2019

High voltage atmospheric cold plasma treatment on soft cheeses for microbial inactivation and quality assessment

Zifan Wan
Iowa State University

High voltage atmospheric cold plasma treatment on soft cheeses for microbial inactivation and quality assessment

by

Zifan Wan

A dissertation submitted to the graduate faculty

in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Food Science and Technology

Program of Study Committee:
Kevin M. Keener, Major Professor
Stephanie Clark
Kurt Rosentrater
Aubrey Mendonca
Hui Wang

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2019

DEDICATION

To my dearest parents, mentors and friends,

thank you for your love, support, inspiration, and encouragement,

which helps me to be brave and fearlessly pursue my dream.

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ACKNOWLEDGMENTS

I would like to sincerely thank my major professor Dr. Kevin Keener for his support, guidance and patience throughout the course of my study. Thank you for believing in me and encouraging me. Also, thank you for always being available for discussion when I encountered difficulties. There were many times when my research did not go as planned, and you have offered insightful suggestions, which helped me to find the solutions from a different perspective. I have really gained a lot of invaluable experience during my time working with you.

I am also very grateful to my committee members, Dr. Stephanie Clark, Dr. Kurt Rosentrater, Dr. Aubrey Mendonca and Dr. Hui Wang. Thank you for giving all the valuable suggestions and guidance. Thank you Dr. Clark for teaching me the cheese-making skills and being my mentor for Prepare Future Faculty program. I have really learned a lot from you. This dissertation would not have been possible without the support from my committee members.

In addition, I would like to thank my labmates, Dr. Shashi Pankaj, Dr. NN Misra and Dr. Bill Colonna, who have always been there when I needed help and encouraged me all the time. Thank you Bill for helping me establishing the microbial analysis protocol and always being responsive when I came with questions. Thank you Shashi for guiding me through my early days of graduate school. While, thank you NN for encouraging and guiding me towards the end of my study. Lastly, I would like to thank my parents and close friends who have always supported and encouraged me to finish my graduate study. Thank you mom for believing in me and encouraging me to pursue the dream I have.

ABSTRACT

Driven by the industrial needs and interests to ensure the safety of soft cheeses, such as queso fresco, my studies have focused on assessing the capability of high voltage atmospheric cold plasma (HVACP), a novel non-thermal technology, in microbial inactivation for its potential application in soft cheese production to remove pathogens from post-processing contaminated cheeses. A variety of treatment conditions were evaluated including three gas blends (air; MA65: 65% O_2, 30% CO_2, 5% N_2; MA50: 50% CO_2, 50% N_2), three voltages (60 kV, 80 kV, and 100 kV) and two modes of exposure (direct vs. indirect). Direct mode of exposure was found to be more efficient in inactivation of *Listeria innocua*, non-pathogenic surrogates for *Listeria monocytogenes*, and matrix with more complex surface structure and higher nutrients was more resistant to microbial inactivation by HVACP treatment. Increased voltage showed higher efficiency for microbial inactivation in queso fresco and HVACP treatment was found to be more effective in inactivation of the gram-negative bacteria (*E.coli* K-12) compared to the gram-positive (*Listeria innocua*). Minimal changes in pH, moisture and color were observed with no significant changes ($p<0.05$) in the texture of queso fresco after HVACP treatment. Moreover, dry air direct HVACP treatment for queso fresco was shown to be more efficient in *Listeria innocua* inactivation compared to MA50 treatment as reactive oxygen species play an important role in microbial inactivation. Lastly, the direct HVACP in dry air demonstrated its ability in inhibiting the growth of *Listeria innocua* and background microflora in queso fresco with extended refrigerated storage. This research has demonstrated the effectiveness of HVACP for decontamination of soft cheeses ensuring the safety as well as extending the shelf-life of the cheese.

CHAPTER 1: GENERAL INTRODUCTION

Dissertation Organization

This dissertation begins with a review of literature focusing on non-thermal processing technology including high pressure processing and cold plasma with an emphasis microbial inactivation and effect of each technology on products' quality. Four manuscripts follow the literature review. Manuscript authors are part of Iowa State University. Dr. Keener is the author for correspondence for all four of the manuscripts. This dissertation ends with a conclusion and recommendation for further work.

Literature Review

Dairy consumption overview

Dairy products contribute greatly to the American diet which provide high-quality protein and are good sources for vitamins A, D and B-12, as well as calcium (U.S. Department of Health Human Services, 2016). In 2017, the average consumption of fluid milk and cheese was around 149 and 39.1 pounds per person in the United States, respectively (USDA, 2017). The 2015 – 2020 Dietary guidelines for Americans recommend Americans to consume 3 to 5 servings of dairy products daily at the 2,000-calorie level consumption pattern (U.S. Department of Health Human Services, 2016).

The exact time when milk of other mammals, such as cow and sheep, was first consumed by human was unable to track, but it was most likely after the domestication of these mammals, for example cattle were domesticated around 10,000 years ago from aurochsen (Götherström et al., 2005). Ever since then, various kinds of dairy products, such as cheese, butter, yoghurt, etc., were discovered which led to dairy products with prolonged shelf-life (Salque et al., 2013). The

production of cheese is a particularly significant development for the processing of milk as it enabled the preservation of fresh milk in a non-perishable and transportable form (Salque et al., 2013). Moreover, the production of cheese made milk more digestible for early prehistoric farmers who had not yet developed lactase persistence (Burger et al., 2007; Itan et al., 2009).

The production of cheese involves a complex process including coagulation of milk, either by enzyme or acid, yielding the curds, removal of liquid whey, and aging (by starter culture) for aged cheeses (Salque et al., 2013). Starter culture plays a very important role during aging of the cheese. Besides contributing to the flavor development during aging and curd formation by lowering the pH, starter culture bacteria play an essential role in controlling harmful/pathogenic microorganisms by acid production and microbial competition during aging (Vedamuthu & Reinbold, 1967). As a type of fresh cheese, queso fresco undergoes a relatively simple cheese making process (shown in Fig. 1.1), and can be made with or without starter culture. Even for queso fresco made from cultured milk, aging process is not involved. Hence, fresh cheeses like queso fresco have a high moisture and low salt content, thus possess a shorter shelf-life and higher potential for pathogen contamination compared to aged cheeses such as Cheddar and parmesan. In the United States, it is required by law that soft and fresh cheeses need to be made from pasteurized milk to ensure the safety of the cheese for consumption (FDA, 2017a). Even though, thermal pasteurization can sufficiently eliminate foodborne pathogens in raw milk, there is still potential risk of post-pasteurization pathogenic contamination during cheese making likely resulted from unsanitary conditions in the production facilities.

Listeria monocytogenes

Listeria monocytogenes (LM) is one of the major foodborne pathogens of concern in ready-to-eat foods (RTE) such as meat, fresh fruits, and dairy products, and has become an

increasing concern worldwide (Farber & Peterkin, 1991; Liu, 2006). Research has indicated that recontamination is the primary source of LM in commercially processed RTE foods (Kousta et al., 2010). LM has the ability to grow at a wide range of temperatures including refrigerated temperatures, can tolerate a high level of salt content, and has the ability to grow at relatively low pH, thus making LM difficult to control during food processing (Farber & Peterkin, 1991). Fresh cheeses like queso fresco are especially vulnerable to LM due to its high moisture, low salt and near neutral pH providing an optimal substrate for the growth of spoilage and pathogenic microorganisms during storage and distribution (Clark et al., 2004).

As mentioned previously, thermal pasteurization effectively eliminates the risk of LM contamination of cheese from raw milk and a detailed and validated thermal processing protocol for milk has been established and recorded in the Pasteurized Milk Ordinance (FDA, 2017b). However, post-manufacturing steps might introduce LM contamination into the cheese (Soni et al., 2012; Tomasula et al., 2014). Without an effective processing method to remove post-manufacturing pathogenic contamination, fresh cheeses like queso fresco could lead to a higher risk of foodborne illnesses such as listeriosis caused by *Listeria monocytogenes*. In the past two years (2017 and 2018), there were 25 recalls due to *Listeria monocytogenes* contamination of various types of cheese such as aged raw milk cheese, fresh cheese, etc. (FDA, 2018). With the high risk of listeriosis, Centers for Disease Control and Prevention (CDC, 2016) advised that pregnant women and immune compromised population should not consume queso fresco and other soft cheeses. Moreover, an estimated 1,600 people get sick from LM each year due to consumption of LM contaminated food products and within that about 260 people died in United States (CDC, 2016). Currently, no effective commercial technology has been identified to reduce microorganisms in queso fresco and other soft cheeses post manufacturing, which introduces a

food safety risk to consumers and limits the shelf-life of fresh soft cheese. Under this circumstance, it is critical to establish an effective commercial technology, which can eliminate post-manufacturing pathogenic contamination, thus ensure the safety as well as maintain the quality of fresh cheeses like queso fresco.

For delicate products like fresh cheeses, thermal treatment is not suitable for post manufacturing treatment, as it would lead to quality degradation making it unacceptable for consumers. Thus, *there is a need to deliver a non-thermal* treatment to remove post-processing pathogenic contamination in products like queso fresco cheese. Over the last two decades, extensive research has been conducted on various non-thermal technologies to produce safe, nutritious and minimal processed foods (Knorr et al., 2002; Rawson et al., 2011; Tiwari et al., 2009). In the following sections, two non-thermal technologies growing in popularity and suitable for fresh dairy products, *high pressure processing* and *cold plasma*, are discussed.

High pressure processing

High pressure processing (HPP) utilizes pressure between 400 MPa and 800 MPa to inactivate microorganisms and denature proteins (enzymes) during processing of food products. HPP effects are governed by Le Chatelier's Principle, which states that under pressure, whenever a stress is applied into an equilibrium system, the system will react in order to counteract the applied stress (Huppertz et al., 2002; Thakur & Nelson, 1998). With high pressure applied, reactions leading to reduced volume will be promoted and these would result in inactivation of microorganisms or enzymes and possibly textural changes in food products (Huppertz et al., 2002; Oey et al., 2008). Researchers have investigated the mechanisms of HPP in microbial inactivation, but the specific mode of action leading to lethality is still unclear. It has been reported that high pressure leads to enzyme inactivation, membrane protein denaturation,

disintegration of ribosomes, disruption of cell membrane and decreasing of intracellular pH, thus results in microbial inactivation (Abe, 2013; Knorr et al., 2010; Smelt et al., 2001).

HPP has been examined for its potential application in non-thermal processing of dairy products such as milk, cheese, yogurt, etc. (Chawla et al., 2011; Datta & Deeth, 1999; Huppertz et al., 2002). Studies have demonstrated the capability of HPP in achieving a > 5 $\log_{10}$ pathogen reduction in milk such as *E.coli* O157:H7 (Vachon et al., 2002), *Listeria monocytogenes* (Dogan & Erkmen, 2004; Erkmen & Dogan, 2004; Vachon et al., 2002), and *Yersinia enterocolitica* (De Lamo-Castellví et al., 2005). The ability of achieving greater than 5 $\log_{10}$ pathogen reduction enables HPP to process milk resulting in comparable microbial inactivation to thermal pasteurization of milk (72 °C, 15 s) which eliminates any pathogens in milk (> 5 $\log_{10}$ reduction) and destroy most spoilage microorganisms (FDA, 2017b).

However, HPP is less effective in spore inactivation compared to inactivation of vegetative cells, which limits the use of HPP for pasteurization purpose as commercial sterilization requires the production of a microbiological stable product at room temperature (Codex Alimentarius, 2004). Papafragkou et al. (2002) have reported no reduction of *Clostridium perfringens* spores after 30 min 500 MPa HPP treatment at 25 °C. McClements et al. (2001) have also observed a minimal 0.5 $\log_{10}$ reduction of *Bacillus cereus* spores in milk after 25 min 400 MPa HPP treatment at 30 °C. To increase the efficiency of HPP in spore inactivation, researchers have introduced mild heating to assist the inactivation of spores along with high pressure treatment. Combined with mild heating, HPP is able to result a significant inactivation of spores (Margosch et al., 2004; Reddy et al., 1999). Reddy et al. (1999) have reported a 5 $\log_{10}$ reduction of *Clostridium botulinum* spore after 5 min 827 MPa HPP treatment at 55 °C. Margosch et al. (2004) have also observed a 5 $\log_{10}$ reduction of *Clostridium*

botulinum spore after HPP treatment at 80 °C, 600 MPa for 6 min. Thus, with the assistance from mild heating, there is even a potential for application of HPP in sterilization of food products.

Beside pathogens and spoilage microorganisms, during thermal pasteurization of milk, indigenous enzyme (lipase) is another important target for inactivation to deliver a good quality pasteurized milk. Lipase is inactivated to prevent lipolysis upon further processing (e.g. homogenization) and during storage and distribution of milk. Lipolysis in milk fat contributes to rancid off-flavors making the milk not acceptable for consumption (Deeth, 2006). High-temperature short-time (HTST) pasteurization is able to efficiently inactivate most of the indigenous enzymes including lipase in milk (Farkye & Imafidon, 1995). However, compared to heat treatment, HPP is less effective in enzyme inactivation. Pandey and Ramaswamy (2004) observed no inactivation of lipase in milk for up to 100 min treatment at 300 MPa, and enhanced lipase activity was found under higher applied pressure (350 MPa and 400 MPa). Seyderhelm et al. (1996) have reported a complete inactivation of lipase after 5 min treatment in a tris buffer system at 45 °C and 700 MPa. However, tris buffer is simple buffer system made from the organic compound, tris(hydroxymethyl) aminomethane, whereas milk is abundant in protein and fat, which would likely lead to less efficient lipase inactivation by HPP compared to in Tris buffer. The inefficient enzyme inactivation after HPP treatment would lead to lipolysis resulting in off-flavor of the milk. Hence, HPP alone cannot sufficiently deliver a good quality pasteurized milk. Thus similar as spore inactivation, mild heating might be adopted to inactivate indigenous lipase in milk prior to HPP treatment.

The combination of HPP and thermal treatment might be efficient to pasteurize or even sterilize the milk prior to yogurt or cheese making. Besides microbial inactivation, HPP also

resulted in modification of protein in the milk (Huppertz et al., 2002). Trujillo et al. (1999a) have observed an enhanced rennet coagulation and higher yield of cheese made from milk after 15 min 500 MPa HPP treatment at 20 °C. Other researchers have also reported an increased yield of cheese made from HPP treated milk with no detrimental effects on cheese flavor (Drake et al., 1997; Trujillo et al., 1999a, 1999b). This increasing in yields is likely due to disintegration of proteins by high pressure treatment, which allows larger surface area for binding during rennet coagulation, thus resulting in less protein loss into the whey (Trujillo et al., 2002). Moreover, Buffa et al. (2001) have reported a firmer and less fracturable cheese made from raw or HPP-treated milk compared to cheeses made from heat-pasteurized milk (Buffa et al., 2001). Furthermore, due to pressure-resistant characteristics of indigenous milk lipase, HPP treated milk leads to enhanced lipolysis in cheese during ripening compared to heat-pasteurized, in which lipase is mostly inactivated during heat treatment (Buffa, Guamis, et al., 2001). With enhanced lipolysis, Buffa, Guamis, et al. (2001) have observed higher score of overall aroma for cheese made from HPP treated milk compared to cheese made from thermal pasteurize milk. This is likely resulted from the breaking down of the lipids into free fatty acids by lipase, which contributes to the unique flavor and aroma of the cheeses. These results demonstrate a potential use of HPP for milk pasteurization prior to cheese production leading to a product with improved quality and enhanced yield.

Improved quality was also observed in yogurt made from HPP treated milk. Johnston et al. (1992); (1993) have found that increasing in pressure during HPP treatment of milk resulted in a formation of firmer acid-induced gels. In addition, they have observed that yogurt made from HPP treated milk has a greater resistance to syneresis (Johnston et al., 1993). Ferragut et al. (2000) have also reported that yogurt made from HPP-treated milk has a higher firmness

compared to yogurt made from heat-pasteurized milk. This increased firmness of yogurt after HPP treatment is likely due to denaturation of whey protein and disruption of casein micelles, which lead to a greater effective area for surface interaction. These results have shown a potential application of HPP treatment for milk prior to yogurt making leading to a product with improved quality.

However, HPP treatment is not suitable for post-contamination treatment during cheese production, as it might lead to undesirable textural changes. Hnosko et al. (2012) evaluated the effectiveness of HPP in inactivation of *Listeria innocua* in queso fresco cheese, and found greater than 5-log reductions after 15, 3, or 1 minutes HPP treatment at 500, 550, or 600 MPa, respectively. However, queso fresco became sticky and unable to crumble after HPP treatment (Hnosko et al., 2012). The crumbliness is an important textural property for queso fresco (Guo et al., 2011; Sandra et al., 2004), as consumers often crush and sprinkle queso fresco on foods before consumption (Hwang & Gunasekaran, 2001). Thus, even with significant inactivation of *Listeria innocua*, non-pathogenic surrogates for *Listeria monocytogenes*, authors still do not recommend HPP for queso fresco applications (Hnosko et al., 2012). Other studies have also observed textural changes of fresh cheese, such as queso fresco, after HPP treatments (Capellas et al., 2001; Okpala et al., 2010; Van Hekken et al., 2013). Thus, HPP treatment is not applicable for elimination of post-manufacturing contamination in cheese.

Last but not the least, cost might be another limitation for the industrial adoption of HPP. With high capital investment and operational and maintenance cost, the cost of HPP is very high compared to the traditional thermal processing. For instance, pasteurization of orange juice on a commercial production scale was estimated to cost 10.7 ¢ per liter, versus 1.5 ¢ per liter for conventional thermal processing (Sampedro et al., 2014). Sampedro et al. (2014) have estimated

that for industrial scale orange juice processing, a capital coast of $132,000 is needed for thermal pasteurization, while $5,090,000 for HPP pasteurization. Moreover, a three times higher labor cost is expected for HPP treatment compared to thermal pasteurization, in which $220,000 is estimated for thermal treatment, whereas $660,000 for HPP (Sampedro et al., 2014). In dairy industry, thermal pasteurization is well established and is the commonplace for raw milk processing. The high capital and labor cost of HPP might limit its use in production of premium dairy products instead of being commonly adopted for processing of dairy products.

