved *($P < 0.05$)* the color retention of CP treated samples (Fig. 4.9).

Table 4.2 Color parameters (L*, a*, and b*) of cut apple samples after treatment with CA or CP or a combination of both after 24 h and 7 days of storage.

	24 h		
Sample	*L**	*a**	*b**
Control	84.08 ± 1.01 ^{e}	-0.76 ± 0.77 ^{bcd}	13.94 ± 0.97 ^{a}
CA 5%	88.23 ± 0.31 ^{b}	-4.88 ± 0.62 ^{h}	10.61 ± 1.27 ^{bc}
CP 3 m	86.99 ± 0.71 ^{c}	-1.82 ± 0.33 ^{ef}	10.17 ± 0.83 ^{bcd}
CA 5 + CP 3	88.22 ± 0.34 ^{b}	-2.51 ± 0.26 ^{f}	9.51 ± 1.3 ^{cd}
	7 days		
Sample	*L**	*a**	*b**
Control	83.94 ± 0.47 ^{e}	-0.15 ± 0.27 ^{ab}	12.8 ± 1.41 ^{a}
CA 5%	88.6 ± 0.22 ^{b}	-4.62 ± 0.28 ^{h}	12.03 ± 0.56 ^{ab}
CP 3 m	86.91 ± 0.79 ^{c}	-0.43 ± 0.26 ^{abc}	8.45 ± 0.6 ^{d}
CA 5 + CP 3	88.56 ± 0.25 ^{b}	-1.11 ± 0.33 ^{cde}	8.34 ± 1.39 ^{d}

Values are the mean ± SD of three independent measurements

4.3.6 Effect of pre-dipping of cut apples in CA on cooling time and cooling loss

Samples dipped in CA took longer to cool irrespective of size (Fig. 4.10). The 1 cm^3 sample normally took 2.5 min to cool from 18 to 1 °C, and this increased to 3 min when they were pre-dipped in 2.5 % CA solution. The difference was more pronounced for the 2 cm^3 samples, where pre-dipping in CA increased the cooling time from 5 min to 7 min. Vacuum cooling times are affected by factors such as sample porosity, bulk density, and surface area available for evaporation (McDonald & Sun, 2000). The initial expectation was that pre-dipping the samples in a CA solution would decrease the cooling times due to the increased availability of water; however, cooling times were significantly increased *($P < 0.05$)* for samples dipped in CA solutions. McDonald & Sun (2001) reported that vacuum-cooled meat joints took longer to cool when injected with a brine solution to reduce moisture loss. They concluded that this was due to the filling of the pores in the product's inner matrix, which reduces the void space available for evaporation. Although dipping in CA could increase cooling time, it improved the yield.

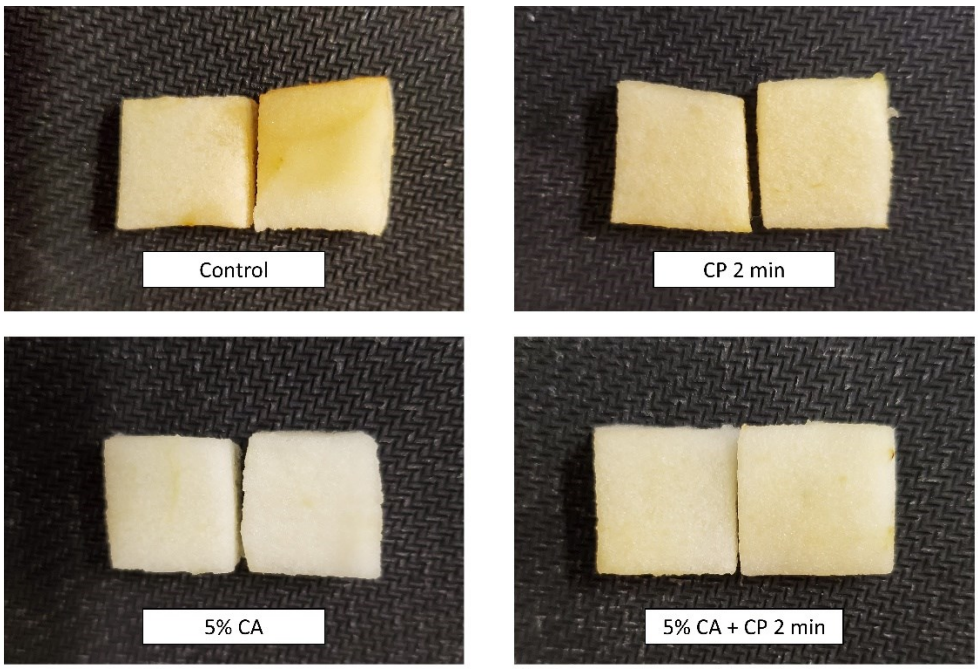

Figure 4.9 Digital images of control and treated cut apples cut apple samples after treatment with CA or CP or a combination of both.