Cold plasma

Plasma is an ionized gas, the fourth state of matter, consisting of ions, free electrons, atoms and molecules in fundamental or excited states with a net neutral charge (Misra, Schlüter, et al., 2016). Based upon thermal equilibrium, plasma can be characterized into two categories: thermal plasma and non-thermal (cold) plasma. In thermal plasma, also referred as equilibrium plasma, all reactive gas species present in a thermodynamic equilibrium in which electrical energy heats up both electrons and the remaining gas molecules, whereas, in cold plasma (non-equilibrium), the electrical energy is only used to heat up the electrons while the remaining gas molecules stay near room temperature (Misra, Moiseev, et al., 2014). This non-equilibrium in thermodynamic enables cold plasma to be equal and close to room temperature, thus making it ideal for non-thermal application in food processing. Moreover, in regards to applied pressure, plasma can also be classified into three categories, including high-pressure, atmospheric pressure and low-pressure plasma. Atmospheric pressure plasma allows the generation of plasma at atmospheric pressure, thus excludes the requirement for the cost-intensive reaction chambers for maintaining the pressure making it more suitable and economical for industrial application.

Over the past decade, cold plasma has gained great interest from the food industry for its potential application in food processing. The novelty of this technology attributes to its nature of non-thermal, economical, adaptable and environmentally friendly (Misra, Schlüter, et al., 2016). Studies have demonstrated the applications of cold plasma in food processing for decontamination (Misra et al., 2011), enzyme inactivation (Misra, Pankaj, et al., 2016), and toxin removal (Misra, 2015). In particularly for microbial inactivation, cold plasma has been shown for its effectiveness against major foodborne pathogens such as *Listeria monocytogenes* (Ziuzina et al., 2014), *Escherichia coli* O157:H7 (Ziuzina et al., 2014) , *Salmonella typhimurium* (Fernandez et al., 2013) and *Staphylococcus aureus* (Kim et al., 2014). Furthermore, studies have demonstrated the cold plasma treatment is very efficient in spore inactivation, such as *Bacillus subtilis, Bacillus pumilus, Bacillus atrophaeus, Geobacillus stearothermophilus*, etc., allowing its applications in non-thermal sterilization of food products (Bourke et al., 2017; Klämpfl et al., 2012; Patil et al., 2014).

Moreover, cold plasma treatment offers distinct economical advantages for decontamination of foods compared to thermal processing including low capital cost and low operational costs (minimal labor). It has already been adopted in industrial processes such as electronics cleaning, bonding of plastics or binding of dye to textile fibers (Grill, 1994), while their potential remains untapped in the food industry. Cold plasma generation at atmospheric pressure is especially of commercial interest to the food industries, because it is very *adaptable* to high speed manufacturing, easy to incorporate into the existing processing system, and can be implemented under *normal* operating condition (Misra, Keener, et al., 2014). Whereas, HPP requires to be operated under high pressure and new operation line is needed for HPP processing.

Microbial inactivation

Cold plasma has demonstrated its significance in inactivation of microorganisms, including bacteria (Niemira & Sites, 2008; Ziuzina et al., 2014), bacterial endospores (Klämpfl et al., 2012; Lee et al., 2006), fungi (Pankaj et al., 2017; Suhem et al., 2013), and biofilms (Ziuzina, Boehm, et al., 2015; Ziuzina, Han, et al., 2015). The exact mode of action for microbial inactivation by cold plasma treatment is still not well understood. However, researchers have identified three major agents, which are responsible for cold plasma induced microbial inactivation, including reactive gas species, charged particles, and ultraviolet radiation (Laroussi, 2005). Figure 1.2 illustrates an overview of the proposed mechanisms for cold plasma microbial inactivation.

Reactive gas species

As mentioned in the previous section, with energy applied, gas can be ionized and broken down resulting in the generation of free electrons. These electrons can then collide with the nearby gas molecules (O_2, N_2, CO_2, etc.) leading to excitation or dissociation of the molecules, forming a quasi-stable gas/plasma, which is composed of electrons, ions, free radicals, metastable molecules, etc. (Misra, Schlüter, et al., 2016). These reactive gas species play a significant role in cold plasma microbial inactivation. As an example, air plasmas are abundant in reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as O, O_2*, O_3, $OH^{\bullet}$, NO, NO_2, NO_3, N_2O_4 etc. (Laroussi, 2005; Moiseev et al., 2014). These ROS and RNS results in a strong oxidative stress to cell membranes and intracellular components (e.g. DNA, proteins, etc.) leading to the inactivation of microorganism by disruption of cell membrane lipids, and leakage of intracellular macromolecules (Laroussi, 2005). Lipids are essential part of cell membrane structure, as it is responsible for the formation of lipid bilayers, which contains

unsaturated fatty acids for maintaining membrane fluidity (Murata & Los, 1997). These unsaturated fatty acids (USFs) are vulnerable to the attack by ROS (such as $OH^{\bullet}$ and $^1O_2$) which can remove an H atom from the methyl groups forming a fatty acid radical (R•), that can be furthered oxidized into lipid hydroperoxide (ROOH) by O_2 (Abramzon et al., 2006; Gavahian et al., 2018). Joshi et al. (2011), Alkawareek et al. (2014) and Yost and Joshi (2015) have observed an increased lipid peroxidation of *E.coli* membrane after cold plasma treatment. Moreover, the products of lipid peroxidation (e.g. malondialdehyde MDA) could result in an irreversible damage to the intracellular DNA and protein via the formation of covalent adducts (Del Rio et al., 2005).

Some studies have demonstrated the ability of ROS permeating the outer membrane, resulting in direct interaction with the intracellular components leading to DNA damage (Han et al., 2016; Yost & Joshi, 2015). Han et al. (2016) have examined the difference of cold plasma inactivation between the Gram-positive (*S. aureus*) and the Gram-negative (*E. coli*) and observed higher cell leakage in *E. coli* compared to *S. aureus* after at 80 kV for up to 5 min in air. The authors proposed that for the Gram-negative *E. coli*, the inactivation is mainly due the oxidation of membrane lipid by ROS, while with the presence of the thick peptidoglycan layer, oxidation of intracellular components by ROS is the major cause for inactivation for the Gram-positive *S. aureus* (Han et al., 2016).

Charged particles

Besides ROS and RNS, the charged particles (positive or negative ions), generated during cold plasma treatment, also play an important role in microbial inactivation by disruption of cell membranes (Laroussi, 2002). Lunov et al. (2015) proposed that the accumulation of charged particles near cell outer surface induces an increase in electrostatic forces on cell membranes,

which can eventually exceed the total tensile force of the membrane, thus resulting in perforation of cell membrane causing lethality of the cell. Cell perforation can induce the formation of pores on cell membranes leading to the release of intercellular components (DNA, ions, etc.) and allow the permeability of reactive gas species, which contribute to the damage of intercellular components (DNA, proteins, etc.), thus eventually leading to cell death (Coutinho et al., 2018; Phan et al., 2017). San-Xi et al. (2010) have observed protein leakage of *B. subtilis* after treatment by an atmospheric pressure plasma jet with Ar or Ar+H_2O_2 vapor mixture, as a result of bombarding action from the charged particles. However, lower protein leakage was found after plasma treatment in Ar+H_2O_2 vapor mixture compared to Ar, which is due to the consumption of protein by OH• radicals (San-Xi et al., 2010). Thus, the deterioration of cell membrane is caused by a synergistic action of charged particles and the reactive gas species (Misra, Schlüter, et al., 2016; San-Xi et al., 2010).

UV radiation

UV light, in the range of 200-300 nm, can lead to dimerization reaction of thymine and cytosine in the same DNA strand resulted in DNA damage and inhibition of DNA replication (Sinha & Häder, 2002). UV radiation can also cause intrinsic photo-desorption, resulting from UV photon, which breaks down chemical bonds in microbes and forms volatile compounds such as CO and CH_x (Lerouge et al., 2000; Philip et al., 2002). Moreover, UV light can accelerate the etching of cell membranes by breaking down the chemical bonds on membrane, thus allowing more reaction sites for the free radicals (Philip et al., 2002). As mentioned in the early section, free radicals (e.g. $OH^{\bullet}$ and $NO^{\bullet}$) are generated during plasma treatment. These free radicals can be absorbed in the cell surface and resulting in the formation of volatile compounds (CO_2, H_2O) leading to etching/erosion of cell membrane (Schlüter & Fröhling, 2014).

Plasma sources

Plasma generation can be carried out using different types of energy which can ionize the gases, such as electrical, thermal, optical (UV light), radioactive (gamma radiation) and X-ray electromagnetic radiation (Pankaj & Thomas, 2016). Within that, electric energy is commonly adopted for cold plasma generation. Various types of plasma sources are available such as corona discharge, dielectric barrier discharge (DBD), and plasma jets. This offers the flexibility of cold plasma application for different industrial purposes. In the following section, three plasma discharge systems are briefly discussed including corona discharge, plasma jet and dielectric barrier discharge (DBD).

Corona discharge is a localized, non-homogenous, and non-thermal discharge which is often employed at atmospheric pressure in a point-to-plane geometry (shown in Fig. 1.3) producing a weak luminous discharge near the sharp point electrode, where ionization of the gas occurs. It is commercially available for ozone production for treatment of fresh fruit and vegetables (Guzel-Seydim et al., 2004). However, due to its configuration, it is limited to small area treatment, which constrains its use for the massive industrial processing.

Among various types of plasma sources, plasma jet and dielectric barrier discharge (DBD) are the most commonly examined for applications in food processing. Prior to application in food processing, plasma jet is employed as a thermal jet system which are used for cutting and welding (Krajcarz, 2014; Lancaster, 1984). While, for food processing, the interest is in atmospheric pressure non-thermal plasma jets. As shown in Fig. 1.4, plasma jet consists of two concentric electrodes, and the inner electrode is generally attached to a radio frequency power at high frequency leading to gas ionization, which then exits the nozzle and results in a ‘jet-like’ appearance. This device enables the generation of a homogenous plasma and guide the reactive

gas into a localized treatment area, which removes the constraint of placing treated target directly in the electric field, thus allowing treatment of objects with various size or dimension. Due to its ability in localization, plasma jet is also utilized for medical application such as skin decontamination (Daeschlein et al., 2012), living tissue treatment (Shashurin et al., 2008), and treatment of chronic wounds (Weltmann et al., 2009).

Dielectric barrier discharge is simple and stable system, which is composed of two metal electrodes with at least one of the electrodes covered with a dielectric barrier (shown in Fig. 1.5). Dielectric barriers are employed to stabilize the plasma, prevent arc transition, thus enable the generation of a homogenous non-thermal plasma at atmospheric pressure. With these advantages, DBD has been utilized for applications in industrial ozone generation (Takayama et al., 2006), surface modification of polymers (Friedrich, 2012), as well as air pollution control (Kim, 2004). DBD system can be easily adapted into the existing industrial production line and produce a homogenous non-thermal plasma, thus it is a particular interest for the food industry (Misra, Keener, et al., 2014).

Moreover, as an extension of DBD, it is worth to note the ‘in-package’ plasma treatment, which has shown a great potential for applications in food processing. In-package plasma treatment allows the ionization of the headspace gas for plasma generation allowing extended exposure of reactive gas species with the food products leading to enhancement of microbial inactivation and avoiding cross-contamination. Furthermore, in-package plasma treatment can be easily scale up into a continuous system making it more feasible for industrial applications.

Cold plasma decontamination of food products

With the efficiency in microbial inactivation, extensive studies have been conducted for evaluation cold plasma application in fresh produces and meat processing for pathogen decontamination as well as potential shelf-life extension (Misra, Schlüter, et al., 2016). Twenty-eight published work related to cold plasma decontamination on fresh produces are summarized in Table 1.1, including application of cold plasma treatment in juices, fresh fruit and vegetables, fresh meat products, and dairy products (four studies for sliced cheese and two for milk).

These studies have demonstrated the significance of cold plasma in controlling of pathogens in food products, such as inactivation of *Salmonella enterica* in orange juice (Xu et al., 2017), *Salmonella typhimurium* in radish sprouts (Oh et al., 2017), *Salmonella* and *E.coli* O157:H7 in apples (Niemira & Sites, 2008), and *Listeria monocytogenes* and *Salmonella* bacon (Kim et al., 2011). Besides the efficacy in pathogen control, cold plasma has also shown the effectiveness in inactivation of spoilage microorganisms in fresh produces, such as inactivation of background microflora in strawberry (Misra, Moiseev, et al., 2014), *Penicillium italicum* in mandarins (Won et al., 2017), *Saccharomyces cerevisiae* in white grape juice (Pankaj et al., 2017), and background microflora in fresh pork (Ulbin-Figlewicz et al., 2015).

Specifically for dairy products, studies in cheeses slices and Cheddar, have shown that the effectiveness of cold plasma in inactivation of pathogens including *Escherichia coli* O157:H7, *Staphylococcus aureus, Salmonella typhimurium,* and *Listeria monocytogenes* (Lee et al., 2012; Song et al., 2009; Yong, Kim, Park, Alahakoon, et al., 2015; Yong, Kim, Park, Kim, et al., 2015).

Song et al. (2009) achieved a > 8 log_{10} reduction of *Listeria monocytogenes* on sliced cheese after 2 min DBD plasma treatment in He at 13.56 MHz. Yong, Kim, Park, Alahakoon, et al. (2015) also observed a significant microbial inactivation after DBD plasma treatment in air at 15 kHz for up to 7 min, in which a maximum of 2.67, 3.10 and 1.65 log_{10} reduction was found for *E.coli, Salmonella* and *Listeria monocytogenes*, respectively. Lee et al. (2012) utilized thin-layer DBD device to produce plasma at 3.5 kV at 50 kHz using He or He+O_2 gas mixer treating a 15x15x2 mm cheese slice for up to 15 min. A maximum reduction of 1.98 log_{10} CFU/ml and 0.91 log_{10} CFU/ml was observed for *E.coli* and *Staphylococcus aureus* after 15 min treatment in He+O_2 (Lee et al., 2012). However, significant reductions in flavor, odor and damage of cheese slices were observed after 10 and 15 min of plasma treatment (Lee et al., 2012). Yong, Kim, Park, Kim, et al. (2015) adopted a thin-layer DBD device and examined the inactivation of *E.coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* Typhimurium in sliced Cheddar cheese by cold plasma treatment at 15 kHz with a 100-W peak power using a N_2/O_2 gas blend for up to 10 min. A maximum of 3.2, 2.1 and 5.8 log_{10} CFU/g reduction of *E.coli* O157:H7, *Listeria monocytogenes* and *Salmonella* was achieved, respectively, after 10 min DBD treatment, with significant reduction in flavor, and increased off-flavor and lipid oxidation.(Yong, Kim, Park, Kim, et al., 2015). Moreover, a significant microbial inactivation was also observed in milk after cold plasma treatment. A maximum of 4.15 log_{10} CFU/ml *E.coli* reduction was achieved after 20 min corona discharge plasma treatment in air at 9 kV with no significant change in pH and color (Gurol et al., 2012). Kim et al. (2015) employed a DBD plasma system treating milk in air for up to 10 min and observed an approximate 2.4 log_{10} CFU/ml reduction of *E.coli, Listeria monocytogenes* and *Salmonella* with decreased pH and a* value, and increased L*, b* and lipid oxidation.

Even though negative impacts of quality was observed in cheese after cold plasma treatment (Lee et al., 2012; Yong, Kim, Park, Kim, et al., 2015), it is worth noting that in these two studies, cheese slices were treated for a prolonged treatment time with a maximum of 15 min at voltage lower than 5 kV. This long treatment time is likely the cause for quality degradation of the cheeses by prolonging the interaction between the sample and the reactive gas species leading to lipid oxidation and potential etching of the sample surface. Thus, a more efficient and optimized cold plasma processing is required for application in cheese processing. With limited literature on the quality attributes of cheese, several cold plasma applications in fresh produce are mentioned here as references to foresee the potential quality changes of cheese after cold plasma treatment. Bermúdez-Aguirre et al. (2013), (Misra, Patil, et al., 2014) and (Ramazzina et al., 2015) reported no significant or minor loss of color after cold plasma treatment of fresh produces such as strawberry, kiwifruits, lettuce and carrot. While, loss of color was observed at higher treatment for blueberry (Lacombe et al., 2015; Sarangapani et al., 2017). Furthermore, many of the reported studies demonstrate the *retention of texture* of food products after cold plasma processing. For fresh fruits and vegetables, no significant difference in texture was reported after cold plasma treatment of strawberry, apple, melons and cherry tomatoes (Misra, Patil, et al., 2014; Niemira & Sites, 2008; Tappi et al., 2016; Ziuzina et al., 2016).

High Voltage Atmospheric Cold Plasma

To achieve a more efficient microbial inactivation without compromising the quality of food, an advanced and enhanced plasma discharge with higher efficiency should be employed to reduce the treatment, while this introduces the application of high voltage atmospheric cold plasma (HVACP). The high voltage atmospheric cold plasma (HVACP) is an improved cold plasma process, which uses high voltages of 30 kV or more to generate plasma (reactive gas

species) in a few seconds to a few minutes. With high voltage applied, HVACP results more efficient inactivation of microorganisms and reduces the effect of cold plasma treatment on products' quality by decreasing the treatment time. Several studies from our group and collaborating group have demonstrated the efficiency of HVACP in microbial inactivation in a few seconds or minutes and minimal changes in quality attributes with great scale-up potentials (Misra, Moiseev, et al., 2014; Xu et al., 2017; Ziuzina et al., 2016; Ziuzina et al., 2014). Xu et al. (2017) achieved a greater than 5 $\log_{10}$ reduction of *Salmonella enterica* after 30 s of HVACP treatment at 90 kV in modified gas (65% O_2, 30% CO_2, 5% N_2) with no significant change in Brix and pH, while minor changes in color. Around 3.0 log reduction of background microflora in fresh strawberry was reported after 5 min HVACP treatment in 65% O_2 + 16% N_2 + 19% CO_2 and 90% N_2 + 10% O_2 at 60 kV, in which higher firmness of treated strawberries was observed than the control (Misra, Moiseev, et al., 2014).

This dissertation offers a general evaluation on the efficacy of HVACP treatment in microbial inactivation on fresh cheese, queso fresco, along with quality measurements including color, texture, pH, moisture content and lipid oxidation. This work could be further elaborated for future studies on HVACP applications in delicate dairy products processing for control of post-manufacturing pathogenic contamination and potential mean for shelf-life extension.

Importance of This Research

With no effective technology to remove post-processing contaminants, fresh cheese possesses a high risk of pathogen contamination and growth during distribution and storage. Since 2011, there are six *Listeria* outbreaks related to cheese, especially fresh soft cheeses (CDC, 2018). A technology, such as HVACP, is needed to resolve this serious issue in post-processing

contamination of fresh cheese, making it safer to eat without compromising products' quality and potentially increasing the shelf-life of fresh cheeses thus reducing food waste caused by spoilage.

Moreover, successful adoption of HVACP might help promote business in dairy export. From 2004 to 2014, the United States became the world's third-largest dairy product exporter and within all the exported dairy products, cheese is the most valued product with a value of $1,388.0 million in 2015 (Cessna et al., 2016). For export purpose, safety and pathogen free of the products is the imperative requirement. Extending the shelf-life of perishable goods would help promote the value of the products, as less spoilage waste would occur.

In addition, as mentioned in the previous section, cold plasma is capable of *spore inactivation*, which makes it a potential processing strategy to inactivate spores, such as *Enterobacter sakazakii* (concerns in infant formula) (Forsythe, 2005), in milk powder. Dry milk powder has an issue on thermophilic spores (Crielly et al., 1994; Scott et al., 2007). Under proper environment, these spores can germinate resulting enzyme and acid production leading to off-flavors in the products, which would cause significant economic loss (Rueckert et al., 2005; Scott et al., 2007). In the United States, milk powder is the second valued export dairy product with a value of $1,385.6 million (Cessna et al., 2016). A solution, such as cold plasma, to overcome this issue of spores in milk power, would help ensure the safety and promote the economical value of the products.

This project is the first study which utilized HVACP application in fresh cheese decontamination. The successful completion of this study enables a general understanding regarding to the efficacy of HVACP in microbial inactivation in fresh cheese and the effect of HVACP in quality attributes of fresh cheese. This study sets a benchmark of HVACP application in cheese processing and invites opportunity for further optimization of HVACP treatment and

future studies to consolidate the findings with a purpose for industrial adoption of HVACP to deliver safe to eat, pathogen-free dairy products.

Project Structure

The first phase of this project focused on examination of the effect of product composition, surface topography and mode of exposure on inactivation of *Listeria innocua*, a non-pathogenic surrogate for LM (Friedly et al., 2008; Hnosko et al., 2012), by HVACP treatment. Three matrices, including commercial queso fresco, cheese model and tryptic soy agar, were examined. Direct and indirect mode of exposure was evaluated. Microbial enumeration was performed to evaluate the efficiency of *Listeria* inactivation by cold plasma treatment. Scanning electron microscope and 3-D microscope was used to assess the structural differences among the three matrices. It was hypothesized that direct treatment was more effective in *Listeria* inactivation, and sample with higher nutrients and more complex surface structure was more resistant to cold plasma treatment for *Listeria* inactivation.

The second phase of this project focused on investigating the effect of voltage, gas composition, and bacteria species on microbial inactivation efficacy of direct cold plasma treatment on sliced queso fresco. Three voltages (60 kV, 80 kV and 100 kV), two gas compositions (dry air and MA65: 65% O_2, 30% CO_2, 5% N_2), and two bacteria species (*Listeria innocua* and *E. coli* K-12) were evaluated. It was hypothesized that higher applied voltage and higher oxygen content of the gas would lead to higher microbial inactivation. Moreover, gram-negative bacteria, *E. coli* K-12, was speculated to be more susceptible to cold plasma treatment compared to the gram-positive *Listeria innocua*.

The third phase of this project focused on evaluating the effect of direct cold plasma treatment on *Listeria innocua* inoculated queso fresco crumbles under two gas compositions (air and MA50: 50% N_2 and 50% CO_2). It was hypothesized that increased surface area would result more efficient *Listeria innocua* inactivation, and lower oxygen content of the gas would lead to decrease of microbial inactivation efficiency, but less lipid oxidation of the cheese.

Optimized cold plasma treatment condition was identified in the third phase of the study and used for the last part of this project. The last phase of this project focused on analyzing the effect of cold plasma treatment in inactivation of *Listeria innocua* and background microorganisms, as well as the quality of queso fresco followed by 28 days of refrigerated storage. It was hypothesized that cold plasma treatment was able to inhibit the growth of *Listeria innocua* and background microorganisms throughout storage with minimal effect on the quality of queso fresco.

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Table 1.1. Summary of effects of cold plasma treatment on microbial inactivation and quality of food products

Sample	Plasma	Microbial observation	Quality observation	References
Sliced soft cheese & Sliced ham	• DBD, 75-150 W, 13.56 MHz • Gas: He • Flow rate: 10 liter per min • Treatment time: 60–120 s • Sample size: 60x60x2 mm	• > 8 log_{10} reduction of *Listeria monocytogenes* in sliced cheese • Up to 1.73 log_{10} reduction in sliced ham	NA	(Song et al., 2009)
Sliced cheese	• DBD, 15 kHz, 250W • Gas: air • Treatment time: 0.5-7 min	• Up to 2.67 log_{10} reduction of *E.coli* • Up to 3.10 log_{10} reduction of *Salmonella* • Up to 1.65 log_{10} reduction of *L.monocytogenes*	NA	(Yong, Kim, Park, Alahakoon, et al., 2015)
Cheese slice	• DBD, 50 kHz, 3.5 kV • Gas: He, He+O_2 • Treatment time: 1-15 min • Sample size: 15x15x2 mm	• Up to 1.47 and 1.98 log_{10} reduction of *E.coli* in He plasma and He+O_2 plasma, respectively. • Up to 0.45 and 0.91 log_{10} reduction of *S. aureus* in He plasma and He+O_2 plasma, respectively.	• Decrease in L* and b* value • Damage of cheese slice after 10 min treatment • Significant reduction in flavor and odor	(Lee et al., 2012)

Table 1 Continued

Sliced Cheddar cheese	• Thin layer DBD, 15 kHz, 100 W • Treatment time:2.5-10 min • Gas: N_2+O_2 • Sample size: 2.5 g	• 3.2, 2.1 and 5.8 log_{10} CFU/g reduction of *E.coli* O157:H7, *L. monocytogenes*, and *Salmonella*, respectively	• Decrease in pH and L* value • Increase in TBARS and b* value • Minimal total color difference and • Significant reductions in flavor and increase in off-flavor	(Yong, Kim, Park, Kim, et al., 2015)
Raw milk	• Corona discharge, 9 kV, 90 mA • Treatment time: 3-20min	• Up to 4.15 log_{10} CFU/ml reduction of *E.coli*	• No significant change in pH and color	(Gurol et al., 2012)
Milk	• DBD, 15 kHz, 250 W • Gas: air • Treatment time: 5-10 min • Sample size: 10 mL	• Up to ~2.4 log_{10} CFU/ml reduction of *E.coli*, *L.monocytogenes*, and *Salmonella*	• Decrease in pH and a* value • Increase in L*, b* value and TBARS	(Kim et al., 2015)
Orange Juice	• DBD, Air/MA65 (65% O_2, 30% CO_2, 5% N_2) • Sample size: 25 and 50 ml • Voltage: 90 kV • 30-120 s • Mode of exposure: direct and indirect	*Salmonella enterica*: • 30s direct and indirect MA65 with no storage (25ml OJ) resulted > 5 log reduction • 120s direct with 24 h storage (50ml OJ): 2.9 log reduction in air and 4.7 log reduction in MA65 • 120 s indirect with 24 h storage (50ml OJ): 2.2 log reduction in air and 3.8 log reduction in MA65	• No significant change in Brix or pH • Vit.C is reduced by 22% in air after 120 s direct treatment • Pectin methylesterase (PME) activity reduced by 74% in air and 82% in MA65 • Maximum total color difference is less than 1.2	(Xu et al., 2017)

Table 1 Continued

White grape juice	• DBD, 60 Hz, 80 kV, air • Treatment time: 1-4 min • Direct mode of exposure	• 7.4 log CFU/ml reduction in *Saccharomyces cerevisiae* after 4 min treatment	• No significant change in pH, acidity and electrical conductivity of the juice. • An increase in non-enzymatic browning was observed with minor total color difference • Decrease in total phenolics, total flavonoids, DPPH free radicals scavenging and antioxidant capacity after plasma treatment • An increase in total flavonols after HVACP treatment.	(Pankaj et al., 2017)
Radish sprouts	• Microwave plasma, 2.45 GHz, 900 W, 669 Pa • Treatment time: 1- 20 min • gas: nitrogen (1 L/min) • sample size: 5 and 25 g.	• 2.6 $\log_{10}$ CFU/g reduction in *Salmonella typhimurium* • 0.8 log CFU/g reduction in total mesophilic aerobes	• No change in color, water activity, ascorbic acid concentration • Moisture content decreases with increased treatment time	(Oh et al., 2017)
Blueberry	• Plasma jet, 47 kHz, 549 W • gas: ambient air • flow rate: 4 and 7 cubic feet/minute (cfm) • treatment time: 0 – 120 s	• After 1 day storage, 0.8 – 1.6 reduction in total aerobic plate count • After 7 days storage, 1.5 – 2.0 $\log_{10}$ CFU/g reduction in total aerobic plate count	• Significant reductions in firmness, anthocyanins and color at higher treatment times	(Lacombe et al., 2015)

Table 1 Continued

Strawberry	• DBD, 60 kV (50 Hz) • electrode gap: 40 mm • gas: ambient air • Treatment time: 5 min • Mode of exposure: indirect	• 2 log_{10} reduction in background microflora (aerobic mesophilic bacteria, yeast and mould)	• No significant change in color, firmness, and respiration rate	(Misra, Patil, et al., 2014)
Strawberry	• DBD, 60 kV (50 Hz) • Gap: 40 mm • gas mixture: 65% O_2 + 16% N_2 + 19% CO_2 and 90% N_2 + 10% O_2 • treatment time: 5 min • mode of exposure: indirect	• ~3.0 log reduction on microflora in both gas mixtures	• Strawberries treated and stored in a high oxygen mixture showed favorable quality results in similar respiration rates and higher firmness than the control • Some change in L* and a* value	(Misra, Moiseev, et al., 2014)
Mandarins	• Microwave plasma, 2.45 GHz, 900 W • gas flow: 1 liters/min • pressure in the chamber: 0.7 kPa • gas: N_2, He, N_2 + O_2 (4:1) • Treatment time: 10 min • sample size: 1 mandarin or 9 mandarin peel samples.	• Significant inhibition of *Penicillium italicum* (84% reduction in disease incidence)	• Increased the total phenolic content and antioxidant activity • No significantly change in CO_2 generation, weight loss, soluble solids, titratable acidity, pH, ascorbic acid concentration, and color	(Won et al., 2017)
Golden delicious apples	• Gliding arc plasma, 60 Hz • gas: air • flow rate: 10, 20, 30 or 40 L/min; • Treatment time: 1-3 min • Sample size: one half of an apple	• ~3.5 log_{10} reduciton in *Salmonella* and *E.coli* O157: H7	• No changes in color or texture	(Niemira & Sites, 2008)

Table 1 Continued

Melon	• DBD, 15 kV, 12.5 kHz • gas: air • treatment time: 30-60 min	• 3.4 and 2 log reductions for mesophilic and lactic acid bacteria, respectively	• Negligible change in titratable acidity, soluble solid content, dry matter, color and texture • 17% and 7% reduction in peroxidase and pectin methylesterase, respectively	(Tappi et al., 2016)
Cherry tomatoes	• DBD, 100 kV • treatment time: 150s • gas: air • sample size: 80 – 100g	• >5 $\log_{10}$ CFU/sample reduction in *E.coli* • 3.5 $\log_{10}$ CFU/sample reduction in *Listeria innocua* • Up to 3.5 $\log_{10}$ CFU/sample reduction on spoilage microflora (mesophiles, yeast and mould)	• No significant difference in color, firmness, pH or total soluble solids.	(Ziuzina et al., 2016)
Fresh fruit (pears) and vegetable (cucumbers and carrots) slices	• Plasma micro-jet, 30 mA, 500 V • Sample size: pear (3 cm x 3 cm x 0.5 cm); cucumbers and carrots (round slice: 3 cm and 0.5 cm thick) • total treatment time: 1-8 min	• 90%, 60% and 40% *Salmonella* inactivation in carrot slice, cucumber slice, and pear slice, respectively	• Less than 5% moisture loss for all three samples after 8 min treatment • Minimal changes in color • 3.6%, 3.2% and 2.8% reduction of Vit.C in cucumber, carrot and pear. respectively	(Wang et al., 2012)
Red chicory	• DBD, 19.15 V, 3.15 A • treatment time: 15 min • sample size: 2 cm x 5 cm	• >4 $\log_{10}$ CFU/cm^2 reduction in *L. monocytogenes* • >5 $\log_{10}$ CFU/cm_2 reduction in VTEC (*E.coli*)	• No detrimental effects on color, freshness and texture. • Odor and overall acceptability of the samples decreased during storage	(Trevisani et al., 2017)

Table 1 Continued

Red chicory (radicchio)	• DBD, 15 kV, 12.5 kHz • treatment time: 15-30 min • gas: air (speed: 1.5 m/s)	• 1.35 $\log_{10}$ MPN/cm^2 reduction in *E.coli* O157:H7 • 2.2 $\log_{10}$ CFU/cm^2 reduction in *Listeria monocytogenes*	• No significant effects in antioxidant activity and external appearance of the sample	(Pasquali et al., 2016)
Romaine lettuce	• DBD, 42.6 kV, 1.5 A • Treatment time:10 min • gap distance between two electrodes: 5.0 cm • gas: air	• 0.4 – 0.8 $\log_{10}$ CFU/g reduction of *E.coli* O157: H7 in the leaf samples in the 1-, 3-, and 5-layer configurations • 1.1 $\log_{10}$ CFU/g reduction in bulk stacking 7 layers	• No significantly change the surface morphology, color, respiration rate, and weight loss	(Min et al., 2017)
Fresh produce: romaine lettuce, baby carrots and cocktail tomatoes	• Atmospheric pressure cold plasma, 3.95-12.83 kV, 60 Hz • gas: argon • treatment time: 0.5-10 min	• 0.5, 1.7 and 1.5 $\log_{10}$ reduction of *E.coli* in carrot, tomato and lettuce, respectively.	• No significant changes in color for all three samples	(Bermúdez-Aguirre et al., 2013)
Bacon	• Atmospheric pressure plasma, 75-125 W, 13.56 MHz • Treatment time: 60-90 s • Gas: He (10 lpm) and a mixture of He and O_2 (10 lpm and 10 sccm) • sample: 1 g	• pathogens studied: *Listeria monocytogenes; Escherichia coli and Salmonella Typhimurium* • Helium plasma reduce the pathogens in 1 – 2 $\log_{10}$ range • Helium/oxygen gas mixture shows a reduction of pathogen in a range of 2 – 3 $\log_{10}$ • 4.53 $\log_{10}$ cfu/g reduction in total aerobic count	• Increase in L*-value of bacon • No differences on pH • Lower TBARS values at Day 0, while after 7 days of storage, plasma treated samples had higher TBARS value than control	(Kim et al., 2011)

Table 1 Continued

Fresh mackerel fillets	• DBD, 70-80 kV, 50 Hz • gas: air (15°C and 50% RH at the time of treatment) • treatment time: 1-5 min • Sample size: ~200g (two fillets).	• No significant reduction in the total aerobic mesophilic count • Significant reduction in psychrotrophic bacteria, lactic acid bacteria and *Pseudomonas*	• No changes in pH, color (except for L* - decrease), fat and moisture content • Higher oleic and eicosapentaenoic acid in plasma treated samples • No significant changes in TBARS values • Decrease in T_{21} (dense myofibrillar network) with increased T_{22} (extramyofibrillar water)	(Albertos et al., 2017)
Fresh and frozen pork	• Plasma jet, 20 kV, 58 kHz, 1.5 A • Gas: Air • Treatment time: 0 – 120 s	• 1.5 $\log_{10}$ reduction in *E.coli* O157:H7 • > 1.0 $\log_{10}$ reduction in *Listeria monocytogenes*	• No significant changes in volatile basic nitrogen, peroxide value and TBARS • No significant impact on the sensory characteristics on frozen pork • Significant changes in color for plasma treated fresh and frozen pork	(Choi et al., 2016)
Fresh pork	• Microwave plasma, 2.45 GHz, 1.2 kW • Gas: air • Treatment times: 5-10 min • gas flow rate: 20 standard liter per min • mode of exposure indirect plasma treatment	• Aerobic viable count remained between 10^2 and 10^3 CFU/g during the storage period of 20 days	• Increased a* values and decreased b* values • Distinct differences in reflectance and fluorescence • Significant changes in pH	(Fröhling et al., 2012)

Table 1 Continued

Fresh pork and beef	• Thin-layer DBD plasma, 100 W • Treatment time: 1-10 min • Sample size: 25 x 25 x 7 mm • Gas: N_2 + O_2	• Up to 2.04 log_{10} CFU/g reduction of *Listeria monocytogenes* • Up to 2.57 log_{10} CFU/g reduction of *E.coli* O157:H7: • Up to 2.67 log_{10} CFU/g reduction of *Salmonella*	• No significant effect on L* values and b* value • Decrease in a* values after 5 min exposures • Significant lipid oxidation after 10 min exposure • Texture of pork and beef was not affected by plasma treatment • Taste was negatively influenced	(Jayasena et al., 2015)
Pork Loin	• DBD, 3 kV, 30 kHz • Gas: He or He + 0.3% O_2 • Treatment time: 5-10 min • Sample size: oval-shaped slices of pork: 85 x 60 x 3 mm • flow rate: 10 standard liter per minute	• Up to 0.55 log_{10} reduction of *E.coli* in He+O_2 plasma • Up to 0.59 log_{10} reduction of *Listeria monocytogenes* in He+O_2 plasma	• Decrease in pH and L*-values with no obvious changes in a* and b* values • Higher lipid oxidation in He+O_2 plasma • Significant reductions in sensory quality parameters (appearance, color, odor, acceptability)	(Kim et al., 2013)
Beef jerky	• RF plasma, 200 W • Gas: Argon • Flow rate: 20,000 sccm • Sample size: 5 cm x 5 cm • treatment time: 0 -10 min	• 1.8 log_{10} reduction of *Staphylococcus aureus*	• No significant change in the fatty acid composition, color and shear force	(Kim et al., 2014)

Table 1 Continued

Pork	• Pulsed low pressure discharge, 0.8 MPa, 20 -100 kHz, 1.2 kVA • Gas: N_2, He and Ar • sample size: thickness 2 cm, length 7 cm, width 7 cm	• Up to 3 log cfu/cm^2 reduction of psychrotroph bacteria, yeast and mold in helium plasma	• No significant differences in color and pH	(Ulbin-Figlewicz et al., 2015)