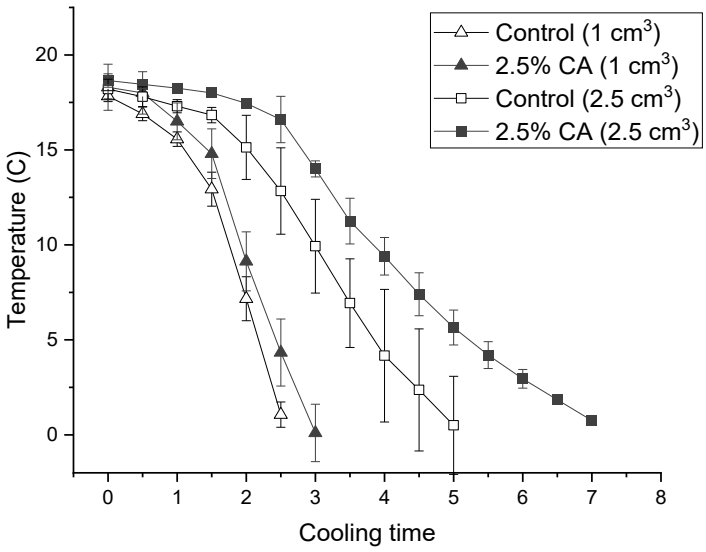

Figure 4.10 Cooling time of apple slices dipped in 2.5% CA and control samples of two sizes (1 and 2 cm^3) during vacuum cooling. Data are the means of 3 independent experimental results ± standard deviations.

Both cooling and CP treatment can cause considerable moisture loss. Pre-dipping in CA solution will not only help improve the microbial reduction and quality parameters such as color and PPO inactivation, but it can also reduce moisture loss. Fig. 4.11 compares the moisture loss in samples per 10 °C reductions in temperature during vacuum cooling. For samples dipped in a 2.5 % CA solution, CP and vacuum cooling had less than half of the moisture loss compared to the samples treated with CP and vacuum cooling without dipping in CA. The final yield for the samples dipped in CA was 93 % of the original weight after 3 min of CP treatment and cooling, compared to 84 % for samples directly cooled and treated with CP without dipping. CA concentration had no effect, so this is most likely due to moisture replacement, since dipping in DW without any CA also resulted in a similar outcome (results not shown).

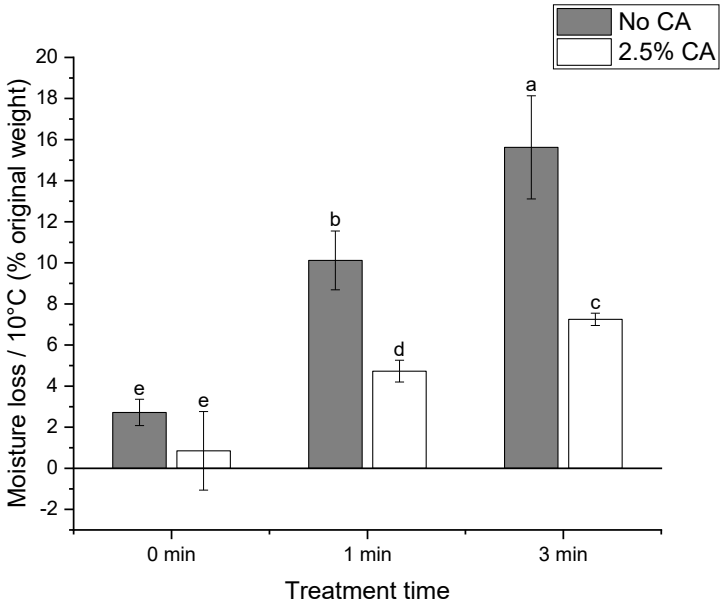

Figure 4.11 Comparison between the measured moisture loss of samples pre-dipped in 2.5% CA and control samples. The results are expressed as % loss of original weight (before dipping in CA) per 10 °C reduction in temperature during vacuum cooling. Data are the means of 3 independent experimental results ± standard deviations. Means with different letters are significantly different *(P < 0.05)*.