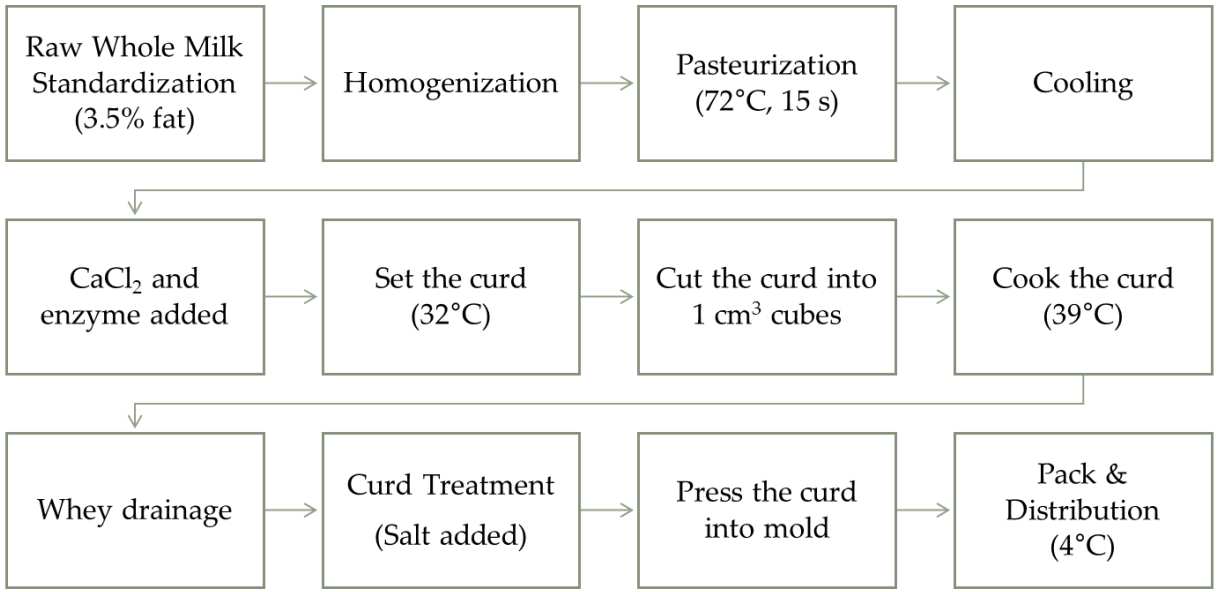

Figure 1.1: Flowchart for queso fresco production adapted from Van Hekken et al

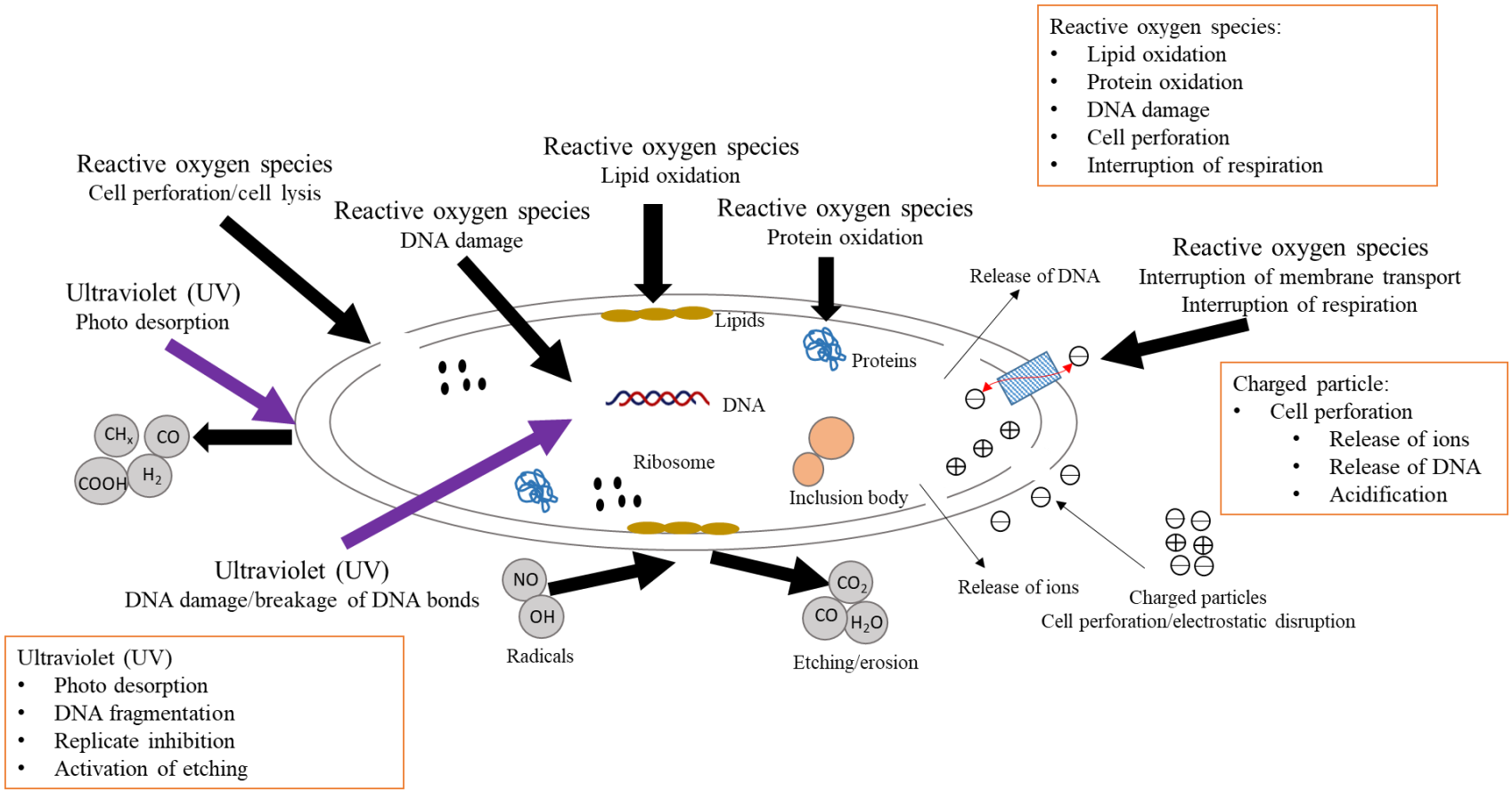

Figure 1.2. Cold plasma microbial inactivation mechanisms. Adapted from Schlüter and Fröhling (2014) and Coutinho et al. (2018)

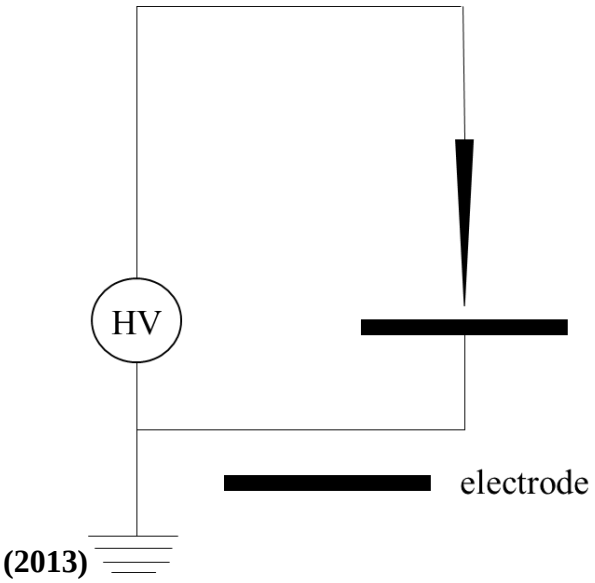

Figure 1.3. Schematic of corona discharge (HV: High voltage)

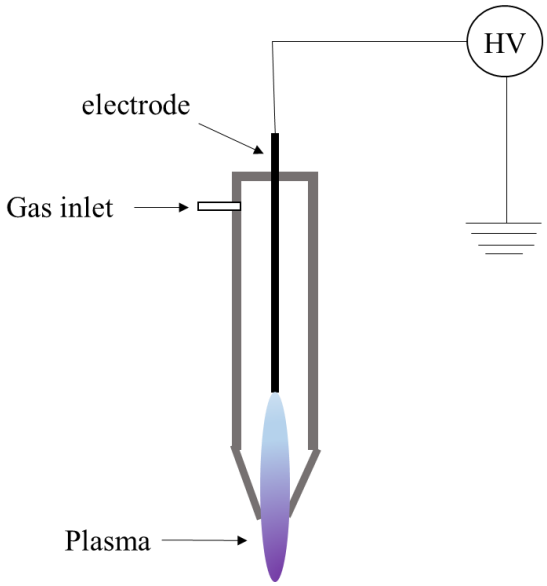

Figure 1.4. Schematic of plasma jet (HV: High voltage)

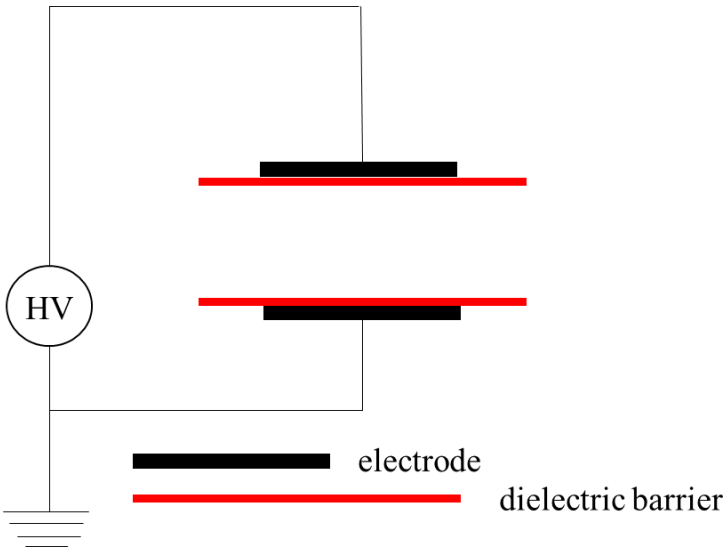

Figure 1.5. Schematic of dielectric barrier discharge (HV: High voltage)

CHAPTER 2: EFFECT OF HIGH VOLTAGE ATMOSPHERIC COLD PLASMA ON INACTIVATION OF *LISTERIA INNOCUA* ON QUESO FRESCO CHEESE, CHEESE MODEL AND TRYPTIC SOY AGAR

A manuscript published by LWT-Food Science and Technology

Zifan Wan[a,b], S.K. Pankaj[b], Curtis Mosher[c], Kevin M. Keener[a,b]*

[a] Food Science and Human Nutrition Department

[b] Center for Crops Utilization Research

[c] Department of Genetics, Development & Cell Biology

*Corresponding Author

Iowa State University

Abstract

High voltage atmospheric cold plasma (HVACP) is a novel technology which has shown promising results on microbial inactivation under low temperature, and thus offers opportunities on decontamination of biological materials. In this study, *Listeria innocua* spot inoculated tryptic soy agar (TSA), queso fresco cheese (QFC) and cheese model (CM) samples were treated with HVACP under direct and indirect mode of exposure in dry air gas environment for 5 min. After direct HVACP treatment, a reduction of 5.0, 3.5 and 1.6 $\log_{10}$ CFU/g was observed for TSA, CM and QFC, respectively. Direct plasma treatment was more effective in *Listeria innocua* inactivation on CM and QFC compared to indirect treatment. Surface analysis showed that the differences in surface roughness and micro-structure of the substrate hugely impact the efficacy of the HVACP treatment by affecting the attachment of the bacteria cells on the surface and providing protective locations for the microbes within the surface.

Highlights:

- Achieved significant inactivation of *Listeria innocua* on QFC, CM and TSA by cold plasma.
- Surface topography significantly affects the inactivation of *Listeria innocua* by cold plasma.
- Direct plasma treatment was found to be more effective for inactivation than indirect treatment.

Key words: Atmospheric cold plasma; queso fresco cheese; *Listeria innocua*; Food surface roughness; Surface microstructure

1. Introduction

Plasma is known as the fourth state of matter consisting of partially or completely ionized gas species in excited or ground states with a net neutral charge, and it can be classified as thermal (equilibrium) and non-thermal (non-equilibrium or cold) plasma. In non-thermal plasma, the electrical energy is only employed to heat up the electrons while the gas (ions, atoms and molecules) stays at ambient temperature, whereas in thermal plasma, the electrical energy is used to heat up both electrons and gas leading to an equilibrium system and higher plasma temperature (Misra et al., 2018).

High voltage atmospheric cold plasma (HVACP) is a novel, non-thermal technology which can be used to treat packaged food products and achieve significant reductions in foodborne pathogens and spoilage organisms without affecting the quality (Misra, Moiseev, et al., 2014; Ziuzina et al., 2014). For obvious reasons, non-thermal atmospheric cold plasma is more useful for food industry applications to maintain the integrity and quality of the food products. Previous studies have shown the effectiveness of HVACP for inactivation of both spoilage and pathogenic organisms on different food products which could lead to extension

of shelf-life and provide an assurance on food safety (Kronn et al., 2015; Misra, Keener, et al., 2014).

An extension of HVACP, in-package plasma processing or encapsulated plasma, emphasizes plasma generation inside a sealed package. This technology utilizes the packaging material as both a dielectric barrier to prevent current transport and a physical barrier to encapsulate plasma reactive species for extended exposure (Pal et al., 2016; Pankaj et al., 2015). This technology could be applied as the last step of processing to secure the safety and extend the shelf-life of the products with minimal risk of post-package recontamination.

Queso fresco cheese (QFC), a type of Hispanic-style soft cheeses, is a popular food in the Latin-American diet (Clark et al., 2004). QFC possess a high risk of post-pasteurization *Listeria monocytogenes* (LM) contamination and there is no effective commercial technology to reduce microorganisms in contaminated QFC and other soft cheeses. Due to its high moisture content, near neutral pH and moderate salt content, QFC provides an optimal substrate for the growth of spoilage and pathogenic microorganisms during storage and distribution. There is an estimated 1,600 people who get sick from LM each year caused by eating food contaminated with LM, and within that about 260 people died in United States (CDC, 2016). This formed the motivation of this work to assess the effectiveness of HVACP as a potential technology for QFC processing to reduce pathogenic microorganisms.

Even though many studies have reported the potential of HVACP for microbial inactivation, there are still unknown variables and potential parameters that could affect the effectiveness of this technology. The aim of this work is to understand the factors affecting the efficacy of this technology in order to better employ it into the food industry. More specifically, effects of product composition, surface topography and micro-structure were explored for *Listeria*

innocua, a non-pathogenic surrogate for LM (Friedly et al., 2008; Hnosko et al., 2012), inactivation by HVACP.

2. Materials and Methods

2.1 Bacterial strain and inoculum preparation

Listeria innocua (ATCC® 33090™) was used in this study and was obtained from the Department of Food Science and Human Nutrition, Iowa State University. Stock cultures were stored with 50% glycerol at -80°C. Fresh working culture was prepared by inoculating 0.1 ml frozen culture in 50 ml Brain Heart Infusion (BHI) broth at 37 °C for 24 h. The culture was spread plated on tryptic soy agar (TSA). After incubation at 37 °C for 48 h, one isolated colony on TSA was transferred into 50 ml BHI broth. The culture was grown under shaking at 160 rpm on an orbital shaker at 37 °C for 18 h. After incubation, *Listeria innocua* cells were harvested by centrifugation at 10,000 rpm for 10 min at 4 °C, and washed twice in sterile phosphate buffered solution (PBS). The washed cells were finally suspended in PBS to a final cell concentration of approximately 8 $\log_{10}$ CFU/ml, which were used as the working inoculum. The concentration of the working inoculum was confirmed by plating serial dilutions on TSA, followed by incubation at 37 °C for 48 h.

2.2 Sample preparation and inoculation

Tryptic soy agar (TSA) was made following the protocol of BD Difco™ TSA. Queso fresco cheese (QFC) was purchased from the local supermarket. Cheese model (CM) was made from the mixture of deionized water, butter (purchased from local grocery store), casein (Sigma-Aldrich, MO, USA), lactose (Fisher Scientific, MA, USA), calcium carbonate (Amresco, OH, USA), sodium chloride (Fisher Scientific, MA, USA) and agar powder

(Becton Dickinson, NJ, USA) resulting approximately composition of CM to 21% protein and fat, 53% moisture, 3.6% sugar, and 0.7% calcium. All three samples were stored at 4°C prior to use. 0.1 ml of 8 $\log_{10}$ CFU/ml *Listeria innocua* inoculum was spot-inoculated onto the surfaces of 10 g of TSA, QFC or CM samples within in a 2*4 cm^2 area (35 spots) placed inside of a sterile petri dish resulting in concentration of 6 $\log_{10}$ CFU/g for each sample. The inoculated samples were dried in the fridge at 4 °C for 1 h to allow the attachment of *Listeria innocua* cells on the surfaces of the samples.

2.3 HVACP treatment

A 10 g spot-inoculated sample was placed in a polypropylene ArtBin® box (273 x 186 x 44 mm) with inoculum facing upward, then pillow sealed with a high barrier Cryovac® B4170 (Sealed Air, North Carolina, USA) film leaving an approximately 2.5 cm opening for gas flushing. The pillow pack (box in bag) as a whole was flushed with dry air (<5% relative humidity) for 2 min before complete heat sealing.

A schematic diagram of the set-up used in this study is presented in Figure 2.1. The HVACP system applied in this study was a dielectric barrier discharge (DBD) with a voltage output of 0 to 130 kV at a frequency of 60 Hz. The distance between two circular aluminum electrodes (outer diameter = 152 mm) was 44 mm which was the height of the polypropylene box. As dielectric barriers, 10 mm plexiglass and 4 mm polypropylene sheets were used to prevent any arc transitions and assure the homogeneity of the plasma during HVACP treatment. The voltage applied to the aluminum electrode was generated by a step-up transformer (Phenix Technologies, Inc., MD. USA) and controlled by a variac. Direct and indirect modes of exposure were used during HVACP treatment. For direct treatment, sample was placed in between two electrodes directly located in the plasma field. For indirect treatment, sample

was placed outside the plasma field. Samples were treated at 100 kV for 5 min. Control samples were also packed under the same condition but without HVACP treatment. After treatment, all samples were stored at 4 °C for 24 h prior to microbial recovery.

2.4 Optical emission spectra measurement

The sealed pack samples were used for optical emission spectra collection. The emission spectra of the HVACP treatment of TSA, QFC and CM were recorded by a computer controlled Ocean Optics spectrometer (Ocean Optics, Inc., Florida, USA). The light from the plasma was delivered by an Ocean Optics optical fiber with a core diameter of 1000 µm. The collimating lenses with 5 mm diameter was used to align the light from plasma that enters the optical fiber. The length from the collimating lenses to the edge of the box containing sample was 15 cm. The emission spectra were collected and saved every 30 seconds. Integration time was five seconds and after six full spectra were collected, the six set of data was averaged and saved.

2.5 Media preparation and microbial enumeration

For microbial enumeration, two types of media (*Listeria* selective and non-selective) were used for the measurement of injured cells (García et al., 2005). Media included tryptic soy agar (non-selective) (TSA, BD Difo™, MD, USA) and *Listeria* selective agar (CM 0856 ThermoFisher Scientific, MA, USA) with *Listeria* selective supplement (SR 0140, ThermoFisher Scientific, MA, USA). Ten grams of sample was removed from the package and placed into a sterile filtered Stomacher bag with addition of 90 ml sterile 2% sodium citrate solution, and was then stomached at 230 rpm for 45 s. After stomaching, the resulting suspension was serial diluted with 9 ml 0.1% (w/w) peptone water. For each dilution,

aliquots were spread plated on TSA and *Listeria* selective agar. Plates were incubated at 37 °C for 48 h before counting. The number of injured cells were calculated by the differences between recovered *Listeria innocua* cells on these two media (García et al., 2005). The limit of detection for the *Listeria* recovery on the samples was 1.0 $\log_{10}$ CFU/g.

2.6 pH measurement

A 1.0 g of HVACP treated or untreated sample was sonicated by Qsonica sonicators with 9.0 ml HPLC grade water for 1 minute. pH of the sample after sonicating was measured by Orion Dual Star pH/SE meter (ThermoFisher scientific, MA, USA). The pH meter was calibrated with the standard buffer solutions pH 4.00, 7.00, and 10.00 at room temperature prior to measurements. The original pH of the samples was calculated based on the pH of the diluted samples.

$$\text{pH (original)} = \text{pH (diluted)} - \log_{10} \text{(dilution factor)}$$

2.7 3-D microscopy

The surfaces of the non-inoculated TSA, QFC and CM were viewed directly under the Sensorfar S neox – Non-contact 3D Surface Profiler microscope (SENSOFAR, Terrassa, Spain) at 50x. Images of the samples' surfaces were captured by SensoScan 6.3 software and analyzed using SensoMAP Standard software.

2.8 Scanning electron microscopy

Non-inoculated or inoculated samples were fixed with 2% paraformaldehyde and 2% glutaraldehyde in cacodylate buffer (0.1M) at pH 7.2 for at least 1 h at 4 °C. After fixation, samples were rinsed three times and each for 15 min in cacodylate buffer (0.1M). Then

samples were post-fixed in 1% osmium tetroxide in cacodylate buffer (0.1M) for 1 h. After several washes with deionized water, samples were dehydrated through graded ethanol series (25%, 50%, 70%, 85%, 95%, 100%) twice each for 15 min. Samples were critical point dried using a Denton Vacuum, Inc. Drying Apparatus, Model DCP-1 (Denton Vacuum, Moorestown, NJ, USA). Dried samples were mounted on aluminum stubs with double-sided tape and colloidal silver paint and sputter coated with palladium using a Cressington HR208 Sputter Coater (Cressington Scientific Instruments, Watford, UK). Images were captured using an Hitachi SU-4800 field emission scanning electron microscope at 10 kV (Hitachi High Technologies America, Inc., IL, USA). The pore diameters for all the samples were obtained from the micrographs using image analysis in ImageJ (Schneider et al., 2012). The mean and standard deviation of the pore size was calculated via fitting of the appropriate distribution (log-normal) to the pore diameter data. In addition, the mean size of the *Listeria innocua* cells was also measured through image analysis and the mean value was reported.

2.9 Statistical Analysis

Statistical analysis was using JMP statistical package (SAS Institute Inc., Cary, NC, USA). Analysis of variance (ANOVA) was used and the data was represented as mean value ± standard deviation. Tukey's means comparison post-hoc test was performed to identify significant differences between treatments ($p \leq 0.05$). Statistical significance was indicated at $p \leq 0.05$.

3. Results and Discussions:

3.1 Optical emission spectra

The emission spectra of the dry air plasma discharge during HVACP treatment for TSA, QFC and CM under direct and indirect mode of exposure are presented in Figure 2.2 and

Figure 2.3, respectively. In all six spectra the highest intensity peaks were observed near UV region (300 - 400 nm), which represents mostly excited nitrogen species, including nitrogen second positive system (SPS) N_2(C-B) and first negative system N_2^+(B-X) (Liu & Zhang, 2014; Machala et al., 2007). These air plasma emission spectra collected were similar and comparable to emission spectra reported in other studies at atmospheric pressure in air (Misra, Pankaj, et al., 2014; Sarangapani et al., 2016). Moreover, at around 723 nm and 740 nm wavelength, two excited oxygen species were found, which represent electron transition of O atom from $2s^22p^2(^3p)4p$ -> $2s^22p^2(^3p)3d$ and $b^1\sum_g^+$ -> $X^3\sum_g^-$, respectively (Ingvar, 1990). In the emission spectra of air plasma, the intensity of the excited oxygen species was fairly low compared to the intensity of reactive nitrogen species, which was most likely caused by the quenching effect of the excited atomic oxygen in air plasma (Walsh et al., 2010). Furthermore, the emission intensity of direct treatment, represented in Figure 2.2, was observed to be lower than indirect treatment, represented in Figure 2.3, which could be due to diffusion and solubilization of reactive gas species into the moisture on the surface of samples during plasma treatment (Misra, Pankaj, et al., 2014). From the results of the optical emission spectra, it is obvious that HVACP is a source of reactive nitrogen species and reactive oxygen species, which have been proven to have antimicrobial properties (Duday et al., 2013; Komine & Tsujimoto, 2013).

3.2 *Listeria innocua* inactivation

The effect of HVACP treatment on *Listeria innocua* inactivation for these three matrices with different mode of exposure is shown in Figure 2.4 and 2.5. Figure 2.4 represents the *Listeria innocua* survival population enumerated on TSA (non-selective media), while Figure 2.5 shows the enumeration on *Listeria* selective agar. During microbial enumeration, one

potential drawback in assessing the antimicrobial effect of any method is the failure to account for the presence of injured cells. In this study, injured cells were considered as the microorganisms which could not grow and were not able to form colonies on selective media for enumeration and the number of injured cells were calculated as the difference between the recovery on selective and non-selective media (García et al., 2005).

Besides on TSA, inactivation of *Listeria innocua* was more efficient for QFC and CM in direct mode of exposure compared to indirect treatment, which was found similar to other reported studies (Dobrynin et al., 2009; Ziuzina et al., 2013). Under direct plasma treatment, 1.6 and 3.5 $\log_{10}$ CFU/g reduction was achieved for QFC and CM, respectively, while 0.8 and 2.2 $\log_{10}$ CFU/g was seen under indirect treatment. This result proves that direct plasma treatment was more effective in terms of bactericidal efficiency than indirect treatment. Direct treatment also led to higher injury of *Listeria innocua* cells compared to indirect treatment. The 0.6 and 0.7 $\log_{10}$ CFU/g injured cells were found under direct treatment while 0.3 and 0.2 $\log_{10}$ CFU/g injured cells were seen under indirect treatment for QFC and CM, respectively. This higher bactericidal inactivation and injury under direct treatment can be accounted by the synergistic effects of electric field, charged particles and the reactive gas species generated during plasma treatment.

Cold plasma generates reactive species with different stability and half-life. Examples of some reactive species with their half-life are shown in supplementary information (Table SI 2.1). Short half-life species were defined as the species with less than 0.5 s half-life. At atmospheric pressure, diffusion coefficients of these gases are around 0.1 cm^2/s (Cussler, 2009), which makes it difficult for them to diffuse and interact with samples under indirect treatment. Moreover, studies have found that these reactive species (such as $O_2^{-}\cdot$, $^1O^2$, $\cdot OH$,

N_2^+, and N_2O^+) would rearrange or go through different transitions before reaching and interacting with sample under indirect treatment (Misra et al., 2013). In those cases, only longer lived reactive species (such as O_3, O_2, NO_2, NO and CO) would reach and interact with the sample in indirect plasma treatment (Laroussi, 2009). In this study, indirect treatment was found less efficient compared to direct treatment, suggesting that short half-life species might be critical in *Listeria innocua* inactivation on QFC and CM during plasma treatment. However, in some previous studies (Patil et al., 2014), indirect treatment was as effective as direct treatment. In order to optimize the treatment conditions, individual applications need to be assessed for the optimal treatment condition.

As for the effect of different matrices on *Listeria innocua* inactivation, Figure 2.4 and 2.5 show that plasma treatment was mostly effective on TSA surface followed by CM surface and QFC surface. Under direct treatment, on inoculated TSA, 5.0 $\log_{10}$ CFU/g reduction was achieved, with 3.5 and 1.6 $\log_{10}$ CFU/g reduction for CM and QFC, respectively. This difference in reduction might be due to the difference in the compositions of matrix. TSA contains the minimal nutrients, which is 2% (w/w) digested protein, whereas CM and QFC have 21% (w/w) protein and 21% (w/w) fat content. Protein and lipid can provide protection during inactivation of *Listeria innocua* by HVACP treatment, since they also act as biological targets for reactive gas species (Gaunt et al., 2006). However, CM and QFC shared the similar nutrient profile but resulted in significantly different ($p<0.05$) *Listeria innocua* inactivation by plasma treatment. This difference is likely the result of different surface topography and microstructure, which will be discussed in the later sections.

3.3 Effect on pH

The effect on pH of samples after HVACP treatment is shown in Table 2.1. Before plasma treatment, the pH of untreated TSA, QFC and CM samples were 6.25, 5.91 and 5.16, respectively. There was a minor decrease in pH after HVACP treatment for QFC and CM, while there was considerable change for TSA. That said, the drop in pH was statistically significant ($p<0.05$) for all three samples. The higher decrease in pH of TSA compared to QFC and CM might due to the buffering capacity of protein present in QFC and CM (Olthuis et al., 1994; Salaün et al., 2005). The shifts in pH due to HVACP treatments could be explained based on the formation of nitric acid (HNO_3) and nitrous acid (HNO_2) by reaction of NO and NO_2, as well as hydrogen peroxide (H_2O_2) (Misra et al., 2015). The chemical reaction can be summarized as follows:

$$N_2 + O_2 + e \rightarrow 2NO + e$$

$$2NO + O_2 \rightarrow 2NO_2$$

$$H_2O + e \rightarrow OH + H + e$$

$$NO_2 + OH \rightarrow HNO_3$$

$$NO + OH \rightarrow HNO_2$$

The decrease in pH was likely due to diffusion and solubilization of the reactive gas species into the aqueous phase, resulting in formation of acids at very low concentrations. However, the increase in acidity from plasma treatment is unlikely to result in *Listeria innocua* inactivation as it can survive even at pH of 4.3 (Le Marc et al., 2002; Rodríguez-Lázaro et al., 2004). It may be noted that the extent of decrease in pH under direct and indirect treatments was statistically insignificant ($p>0.05$). This is because the acid formation occurs due to long lifetime species (NO, NO_2) that could freely diffuse inside the package.

3.4 Effect of surface roughness on plasma inactivation of *Listeria innocua*

The surface of TSA, QFC and CM is shown in Figure 2.6, as viewed under a confocal microscope at 50x. Surface roughness for TSA, CM and QFC was 24.753 nm, 8.569 μm and 22.178 μm, respectively. The microbial results have shown that *Listeria innocua* inactivation efficiency was in a descending order for TSA, CM and QFC. This decrease in microbial inactivation efficacy from TSA to QFC by plasma treatment was found to be linearly related to the increase in surface roughness (see Figure SI. 2.1 of the supplementary information). The increases in surface roughness might provide better protection of bacteria cells from plasma gas reducing the inactivation by cold plasma.

3.5 Effect of surface microstructure

The micro-structure of TSA, CM and QFC are shown in Figures 2.7(a), 2.7(c), and 2.7(e), depicting the surface complexities among the substrates. This difference in surface micro-structure has led to the difference in *Listeria innocua* inactivation by plasma treatment for these three matrices. Figure 2.7(b) shows that for TSA, *Listeria innocua* cells were merely attached on the smooth surface of the TSA. The pores on the TSA surface were much smaller (diameter = 0.27 μm ± 0.08 μm) than *Listeria innocua* cells (length = 1.36 μm ± 0.21 μm) which restricted the cell migration inside the substrate. However, for CM (diameter = 1.94 μm ± 1.52 μm) and QFC (diameter = 1.94 μm ± 1.35 μm), represented in Figure 2.7(d) and 2.7(f), the size of the pores were large enough for *Listeria innocua* cells to embed and migrate deeper in the matrix, which might give some protection against the attack of the reactive gas species during HVACP treatment. This difference in surface micro-structure could be the reason for the difference in *Listeria innocua* reduction among inoculated TSA, QFC and CM. This result of the effect of the surface features on the efficiency of plasma

inactivation was also supported by other works (Fernandez et al., 2013; Noriega et al., 2011) which have found the significant influence of surface micro-structure cold plasma inactivation efficacy.

4. Conclusions:

This study demonstrated the application potential of HVACP for decontamination of food products to ensure the safety of food products. HVACP treatment at 100 kV for 5 min in dry air of inoculated *Listeria innocua* achieved f 5.0, 3.5 and 1.6 $\log_{10}$ CFU/g reductions on TSA, CM and QFC, respectively. The direct mode of HVACP treatment has shown the greatest impact on microbial inactivation and injury of *Listeria* cells. The micro-structure of the substrate was found to significantly influence the antimicrobial efficacy of cold plasma with a rougher surface, in which surface of QFC was 896 times rougher compared to TSA, resulting in significantly less reduction. With the difference in surface topography including roughness and micro-structure, the efficacy of cold plasma was greatly influenced. These observation suggests that during the application of HVACP on food products, treatment parameters need to be optimized for individual product based on its surface topography.

Acknowledgement

Research funding support provided by the National Dairy Council and Innovation Center for US Dairy.

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Table 2.1. pH of queso fresco cheese (QFC), cheese model (CM) and Tryptic soy agar (TSA) after HVACP treatment. Same small letter beside each pH indicated no significant difference among the same sample. ($p<0.05$)

	QFC	CM	TSA
Control	5.91 ± 0.03^{a}	5.16 ± 0.03^{a}	6.25 ± 0.02^{a}
Direct	5.71 ± 0.07^{b}	5.06 ± 0.04^{b}	5.35 ± 0.15^{b}
Indirect	5.74 ± 0.05^{b}	5.07 ± 0.04^{b}	5.38 ± 0.10^{b}

Table SI 2.1. Examples of the half-life of reactive oxygen and nitrogen species generated during HVACP treatment

Reactive species	Half-life	References
NO (nitric oxide)	15 seconds	(Dhawan, 2014)
NO_2 (nitrogen dioxide)	13 minutes under 1.0 ppm ozone	(Thomas et al., 1956)
O_3 (ozone)	99 minutes at refrigeration	(Klockow & Keener, 2009)
O_2 ($^1\Delta g$) (metastable oxygen)	45 minutes	(Barry, 1980)
CO (carbon monoxide)	250 minutes under air	(Meredith & Vale, 1988)
O_2^- (superoxide)	10^{-6} second	(Sies & Stahl, 1995)
$^1O_2$ (singlet oxygen)	10^{-6} second	(Sies & Stahl, 1995)
OH (hydroxyl)	10^{-9} second	(Sies & Stahl, 1995)
N_2^+	7×10^{-7} second	(Wakenne & Momigny, 1971)
N_2O^+	9×10^{-8} second	(Newton & Sciamanna, 1970)

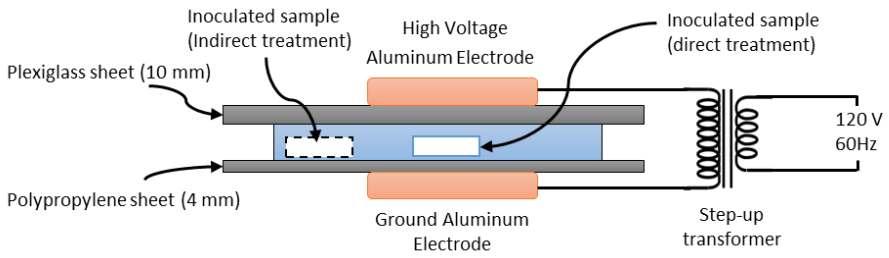

Figure 2.1. Schematic diagram of HVACP treatments

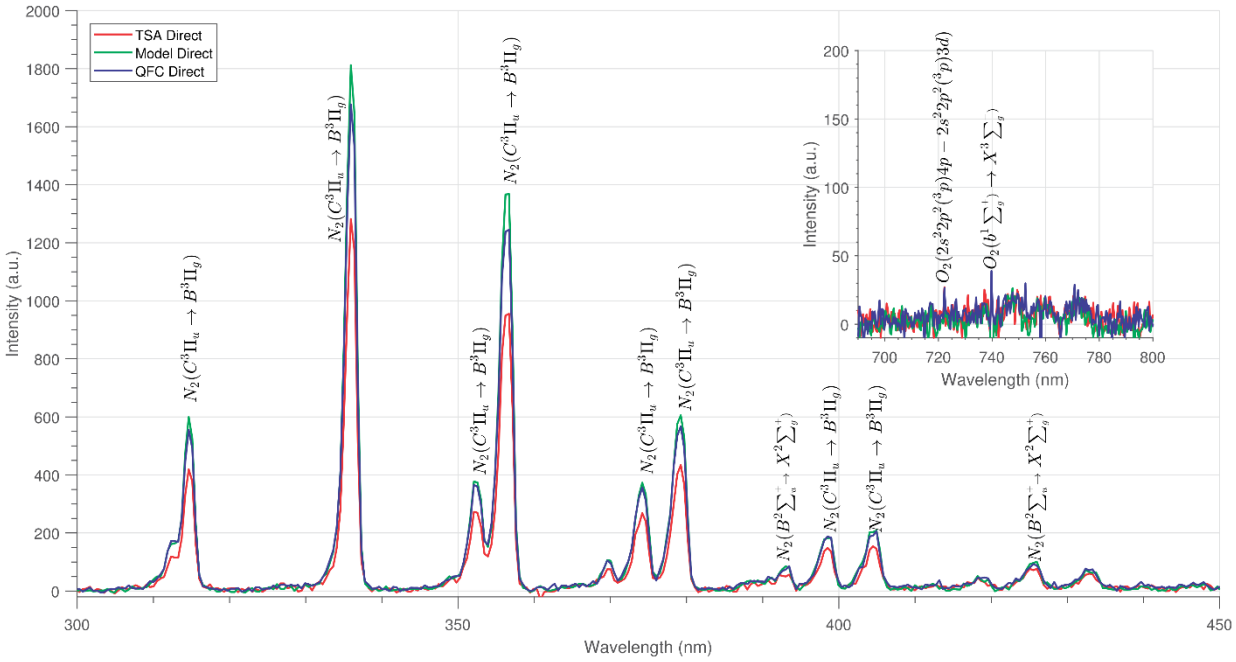

Figure 2.2. Optical emission spectra of direct HVACP treatment of tryptic soy agar (TSA), cheese model (CM) and queso fresco cheese (QFC) at 100 kV