4.4 Conclusions

The effects of sequential citric acid (CA) and cold plasma (CP) treatments on the inactivation of *Salmonella*, polyphenol oxidase inhibition, color changes, and total phenolics content of Granny Smith apples during plasma integrated low-pressure cooling (PiLPC) were evaluated. A combined treatment of CP and CA significantly increased the inactivation of *Salmonella* and the inhibition of PPO and improved the color without affecting the total phenolics content. In addition, dipping the samples in CA prior to CP and cooling prevented excessive moisture loss during CP treatment and vacuum cooling. The outcomes of this study are promising in terms of microbial safety and color maintenance of fresh-cut fruits. In the future, a wider variety of food items that are commonly vacuum cooled, such as leafy greens, cooked meat, and fish products could be evaluated.

Chapter 5: Conclusions and recommendations

5.1 Overall Conclusions

In this research, a new PiLPC system was designed and tested with the intention of achieving rapid cooling and microbial inactivation at low pressures. There are several studies in the literature on the microbial inactivation efficacy of CP at very low pressures but only a few studies conducted plasma treatment in the range between 20 – 600 mbar, which is the pressure range where many food processes like vacuum cooling and vacuum drying take place. A clear understanding of how CP's inactivation efficacy and discharge characteristics at these pressures compare to those at atmospheric pressure is required to integrate it into these common food processes successfully.

In chapter 3 of this study, the inactivation of *Salmonella* on cut-apples increased with CP treatment time. At sub-atmospheric pressures, the inactivation was not significantly lower than that at atmospheric pressure initially but started to decrease at 50 mbar pressure.

The emission spectra of CP were taken at atmospheric, 200, and 50 mbar. No differences in the peaks were observed, indicating that similar RONS were present at atmospheric and reduced pressures. However, their quantities may be affected at lower pressures, especially considering that RH was reduced inside the vacuum chamber at lower pressures, which may reduce the abundance of crucial ROS like hydroxyl radical and partially explain CP's reduced efficacy at lower pressures.

Also, two protocols were developed to compare and identify the optimal window for CP inactivation. Samples treated with CP at 200 mbar before they were exposed to any pressure increase had significantly higher reduction rates than samples treated with CP after a pressure increasing step.

Rapid cooling of the samples was achieved with cooling times ranging from 3 to 7 min. The cooling rate of the samples directly correlated with their SA: V ratio. The major disadvantage of this integrated system was high moisture loss. However, moisture loss from evaporative cooling was considerably higher than the theoretical amount. More optimization in the sample size and pressure reduction rate can significantly reduce it.

In chapter 4 of this study, the aim was to improve the inactivation rate of *Salmonella* to more than *5-log reduction*, which is the standard used by the FDA to evaluate sanitization processes for multiple food products, without increasing the CP treatment time. Such a process could also help with color retention, PPO inhibition, and moisture loss.

Dipping the samples in CA prior to CP treatment significantly improved inactivation. A maximum inactivation of 5.68 log CFU/g was obtained for samples treated with CP for 3 min after dipping in 5% CA, and all samples had greater reduction of *Salmonella* during storage.

The residual activity of PPO enzyme was only 22% of what it was in the control samples, when the samples were treated with a combined 5% CA and 3 min of CP, and it correlated with citric acid concentration and CP treatment time. This hybrid treatment also improved color retention and final yield by reducing moisture loss by more than half.

PiLPC could help the fresh produce industry meet their pathogen reduction and cooling targets along with improved color retention and considerable inhibition of endogenous food enzymes. Additionally, this process could be further tailored to bring the benefits of CP in terms of decontamination to other high moisture products that require fast cooling such as leafy greens like lettuce and spinach, fishery products, and meat joints.