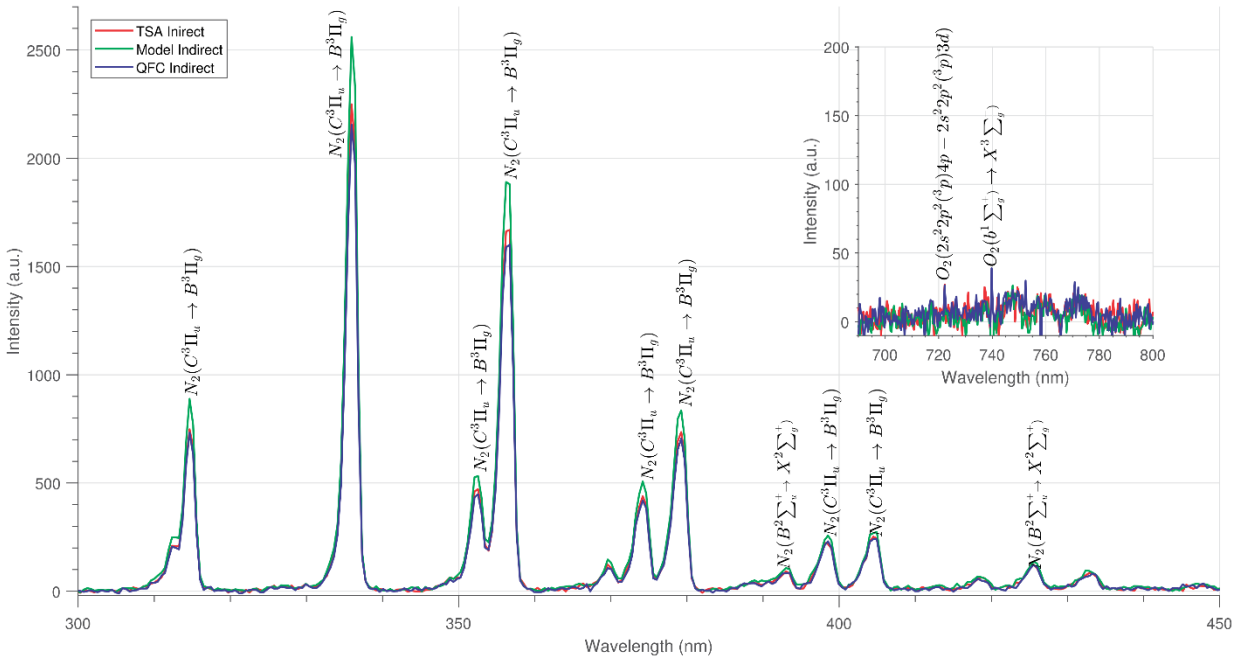

Figure 2.3. Optical emission spectra of indirect HVACP treatment of tryptic soy agar (TSA), cheese model (CM) and queso fresco cheese (QFC) at 100 kV

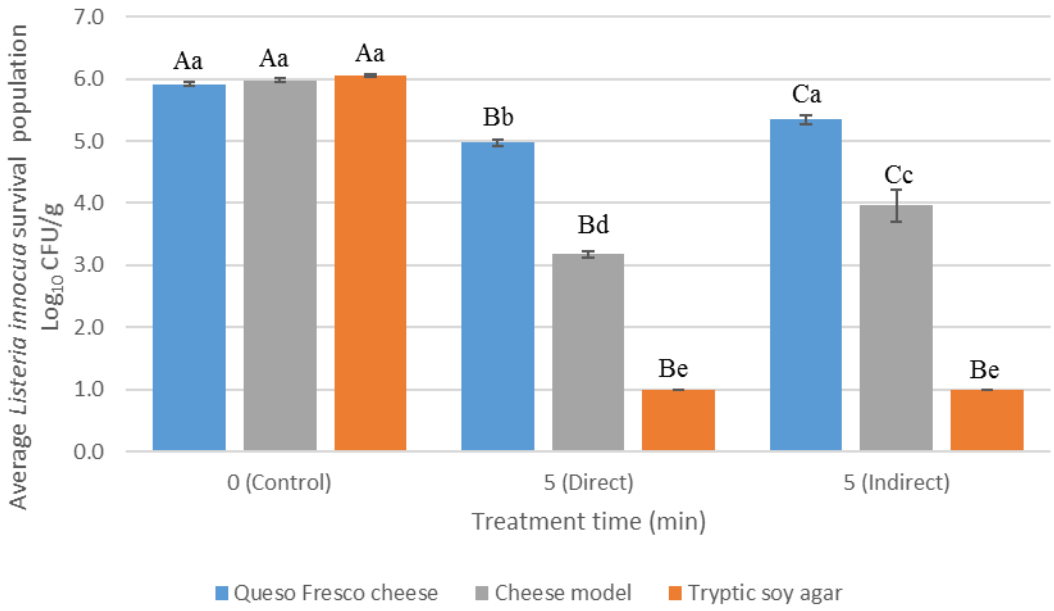

Figure 2.4. Survival of *Listeria innocua* population (log_{10} CFU/g) exposed to HVACP treatments for 5 min and recovered on TSA. The detection limit of the applied enumeration method was 1.0 log_{10} CFU/g. Same capital letter on top of each bar indicated no significant difference among each bar with the same sample. Same small letter on top of each bar indicated no significant difference among the same treatment time. ($p<0.05$)

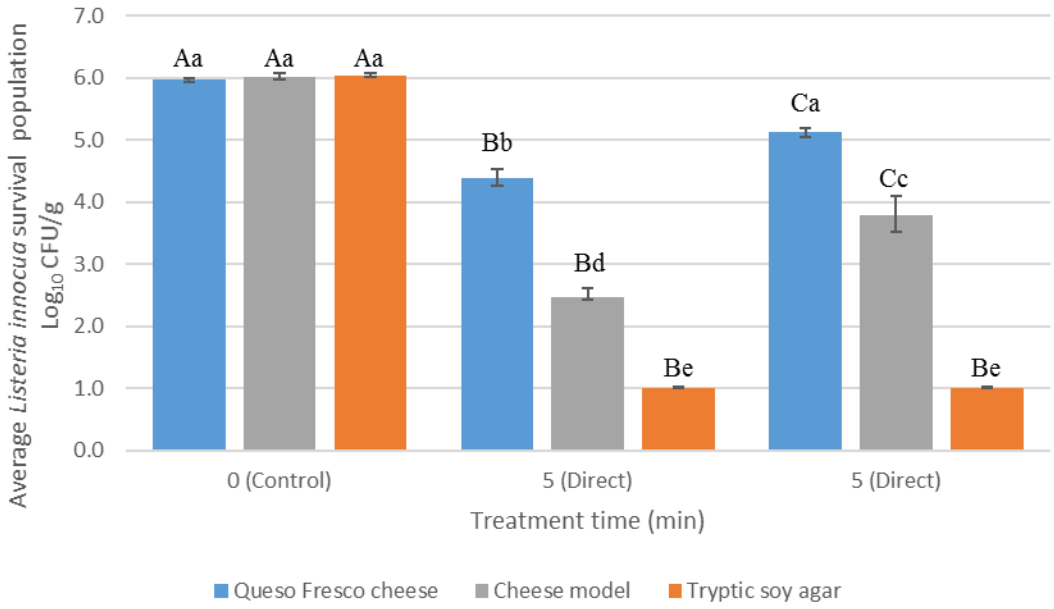

Figure 2.5. Survival of *Listeria innocua* population (log_{10} CFU/g) exposed to HVACP treatments for 5 min and recovered on *Listeria* selective agar. The detection limit of the applied enumeration method was 1.0 log_{10} CFU/g. Same capital letter on top of each bar indicated no significant difference among each bar with the same sample. Same small letter on top of each bar indicated no significant difference among each bar with the same treatment time. ($p<0.05$)

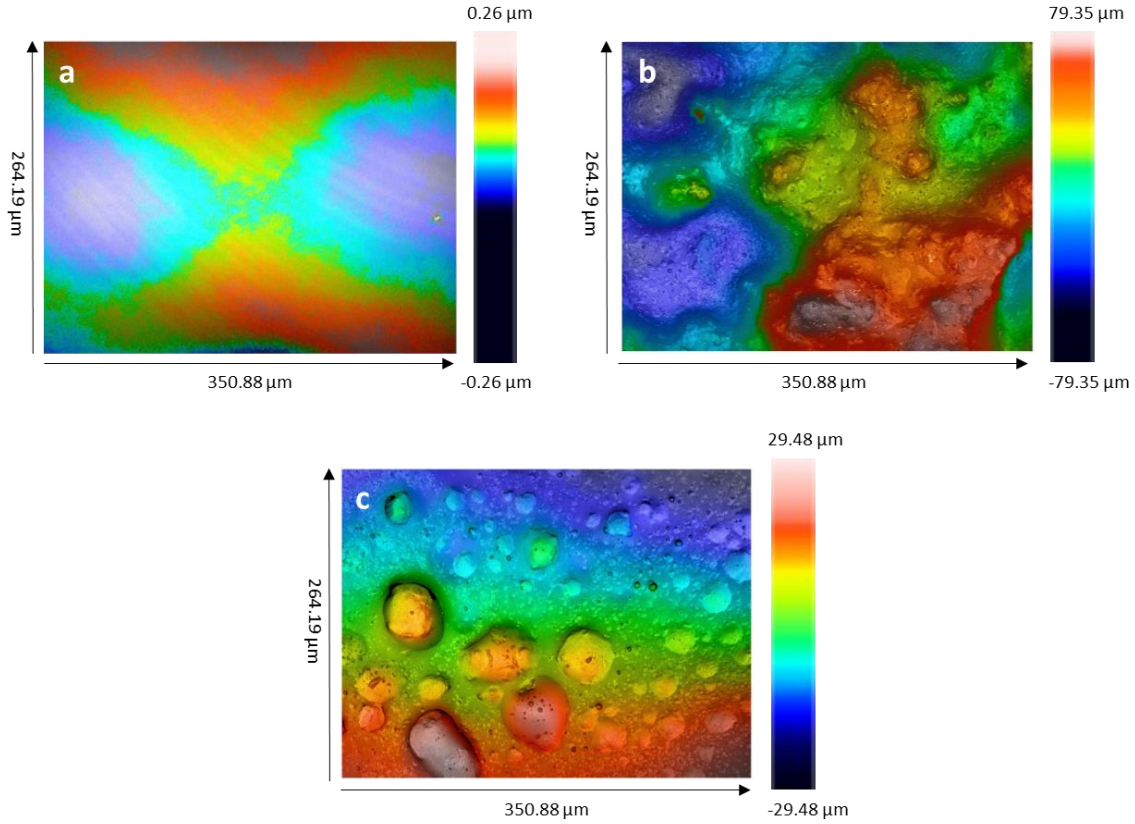

Figure 2.6. 3-D microscope images of sample surface at 50x (a: tryptic soy agar surface; b: queso fresco cheese surface; c: cheese model surface)

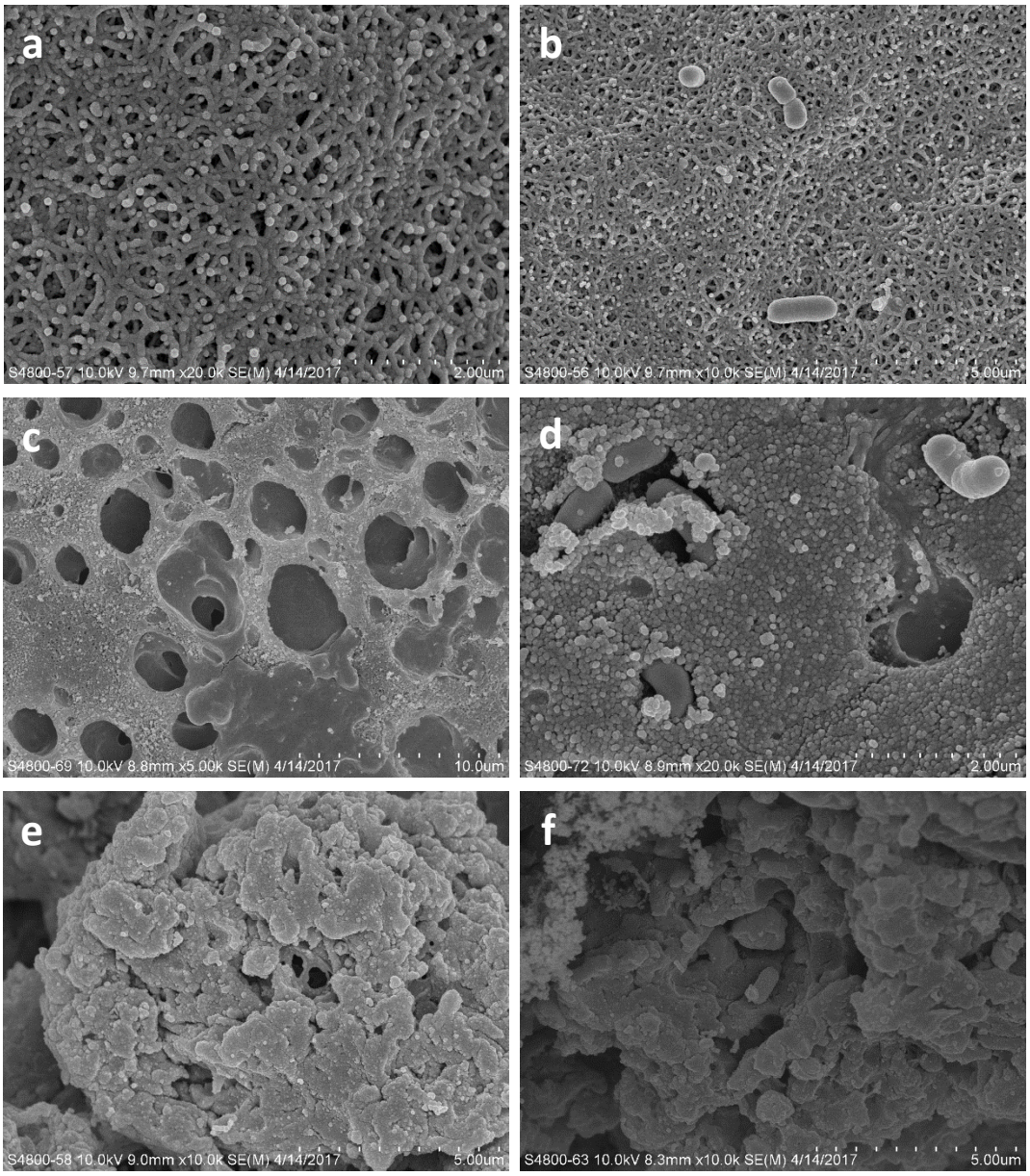

Figure 2.7. Scanning electron microscopy images for *Listeria innocua* inoculated and non-inoculated samples (a: surface of non-inoculated Tryptic soy agar; b. surface of inoculated Tryptic soy agar; c: surface of non-inoculated cheese model; d: surface of inoculated cheese model; e: surface of non-inoculated queso fresco cheese; f: surface of inoculated queso fresco cheese)

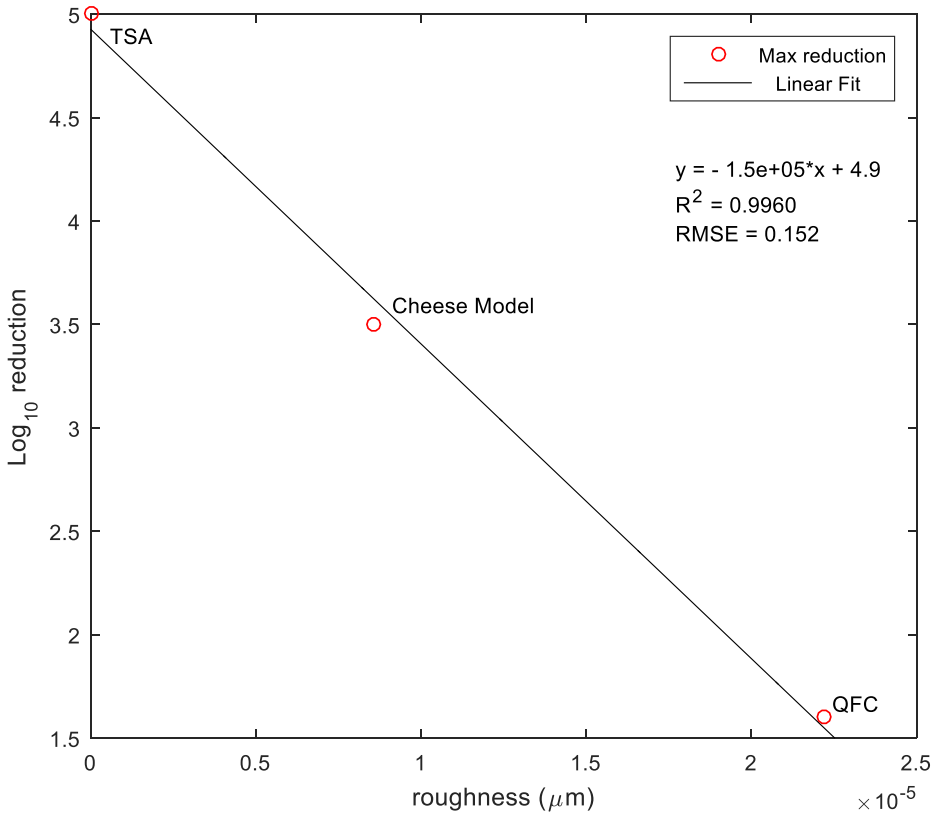

Figure SI 2.1. The linear relationship between the surface roughness versus the *Listeria* reduction efficacy of cold plasma for the cheese and model systems evaluated.

CHAPTER 3: HIGH VOLTAGE ATMOSPHERIC COLD PLASMA TREATMENT OF *LISTERIA INNOCUA* AND *ESCHERICHIA COLI* K-12 ON QUESO FRESCO CHEESE

A manuscript prepared for submission to Innovative food science and emerging technologies

Zifan Wan[a], NN Misra[b], Guo Li[a], Kevin M. Keener[a,b]*

[a] Food Science and Human Nutrition Department

[b] Center for Crops Utilization Research

*Corresponding Author

Iowa State University

Abstract

High voltage atmospheric cold plasma (HVACP) is a non-thermal technology that demonstrates effectiveness in reducing microbial populations in food products leading to improved safety and increased shelf-life. Queso fresco cheese is a food product prone to *Listeria monocytogenes* contamination. Once queso fresco gets contaminated during production, there is no additional treatment to remove pathogen contaminants. In this study, *Listeria innocua* and *E.coli* K-12 were spot inoculated on queso fresco, then treated with HVACP under direct mode of exposure in dry air and MA65 (65% O_2, 30% CO_2, 5% N_2) gas blends for up to 5 min at 60 kV, 80 kV, and 100 kV. After five minutes HVACP treatment in dry air at 100 kV, a reduction of 1.4 $\log_{10}$ CFU/g and 3.5 $\log_{10}$ CFU/g was observed for *Listeria innocua* and *E.coli* K-12 after 24 h storage at 4 °C, respectively. Increasing the voltage for the direct HVACP treatment from 60 kV to 100 kV resulted in greater bacterial reductions and minimal quality change. Overall, minimal quality changes in pH, moisture and color were observed and there was no significant ($p<0.05$) difference in the cheese texture after plasma treatment.

Highlights

- Significant inactivation of *Listeria innocua* and *E.coli* K-12 was achieved by cold plasma treatment
- Higher applied voltage led to more efficient microbial inactivation by cold plasma treatment.
- Minimal or no significant effects were observed in the quality of queso fresco after cold plasma treatment.

Key words

Cold plasma, cheese, adsorption spectroscopy

1. Introduction

The exploration of suitable non-thermal technologies for food processing has been ongoing for decades. Consumers are demanding safe, high quality, and less "processed" foods (Pereira & Vicente, 2010; Raso & Barbosa-Cánovas, 2003). Cold plasma is a novel technology that is non-thermal and can significantly reduce microbial populations without compromising product quality (Misra et al., 2019). Plasma is referred to by physicists as the fourth state of matter, consisting of partially or completely ionized gas species in excited or ground states with a net neutral charge. It is comprised of reactive gas species including electrons, atoms, positive and negative ions, and metastable molecules (Misra, Moiseev, et al., 2014; Misra et al., 2019). Depending on thermodynamic equilibrium, plasma can be categorized into thermal (equilibrium) plasma and non-thermal (non-equilibrium or cold) plasma. In cold plasma, electrons are heated up by the coupled electrical energy, which heats up while the remaining gas (ions, atoms, and molecules) stays at ambient temperature. In thermal plasma, electrical energy is used to heat up both electrons and gas leading to a thermodynamic equilibrium and higher plasma temperature (Misra, Patil, et al., 2014; Misra

et al., 2016). This non-equilibrium of cold plasma allows it to be considered as a potential non-thermal technology for food processing.

High voltage atmospheric cold plasma (HVACP) is an advanced cold plasma technology, which utilizes voltage greater than 30 kV to achieve significant reductions in foodborne pathogens and spoilage microorganisms without compromising products' quality (Wan et al., 2017; Xu et al., 2017; Ziuzina et al., 2014). The in-package HVACP, an extension of HVAVP, highlights the plasma generation inside a sealed package, which utilizes the packaging material as a dielectric barrier to prevent electric arc, thus ensuring the uniformity of the plasma discharge, and the barrier can encapsulate reactive gas species for extended exposure (Misra, Keener, et al., 2014a; Misra et al., 2019). In-package HVACP technology can be applied as the last step of processing to secure the safety and extend the shelf-life of products and minimize the risk of recontamination.

Queso fresco cheese (QFC) is a type of fresh Mexican style soft cheese with high moisture content, low salt, near neutral pH thus providing the optimal substrate for the growth of spoilage and pathogenic microorganisms (Hnosko et al., 2012; Holle et al., 2018). In particular, QFC possesses a risk of *Listeria monocytogenes* (LM) post-pasteurization contamination, which is one of the major pathogens in ready-to-eat foods with the ability to grow under refrigerated temperature (Farber & Peterkin, 1991). Currently, no effective commercial technology has been identified to reduce pathogens in contaminated QFC and other soft cheeses post manufacturing. The Centers for Disease Control (CDC, 2016) recommend people with weakened immune system, such as pregnant women, avoid consumption of fresh cheeses like QFC. Several LM outbreaks were found to be related to fresh cheeses (CDC, 2018). This situation drives the work on finding an effective and

promising non-thermal technology to deliver safe and high-quality fresh cheeses, such as queso fresco, allowing consumers to enjoy them without concerns for foodborne illness.

The objective of this study is to evaluate the efficacy of HVACP in microbial inactivation as well as its effect in quality of the QFC cheese in order to assess its potential for future industrial application of in fresh cheese production. In this study, three voltages, two gas compositions and two strains of bacteria were assessed, which include *E.coli* K-*12* (gram-negative) and *Listeria innocua* (gram-positive). *Listeria innocua* was selected as a non-pathogenic surrogate for LM (Friedly et al., 2008; Hnosko et al., 2012). Both gram positive and negative strains that can lead to potential contamination of QFC are covered in this study.

2. Materials and Methods

2.1 Bacterial strain and inoculum preparation

Listeria innocua (ATCC® 33090™) and *Escherichia coli* K12 (ATCC® 25922™) were obtained from the microbiology stock culture of the Department of Food Science and Human Nutrition, Iowa State University. Stock cultures were stored with 50% glycerol at - 80°C. Fresh working culture was prepared by inoculating 0.1 ml frozen culture in 50 ml Brain Heart Infusion (BHI) broth and tryptic soy broth (TSB) at 37 °C for 24 h for *Listeria innocua* and *E.coli* K-12, respectively. The culture was spread plated on tryptic soy agar (TSA) and incubated at 37 °C for 24 h and 48 h for *E.coli* K-12 and *Listeria innocua*, respectively. After incubation, one isolated colony on TSA was transferred into 50 ml BHI broth or TSB broth for *Listeria innocua* and *E.coli* K-12. The culture was grown under shaking at 160 rpm on an orbital shaker at 37 °C for 18 h for both *Listeria innocua* and *E.coli* K-12. After incubation, bacteria cells were harvested by centrifugation at 10,000 rpm for 10 min at 4 °C, and washed

twice in sterile phosphate buffered solution (PBS). The washed cells were finally suspended in PBS to a final cell concentration of approximately 8 $\log_{10}$ CFU/ml, which was used as the working inoculum. The concentration of the working inoculum was confirmed by plating serial dilutions on TSA, followed by incubation at 37 °C for 48 h for *Listeria innocua* and 24 h for *E.coli* K-12.

2.2 Sample preparation

Queso fresco cheese was purchased from a local grocery store and cut into 2*4*1 cm^3 slice resulting in approximately 10 g of weight. Cheese slice was then spot-inoculated with *Listeria innocua* or *E.coli* k-12 inoculum resulting in ca. 6 $\log_{10}$ CFU/g population. Inoculated cheese slices were allowed to dry under refrigeration temperature for 1 h to allow cell attachment.

2.3 HVACP treatment

Figure 3.1 presents the schematic diagram for HVACP treatment set-up. The distance between the two circular aluminum electrodes (outer diameter = 152 mm) was 28 mm, the height of the polypropylene box. The spot-inoculated cheese slice was placed inside a polypropylene ArtBin® box (168*121*28 mm^3) with inoculum facing upward, and the box was then sealed with a high barrier film leaving an opening for gas flushing. The pillow pack (box in bag) as a whole was flushed with MA65 (65% O_2, 30% CO_2, 5% N_2) or dry air, and was then sealed using an impulse sealer. The sealed pack samples were treated, in duplicates, under direct mode of exposure at 60, 80, or 100 kV for up to five minutes. Control samples, in duplicates, were also packed under the same condition but without HVACP treatment. Treated and control samples were stored at 4° C for 24 h prior to microbial recovery and quality analysis.

2.4 Media preparation and microbial enumeration

For this study, selective agar (Oxoid CM856 for *Listeria innocua*, Eosin Methylene Blue (EMB) agar for *E.coli* K-12) and thin agar layer (TAL) method was employed for microbial enumeration. Thin agar layer method is used to recover bacteria cells as well as differentiate the target species, *Listeria innocua* or *E.coli* K-12, from mixed population. A thin layer of Tryptic soy agar (TSA) was laid on top of *Listeria* selective agar (CM856) and EMB agar for enumeration of *Listeria innocua* and *E.coli* K-12, respectively. In TAL method, sample aliquot was plated on top of the TSA layer, which allowed injured bacteria cells to recover. During incubation, selective agents diffused from the selective agar layer to the top thin TSA layer and allowed for selective growth of *Listeria innocua* and *E.coli* K-12 (Kang & Fung, 1999; Wu et al., 2001).

After 24 h storage, 10 g QFC sample was removed from the package and placed into a sterile filtered Stomacher bag with addition of 90 ml sterile 2% sodium citrate solution, and was then stomached at 230 rpm for 45 s. After stomaching, the resulting suspension was serially diluted with 9 ml 0.1% peptone water. For each dilution, aliquots were spread plated on TAL agar selective agar CM856 and EMB agar for *Listeria innocua* and *E.coli* K-12 enumeration, respectively. Plates were incubated at 37 °C for 24 h and 48 h before counting for *E.coli* K-12 and *Listeria innocua*, respectively. The number of injured cells were calculated by the differences between recovered cells on these two media (selective and TAL). The limit of detection for bacteria recovery on the samples was 1.0 $\log_{10}$ CFU/g.

2.5 Lipid peroxidation

Lipid peroxidation test is modified from method described in Jung et. al. (2015). 3 g of QFC sample was homogenized with 9 ml HPLC grade water and 7.2% BHT in ethanol for 30 s at

16,000 rpm. The homogenate was centrifuged at 10,000 g for 15 min at 4 °C. 1.0 ml supernatants was mixed with 2.0 ml 20 mM thiobarbituric acid ((TBA) in 15% trichloroacetic (TCA) reagent in a 15 ml centrifuge tube. After mixing, the tubes were heated in a 90 °C water bath for 30 min and cooled in ice for 10 min. After cooling, the test tubes were centrifuged at 3,000 rpm for 10 min at 4 °C followed by 30 min stabilization at room temperature prior to absorbance spectroscopy measurement at 532 nm.

2.6 Color and TPA

The color of QFC samples was measured, in quadruplicates, using a Hunter lab bench-top colorimeter based on L*, a*, b* color coordinate system at room temperature. The color values were expressed as L*(whiteness or darkness), a* (redness/greenness) and b* (yellowness/blueness). The total color difference (ΔE) was also calculated using the below equations:

$$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2}$$

Texture profile analysis (TPA) was also performed for HVACP treated and control QFC samples. Before analysis, QFC samples were cut in half to 2*2*1 cm^3 in size and kept at room temperature for 15 min. All measurements were completed within 10 min of cutting. TPA text protocol was modified from method described in Bermúdez-Aguirre and Barbosa-Cánovas (2010) and Sandra et al. (2004). For this study, series of compression tests were performed to determine the conditions most similar to the manual crumbling of QFC. The results depict the peak force at 70% compression being the most representative stimulation of the manual crumbling of QFC. Measurements were done in quadruplicates. A 45 mm diameter probe was used and each sample was compressed twice in texture analyzer with a 25 kg load cell. 1 mm/s was employed as the speed of pre-test, test, and post-test. 70%

compression distance was adopted and there was 5 seconds interval between 1st and 2nd compression.

2.7 pH and moisture content measurement

A 1.0 g of HVACP treated or untreated sample was homogenized with 9.0 ml HPLC grade water for 30 seconds at 16,000 rpm (Lee et al., 2012). Orion Dual Star pH/SE meter was used for pH measurement. Before measurement of pH, the pH meter was calibrated with the standard buffer solutions pH 4.00, 7.00, and 10.00 at room temperature. For moisture content measurement, 3 g QFC sample was weighed and oven dried at 102 °C for moisture content analysis based upon AOAC method 984.12 (AOAC, 1995).

2.8 Optical emission spectroscopy

To characterize reactive oxygen and nitrogen species (RONS) generated inside the package during HVACP treatment, the sealed pack samples were used for optical emission spectra collection. The emission spectra of the HVACP treatment of QFC was recorded by a computer controlled Ocean Optics spectrometer (Ocean Optics, Inc. Florida, USA). The light from the plasma was delivered by an Ocean Optics optical fiber with a core diameter of 1000 µm. To maximize the light captured from the plasma entering the optical fiber, collimating lenses with 5 mm diameter was used. The collimating lenses was placed 15 cm away from the edge of the box containing sample. The integration time was 5 seconds and after six full spectra were collected, the six set of data was averaged and saved, thus the emission spectra were saved every 30 seconds.

2.9 Optical absorption spectroscopy

To provide more details about the plasma-chemical kinetics, direct measurements of the concentrations of active reactive oxygen species (ROS) and reactive nitrogen species (RNS)

inside the package were conducted using optical absorption spectroscopy (OAS), which enables the measurement of spatially averaged concentration of the post-discharge long-lived reactive species inside the package. The incident and transmitted spectral intensity I_0 (λ=200-800 nm) and I_T (λ=200-800 nm) of a beam from a UV-Vis tungsten-deuterium-halogen light source (BDS 130A, BW Tek, Delaware, USA) crossing the post-discharge gas inside the package was measured through an imaging spectrograph, equipped with a charge coupled device (CCD) camera (HR 2000+, Ocean Optics, Inc., Florida, USA). The concentration of RONS is calculated by the Lambert-Beer law which demonstrates the relationship between the intensities and the RONS concentration–

$$I_T(\lambda) = I_0(\lambda)e^{-\sum_i \sigma_i(\lambda)C_i L} \quad (1)$$

where L is the path length (cm), $\sigma_i(\lambda)$ is the wavelength dependent absorption cross-section of each species (cm^2/molecule), and C_i is the number density of each species (cm^{-3}). The open path-length was set at 2.1 cm. The concentrations of ROS and RNS were determined via numerical deconvolution. The ROS and RNS species, O_3, NO_2, NO_3, N_2O_4, and N_2O_5 were measured.

2.10 Statistical analysis

Statistical analysis was done by JMP statistical package. Analysis of variance was used and the data were represented as mean value ± standard deviation. Tukey's multiple sample comparison tests were done to deliver assess the significance of difference between treatments. Statistical significance was indicated to evaluate at a confidence level of 95% ($p \leq 0.05$).

3. Results and Discussions

3.1 Optical emission spectra (OES)

Optical emission spectroscopy was used to characterize the reactive gas species generated inside the package during cold plasma treatment. The emission spectra of the dry air and MA65 at 60 kV, 80 kV, and 100 kV are presented in Figure 3.2 and 3.3, respectively. In dry air plasma, the majority of the peaks were observed near the UV region (300 - 400 nm), which represents mostly excited nitrogen species including nitrogen second positive system (SPS) N_2(C-B) and first negative system N_2^+(B-X) (Liu & Zhang, 2014; Sarangapani et al., 2016). At 740 nm, an excited molecular oxygen peak was observed (Ingvar, 1990). The emission intensity was observed to increase with an increase in applied voltage. For MA65 plasma, the excited nitrogen species were also observed in the near UV region, but at relatively lower emission intensities compared to dry air plasma. This was obviously due to the less amount of N_2 in MA65 gas blend. Two excited molecular oxygen species were detected at 720 nm and 740 nm (Ingvar, 1990). However, relatively low intensities of atomic oxygen peaks were shown in OES which is likely due to the self-quenching of $O(^3P)$ and $O(^5P)$ in the air plasma (Fridman, 2008; Walsh et al., 2010). The singlet oxygen can further undergo self-recombination or react with ozone (O_3) resulting in the formation of molecular oxygen (Fridman, 2008):

$$O + O + M \rightarrow O_2 + M \quad (2)$$

$$O + O_3 \rightarrow O_2 + O_2 \quad (3)$$

As in dry air plasma, the emission intensity was observed to increase with the increase in applied voltage in MA65 plasma. The emission peaks observed through OES indicate the collision of electrons with oxygen and nitrogen molecules, resulting in the formation of

reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Fridman, 2008). It may be noted that no plasma emission peaks were observed under 300 nm of the UV region; therefore, the antibacterial action of the HVACP was concluded to be dominated by the reactive oxygen and nitrogen species, with a negligible role of the UV radiation (Laroussi, 2002).

3.2 Optical absorption spectra (OAS)

Optical absorption spectroscopy (OAS) was utilized to assess the post-discharge plasma composition, in which the concentration of O_3, NO_2, NO_3, N_2O_4, and N_2O_5 was assessed in this study. Figure 3.4 and Figure 3.5 represent the concentration of ROS and RNS after plasma treatment at 60 kV, 80 kV and 100 kV in dry air and MA65, respectively. For both gas blends, the concentration of ROS and RNS was found to increase with an increase in the applied voltage. Moreover, a higher concentration of ozone (O_3) was observed in MA65 due to the higher oxygen content in MA65. Ozone formation in plasma discharge occurs via a two-step, three body collision reaction (Fridman, 2008):

$$e + O_2 \rightarrow O^{\bullet} + O^{\bullet} + e \quad (4)$$

$$O^{\bullet} + O_2 + M \rightarrow O_3 + O_2 \text{ (where M = } O_3 \text{ or } O_2) \quad (5)$$

As discussed in the OES section, excited nitrogen species including nitrogen second positive system (SPS) N_2(C-B) and first negative system N_2^+(B-X) were produced during HVACP treatment. N and O produced from electron collision reactions participate the synthesis of N_xO_y species (Fridman, 2008) which are highly toxic and can result in DNA damage of the cell thus leading to cell death (Davies et al., 2011):

$$N + O_2 \rightarrow NO + O \quad (6)$$

$$O + N_2 \rightarrow NO + N \quad (7)$$

NO can be further oxidized by the atomic oxygen to form NO_2 via a three-body reaction (reaction 8) or oxidized by ozone (reaction 9) (Fridman, 2008):

$$NO + O + M \rightarrow NO_2 + M, \text{ where } M = N_2/O_2/NO/NO_2 \quad (8)$$

$$O_3 + NO \rightarrow O_2 + NO_2 \quad (9)$$

The N_2O_4 can be formed by NO_2 recombination, which is a reversible reaction (Hall Jr & Blacet, 1952):

$$NO_2 + NO_2 \leftrightarrow N_2O_4 \quad (10)$$

The N_2O_5 forms via a reversible reaction from NO_3 and NO recombination (Hjorth et al., 1992):

$$NO_3 + NO + M \leftrightarrow N_2O_5 + M, \text{ where } M = N_2/O_2 \quad (11)$$

A large deviation in N_2O_5 concentration was observed which is likely due to the presence of peroxide during plasma treatment as N_2O_5 and peroxide shares similar cross-section (Moiseev et al., 2014). Thus, the measured N_2O_5 concentration may include peroxide content as well resulting in the large deviation. The OAS results demonstrate that ozone is not the sole bactericidal agent in plasma, The increased concentrations of N_xO_y and subsequent reactions for the formations of HNO_x may also have the significance in bactericidal effects (Gentile & Kushner, 1995; Moiseev et al., 2014).

$$NO_2 + OH \rightarrow HNO_3 \quad (12)$$

$$NO_2 + HO_2 \leftrightarrow HO_2NO_2 \quad (13)$$

The high voltage applied results in a complex plasma chemistry kinetic. As shown in Figure 3.5, MA65 plasma post-discharge has shown a decrease of O_3 with increased treatment time, in which after 5 min treatment, O_3 concentration dropped from 6,000 ppmv (1 min) to around

3,100 ppmv. This might be caused by recombination O_3 leading to formation of reactive gas species which can not be captured by OAS measurement.