5.2 Recommendations

The main objective of this research was to develop a plasma integrated low-pressure cooling

(PiLPC) process that can simultaneously cool and decontaminate fresh food products and to optimize that process for maximum yield and decontamination rates. The following are areas that can be further investigated in the future:

1) The main peaks of air plasma at different pressures were identified and found to be identical. However, an accurate measurement of how the concentrations of different CP reactive species are affected at reduced pressures and relative humidity levels is needed for better optimization of the process,
2) Use of a wider variety of food items that are commonly vacuum cooled, such as leafy greens, cooked meat, and fish products,
3) Quantification of the effect of reactive oxygen species on lipid oxidation and their impact on the quality of lipid-rich products,
4) Monitoring of product temperature throughout the whole process, especially during CP treatment, using suitable thermometers or fiber optic temperature sensors,
5) Most microorganisms in the natural environment come in the form of biofilms rather than in a single species planktonic form. It is important to investigate how the pressure fluctuations and process sequence in PiLPC can affect the efficacy of the process,
6) Develop more accurate modeling of moisture loss during PiLPC, that takes into account how variables such as rates of pressure reduction and re-pressurization, and temperature and RH of the air during re-pressurization could help in further optimizing the process to reduce moisture loss,
7) Instead of dipping in CA solution prior to CP treatment, an automatic sprayer/mist system inside the vacuum chamber for spraying/misting of antimicrobial/antioxidant solutions on food products during PiLPC could make this a one batch process,

8) Understanding the microbial inactivation mechanisms at low-pressure CP process in comparison to atmospheric pressure, and the combined effects of antimicrobial/antioxidant solutions with CP on microbial inactivation mechanisms,
9) Understanding the potential of PiLPC process for fungal inactivation and spoilage reduction of fresh food products, and
10) Integrating CP with other low-pressure food processing methods such as vacuum and freeze-drying could provide multiple benefits such as microbial inactivation along with drying.

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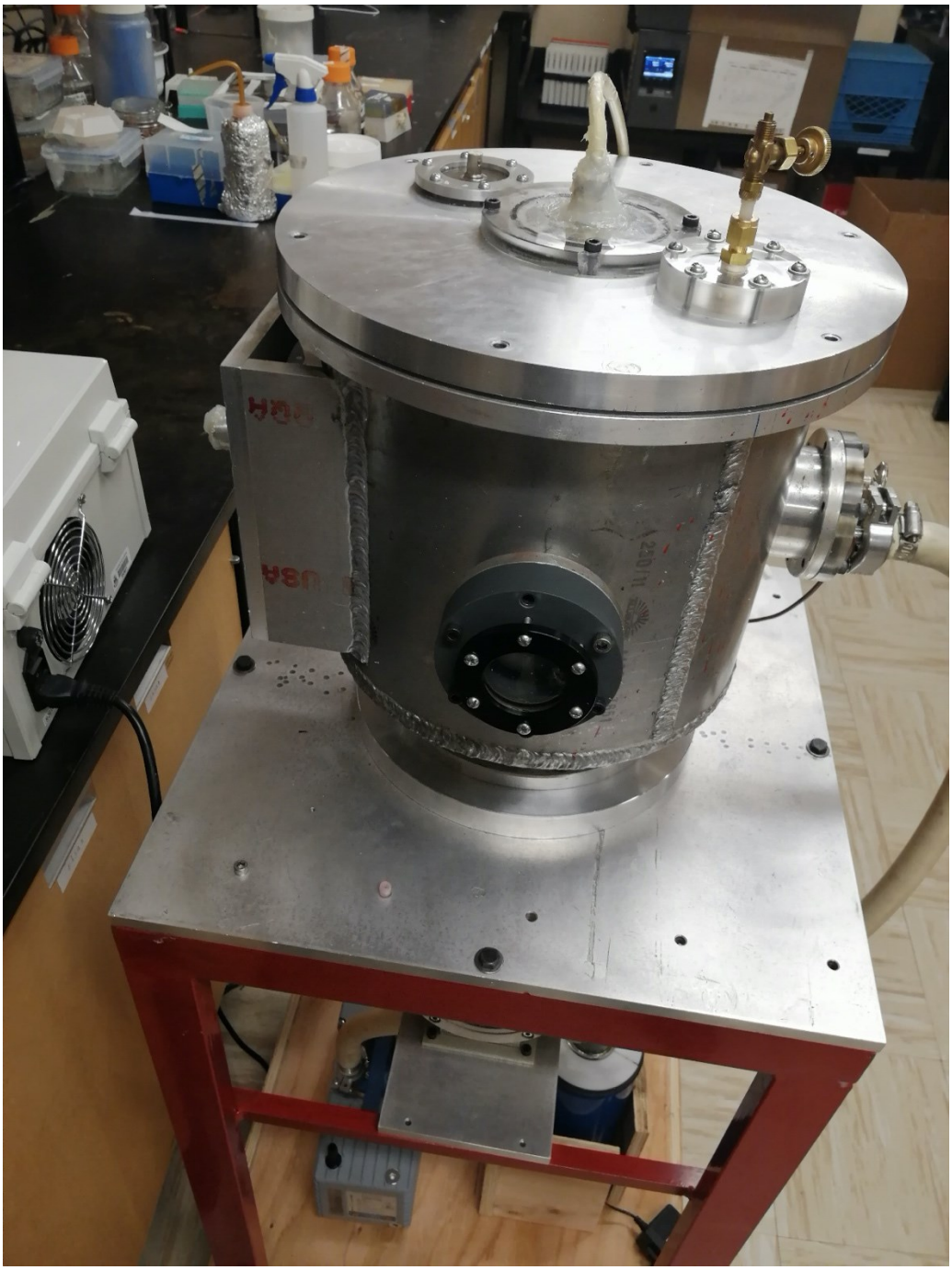
APPENDIX - I. Digital images of the PiLPC system


SHARPS
WASTES



APPENDIX - II. D PiLPC: User Manual

Scope and Purpose

This manual provides instructions for the assembly and operation of the Plasma integrated Low-Pressure Cooling (PiLPC) system at the Food Safety and Sustainability Engineering Lab. Please, read this manual and its supporting documents before you use or operate this system.

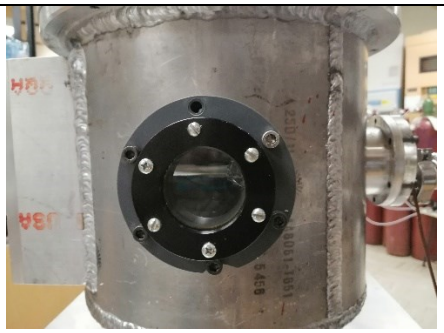
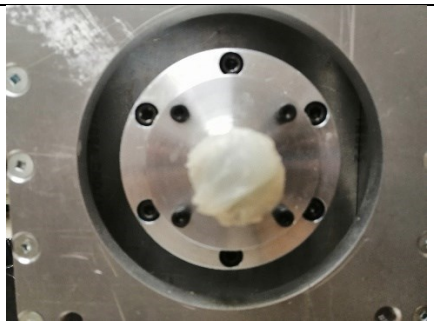
Getting started

Description of the system

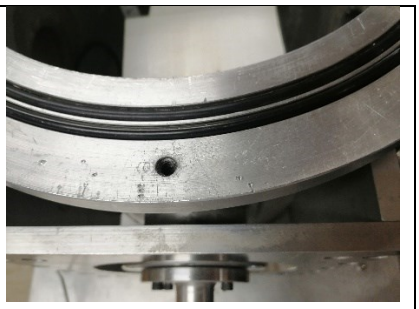
This system is composed of a stainless-steel chamber connected to a vacuum pump and a cold trap to obtain vacuum conditions. Inside the chamber, two electrodes are connected to a plasma generator through two feedthroughs. In addition to this, the chamber has multiple portals that have sensors and valves for process control.

Please refer to Appendix I for an overall diagram of the system. Below is a list of the different components of the system and their installation points.

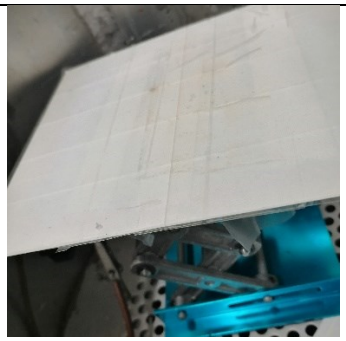
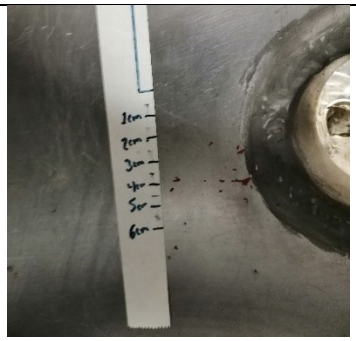
Side view

	
Pressure Sensor.	The window for spectra measurement.
	
Ground electrode and pump connection.	Spare window.

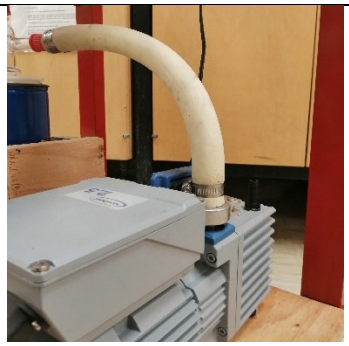
Top view

		
Air valve.	Top electrode connection.	The top cover and its O-rings.

Interior view

		
The powered electrode.	Ground electrode.	Ruler for gap measurement.

Bottom view

	
Cold Trap	Vacuum pump

How to use the system

Cold plasma treatment:

Before starting the cold plasma machine, determine the pressure you will be treating the samples and adjust the gap between the two electrodes to the appropriate one.

After you reach the target pressure at which you will operate the cold plasma, take into account that the pressure will slightly fluctuate, so establish a margin of error, i.e., ± 10 mbar, and if the pressure approaches the higher end of your margin of error briefly start the pump to keep it from going higher.

Cooling and Temperature Measurement

To measure the temperature during cooling, you can use one of two options:

1. Insert extremely thin thermocouples (5srtc-tt-k-40-36) under the O-ring of the vacuum chamber cover. These thin thermocouples have a marginal impact on the pressure. However, due to their very small diameter, they can be easily cut, so if you are to use the more normal thermocouples, use method 2.
2. Use the spare side window by utilizing adhesives or by adding a new NPT connection.

If you are targeting refrigeration temperatures (< 4 °C), consider that the temperature will rise during re-pressurization, thus setting your target temperature at 1 or 2 °C is recommended.

Acquisition of the emission spectra

All the windows in the system are made of quartz glass except the one mentioned in the system description, the side view section, which is made of appropriate glass to acquire the emission even at low wavelengths that are filtered out by the quartz.

Tips and Warnings

Leakage

Since this is a vacuum system, leakage is the most likely problem. Some of it is to be expected since the vacuum chamber has many connections, but if extra leakage is spotted, consider:

1. Looking at the tube connections, especially those connected to the two sides of the cold trap, they are the most likely to leak, also check the top cover of the cold trap itself; sometimes, its O-ring can get displaced.
2. Check the silicone sealants. Sometimes a small, barely visible opening can develop and cause problems.

High Final Pressure

Theoretically, the final pressure that the vacuum pump can reach is 0.5 mbar; the pump was capable of reaching this value when the vacuum chamber had no outer connections. However, if the pressure reduction stops at higher pressures (5 or 6 mbar), its to be expected due to:

1. The multiplicity of outer connections.
2. The presence of high moisture samples.

However, placing enough dry ice in your cold trap and making sure the vacuum chamber is as dry as possible always helps reach a lower final pressure.

Sparking

Sparking can happen more at lower pressures and may damage the ground electrode and, more seriously, the powered electrode.

Usually, this starts happening if the ground electrode is not covered with enough dielectric material (two layers of glass is recommended) or the dielectric is not aligned well.

1. When there is a misalignment, sparking will start, and if it's caught early enough, the top electrode will not be damaged, which is why it is recommended to keep an eye on the plasma during the treatment (at least looking frequently).
2. If the top electrode is damaged, apply epoxy on the damaged spot while keeping away from the central ring where the plasma is generated (to not affect the uniformity of the plasma).

Safety

1. Avoid inhaling the ozone after opening the vacuum chamber.
2. Put an electrically nonconductive material between the stainless steel and the cable of the powered electrode.

Disclaimer: this user manual does not stand in for reading and following the safe use instructions and user manuals of the different parts comprising the PiLPC system.

Please refer to the resources below:

Resources

The Vacuum Pump manual *(Hard copy in the lab and soft copy on the lab desktop).*

The Cold Plasma Generator manual *(soft copy on the lab desktop).*

The Cold Trap manual *(soft copy on the lab desktop).*

The Pressure Sensor manual *(Hard copy in the lab and soft copy on the lab desktop).*

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