3.3 *E.coli* K-12 inactivation

Figure 3.6 and 3.7 present the *E.coli* K-12 population after cold plasma treatment in dry air for 60 kV, 80 kV and 100 kV recovered on EMB agar and by TAL method, respectively. These results demonstrate that an increase in treatment voltage and treatment time results in increasing *E.coli* K-12 inactivation ($p<0.05$). The explanation for this enhanced effect follows from the fact that a higher voltage results in the formation of higher levels of ROS and RNS that have established antibacterial properties (Liao et al., 2017; Misra et al., 2015). This is confirmed by the OAS measurement discussed in the previous section in which increased ROS and RNS concentration was observed with higher applied voltage. After five minutes of treatment in dry air, 2.1 $\log_{10}$ CFU/g, 3.1 $\log_{10}$ CFU/g and 3.5 $\log_{10}$ CFU/g of *E.coli* K-12 reduction was observed, based on recovery on EMB agar in 60 kV, 80 kV and 100 kV treatments, respectively. In dry air treatment, the D-value for *E.coli* K-12 was calculated to be 2.26 min, 1.78 min and 1.59 min at 60 kV, 80 kV and 100 kV, respectively. Moreover, a regression model is created to estimate the *E.coli* K-12 population under different treatment time and voltage in dry air (14) with $R^2 = 0.81$ and $p < 0.0001$. Figures SI 3.1 and 3.2 demonstrate the actual by predicted plot and residual plot for this regression model, respectively. The residual plot illustrates that the residual points (observed value – predicted value) are randomly dispersed around the horizontal axis, thus the linear regression model is appropriate for predicting the *E.coli* K-12 population based on the applied voltage and treatment time. Table SI 3.1 shows the parameter estimates of this model with $p<0.0001$ for both of the coefficients for voltage and treatment time. As this model indicated, *E.coli* K-

12 population is negatively and significantly ($p<0.0001$) correlated with the applied voltage and treatment time {T: treatment time (> 0 min); V: voltage (>60 kV)}, demonstrating that higher applied voltage and increased treatment time leads to a reduced *E.coli* K-12 population after HVACP in dry air. As discussed in the previous section, increased voltage and treatment time resulted in a higher concentration of ROS and RNS, which contribute to microbial inactivation. Thus, at higher applied voltage and increased treatment time, higher microbial inactivation is expected.

E.coli K-12 population ($\log_{10}$ CFU/g) = -0.51*T – 0.024*V+ 7.33 *(R^2=0.81)* (14)

Figures 3.8 and 3.9 depicts the *E.coli* K-12 population after cold plasma MA65 (65% O_2, 30% CO_2, 5% N_2) at applied voltage of 60 kV, 80 kV and 100 kV. Similar to dry air treatments, MA65 HVACP treatments have shown an enhanced inactivation of *E.coli* K-12 at higher applied voltage and longer treatment time ($p<0.05$). A 2.1 $\log_{10}$ CFU/g, 2.1 $\log_{10}$ CFU/g and 2.9 $\log_{10}$ CFU/g *E.coli* K-12 reduction was found after five minutes treatment at 60 kV, 80 kV and 100 kV, respectively. The D-value for *E.coli* K-12 in MA65 treatment was found to be 2.41 min, 2.54 min and 2.12 min at 60 kV, 80 kV and 100 kV, respectively. A regression model is used to estimate the *E.coli* K-12 population under different treatment time and voltage in MA65 (15) with $R^2 = 0.75$ and $p < 0.0001$. The actual by predicted plot and residual plot for this regression model is shown in Figure SI 3.3 and 3.4, respectively. As shown in Figure SI 3.4, the residual points are randomly distributed around the horizontal axis, thus linear regression model is appropriate for analyzing the *E.coli* K-12 population under various voltages and treatment time. Table SI 3.2 presents the parameter estimates of this model with $p<0.0001$ for both of the coefficients for voltage and treatment time. Similar to dry air treatment, as shown in this model, *E.coli* K-12 population is significantly

($p<0.0001$) and negatively correlated with the applied voltage and treatment time {T: treatment time (> 0 min); V: voltage (>60 kV)}. Thus, increased applied voltage and treatment time leads to higher inactivation of *E.coli* K-12 by HVACP treatment in MA65.

E.coli K-12 population ($\log_{10}$ CFU/g) = -0.44*T – 0.019*V+ 6.95 *(R^2=0.75)* (15)

Minimal injury in the surviving population of *E.coli* K-12 was observed for both dry air and MA65 treatment by comparing *E.coli* K-12 population detected via TAL method and EMB agar with a maximum of 0.3 $\log_{10}$ CFU/g injury after five minutes dry air treatment at 100 kV. Moreover, at 100 kV, after five minutes treatment, inactivation of *E.coli* K-12 was found to be efficient in dry air plasma compared to MA65 plasma treatment ($p<0.05$). This is unlike earlier studies where MA65 was found to result in higher bacterial inactivation compared to dry air (Han, Ziuzina, et al., 2016; Wan et al., 2017), likely due to formation of higher concentrations of ROS (Misra, Moiseev, et al., 2014). Thus, in the present study, the *E.coli* K-12 inactivation might not be limited by the total ROS concentration. ROS and RNS, such as O, O_2*, O_3, $OH^{\bullet}$, NO, NO_2, NO_3, and N_2O_4, can result in oxidative stress to the cell membrane and also intracellular components leading to cell death by disruption of cell membrane lipid and damage of the intracellular components (e.g. DNA, proteins) (Laroussi, 2005; Liao et al., 2017). Lipid peroxidation of *E.coli* membrane after cold plasma treatment was reported by Joshi et al. (2011), Alkawareek et al. (2014) and Yost and Joshi (2015).

3.4 *Listeria innocua* inactivation

Figures 3.10 and 3.11 show the *Listeria innocua* population after HVACP treatment in dry air for 60 kV, 80 kV and 100 kV recovered on LSA and by TAL method, respectively. Similar to *E.coli* K-12 inactivation, results indicate that increasing applied voltage and treatment time leads to higher *Listeria* inactivation. After five minutes of treatment in dry air,

a 0.7 $\log_{10}$ CFU/g, 1.2 $\log_{10}$ CFU/g and 1.4 $\log_{10}$ CFU/g of LI reduction was observed, based on recovery on *Listeria* selective agar (Oxoid CM856), in 60 kV, 80 kV and 100 kV treatments, respectively. No significant injury was observed by comparing *Listeria* population detected via TAL method and *Listeria* selective agar. This implies that under the studied treatment conditions, the injured cell population was practically negligible. In other words, HVACP treatment with 24 hours storage was effective in causing complete inactivation of the *Listeria* cells. The D-value for LI in dry air treatment was found to be 7.48 min, 4.12 min and 4.01 min at 60 kV, 80 kV and 100 kV, respectively. A regression model is created to estimate the LI population under different treatment time and voltage in dry air (16) with $R^2 = 0.83$ and $p < 0.0001$. Similar to the regression models built for *E.coli* K-12 inactivation, LI population is also significantly ($p<0.0001$) and negatively correlated with the applied voltage and treatment time {T: treatment time (> 0 min); V: voltage (>60 kV)}. Thus, increased voltage and treatment time is expected to result in higher inactivation of LI by HVACP treatment in dry air. Table SI 3.3 demonstrates the parameter estimates of this model with $p<0.0001$ for both of the coefficients for voltage and treatment time. The actual by predicted plot and residual plot for this regression model is presented in Figure SI 3.5 and 3.6, respectively. The residual points are randomly dispersed around the horizontal axis, which indicates that linear regression model is appropriate for predicting LI population based on voltage and treatment time.

$$\text{LI population } (\log_{10} \text{CFU/g}) = -0.21*T - 0.008*V + 6.57 \quad (R^2=0.83) \tag{16}$$

Figures 3.12 and 3.13 illustrate the *Listeria* population after HVACP treatment in MA65 at applied voltage of 60 kV, 80 kV and 100 kV. A 0.5 $\log_{10}$ CFU/g, 1.0 $\log_{10}$ CFU/g and 1.1 $\log_{10}$ CFU/g LI reduction was found after 60 kV, 80 kV and 100 kV, respectively. In MA65

treatment, the D-value for LI in dry air treatment was found to be 8.57 min, 4.89 min and 5.52 min at 60 kV, 80 kV and 100 kV, respectively. As shown in equation (17), a regression model (R^2 = 0.77, $p<0.0001$) is applied to estimate the LI population under different treatment time and voltage in MA65 HVACP treatment. Similarly, under MA65 treatment, LI population is found to significantly ($p<0.001$) and negatively correlated with applied voltage and treatment time {T: treatment time (> 0 min); V: voltage (>60 kV)}. Figures SI 3.7 and 3.8 present the actual by predicted plot and residual plot for this regression model, respectively, while the parameter estimates of this model is shown in Table SI 3.4 ($p<0.0001$ for both of the coefficients for voltage and treatment time). The residual plot indicates that linear regression model is appropriately applied as the residual points are randomly distributed around the horizontal axis. All the four models {(14), (15), (16) (17)} imply that increased voltage and treatment time results in enhanced the microbial inactivation (*E.coli* K-12 and LI) by HVACP treatment in both dry air and MA65.

$$\text{LI population } (\log_{10} \text{CFU/g}) = -0.167*T - 0.0097*V + 6.50 \quad (R^2=0.77) \tag{16}$$

Furthermore, *Listeria* cell injury was not observed under MA65 treatments in this study. Similar to *E.coli* K-12 inactivation, there was no significant difference in *Listeria* inactivation between dry air and MA65 treatment. In light of this observation, air being inexpensive, was used for subsequent *Listeria* inactivation studies. In addition, after comparing the inactivation results between *E.coli* and *Listeria*, it is clear that cold plasma treatment leads to a more efficient inactivation in *E.coli* K-12 compared to *Listeria innocua.* This is likely due to the thick peptidoglycan layer in the gram-positive *Listeria innocua* which prevents cell leakage resulting from reactions of ROS and RNS with cell components (Han, Patil, et al., 2016). Thus, instead of disruption of cell membrane, Han, Patil, et al.

(2016) proposed that the inactivation of gram-positive microorganisms by cold plasma treatment is likely due to the oxidation of intracellular components.

3.5 pH and moisture measurement

Tables 3.1 and 3.2 summarize the effects of HVACP treatment at 60, 80 and 100 kV on pH and moisture content of QFC after dry air and MA65 treatments. After dry air HVACP treatments at 80 kV and 100 kV, no significant difference ($p<0.05$) was observed in pH and moisture content of QFC. Although minimal, changes in pH and moisture content were found between control and HVACP treated samples after dry air treatment at 60 kV. Yong et al. (2015) and Kim et al. (2015) have also reported a minor decrease of pH of cheese slices and milk, respectively after cold plasma treatment. This is likely due to the buffering capacity of protein in dairy products (Olthuis et al., 1994). In dry air treatment at 60 kV, the pH for control and treated samples ranged between 5.69 and 5.83. Irrespective of the treatment conditions, no significant ($p<0.05$) loss in moisture and minimal change in pH was observed with MA65 plasma. Therefore, it was concluded that under the process conditions employed, HVACP treatments in dry air and MA65 did not result in drying and loss of moisture in QFC.

3.6 Texture and color measurement

Tables 3.3 and 3.4 summarize the effects of direct HVACP treatment at 60, 80 and 100 kV on the hardness, cohesiveness and springiness of QFC after dry air and MA65 treatments, respectively. Simulation of cheese crumbling was performed by compressing a sample to 70% compression and recording the peak force. This measurement represents the crumbliness of QFC. It is to be noted that crumbliness is an important textural parameter of QFC (Hnosko et al., 2012). Irrespective of the treatment conditions, no significant

differences ($p<0.05$) were observed between the control and HVACP treated QFC for all the three texture parameters.

Table 3.5 and 3.6 provide the summary of the effect of direct HVACP treatments at 60, 80 and 100 kV on color of QFC after dry air and MA65, respectively. No significant difference ($p<0.05$) in L* was found in samples after 60 kV and 80 kV HVACP treatment in dry air, as well as 100 kV HVACP treatments in MA65. This indicates that the HVACP process did not result in a browning effect as was reported in earlier studies with plasma jet and radio-frequency barrier discharge plasma treatment of cheeses (Lee et al., 2012; Yong et al., 2015). A decrease in a* values and an increase in b* values were observed after the plasma treatment at 60 kV, 80 kV and 100 kV in dry air and MA65. It suggests that the color of QFC shifted towards a yellower shade after the plasma treatment in dry air and MA65. In the past, it was shown that oxidation of milk proteins is effected by HVACP (Segat et al., 2015), which is known to induce a minor yellowing effect that could be picked on a colorimeter. Differences in perceivable color can be analytically classified as very distinct ($\Delta E > 3$), distinct ($1.5 < \Delta E < 3$) and small difference ($\Delta E < 1.5$) (Misra, Keener, et al., 2014b). Although, HVACP treatments in dry air and MA65 induced some color change in QFC samples, they were not perceptible with the naked eye due to the low total color difference ($\Delta E < 3$).

3.7 Lipid peroxidation

Ozone generated by HVACP can react with lipids in QFC, resulting in the production of malondialdehyde, a primary product of hydroperoxide homolytic cleavage during oxidation (Gavahian et al., 2018). Tables 3.7 and 3.8 provide a summary of the malondialdehyde (MDA) content in QFC after direct HVACP treatment in dry air and MA65, respectively. For

both dry air and MA65 treatments, higher MDA content was observed at the lowest voltage (60 kV) applied, while there was no significant difference ($p<0.05$) between 80 kV and 100 kV. Moreover, for MA65 treatment, 5 min treatment resulted in the least MDA formation, which can be explained by the OAS results, in which lowest ozone concentration was observed after 5 min treatment. Meanwhile, the reactive gas species in MA65 generated after 5 min treatment might not favor the oxidative reaction of lipids. After dry air treatment, even at 60 kV, the amount of MDA produced was less than 2.0 mg/kg, which is very minimal. Furthermore, MA65 treatment induced higher ($p<0.05$) MDA formation in QFC compared to dry air treatment due to a higher ROS production in MA65 plasma. As measured by OAS, at 100 kV, air plasma contained a maximum of ca. 2750 ppmv of ozone, while MA65 resulted in ca. 6,000 ppmv within 5 min. It should be noted that despite the higher oxygen to start with for reactive oxygen production, MA65 plasma at 80 kV and 100 kV resulted in minimal MDA formation (less than 2.0 mg/kg MDA).

4. Conclusions

This study demonstrates that the application potential of HVACP for decontamination of queso fresco ensuring the safety of fresh cheeses like queso fresco. HVACP treatment at 100 kV for 5 min in dry air, a maximum of 1.4 and 3.5 $\log_{10}$ CFU/g reductions in *Listeria innocua* and *E.coli* K-12, respectively, were observed. The higher inactivation efficacy in *E.coli* K-12 by cold plasma treatment is likely due to the thin peptidoglycan in the gram-negative *E.coli* cells resulting in cell leakage after reactions with ROS and RNS. Increasing the applied voltage led to higher production of ROS and RNS, thus higher efficiency of microbial inactivation. Minimal changes were observed in the pH, color and lipid oxidation of queso fresco after HVACP treatment. No significant ($p<0.05$) changes in texture were

found with no effect on the crumbliness of the cheese after plasma treatment. These observations suggest HVACP may be a potential non-thermal technology for fresh cheese processing to prevent post-manufacturing pathogen contamination with minimal effects on products' quality.

Acknowledgement

Research funding support provided by the National Dairy Council and Innovation Center for US Dairy.

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Table 3.1. Moisture content and pH of QFC after HVACP treatment in dry air. Same small letter beside each measurement indicated no significant (p>0.05) different within the same treatment voltage

Treatment time (min)	60 kV		80 kV		100 kV	
	pH	Moisture content% (wet basis)	pH	Moisture content% (wet basis)	pH	Moisture content% (wet basis)
0	5.69^{b}	49.64^{c}	5.69^{a}	49.64^{a}	5.69^{a}	49.64^{a}
1	5.81^{a}	50.40^{a}	5.79^{a}	49.76^{a}	5.61^{a}	50.44^{a}
2	5.82^{a}	50.23^{ab}	5.74^{a}	49.80^{a}	5.60^{a}	48.29^{a}
3	5.83^{a}	49.89^{abc}	5.79^{a}	49.83^{a}	5.66^{a}	47.80^{a}
4	5.82^{a}	49.78^{bc}	5.72^{a}	49.42^{a}	5.66^{a}	49.71^{a}
5	5.77^{ab}	49.66^{c}	5.68^{a}	49.21^{a}	5.62^{a}	48.93^{a}

Table 3.2. Moisture content and pH of QFC after HVACP treatment in MA65. Same small letter beside each measurement indicated no significant (p>0.05) different within the same treatment voltage

Treatment time (min)	60 kV		80 kV		100 kV	
	pH	Moisture content% (wet basis)	pH	Moisture content% (wet basis)	pH	Moisture content% (wet basis)
0	5.73^{a}	49.18^{a}	5.73^{a}	49.18^{a}	5.73^{a}	49.18^{a}
1	5.68^{ab}	49.13^{a}	5.61^{b}	49.29^{a}	5.69^{a}	48.35^{a}
2	5.61^{abc}	49.01^{a}	5.59^{b}	48.86^{a}	5.66^{a}	49.00^{a}
3	5.43^{c}	49.39^{a}	5.56^{b}	49.20^{a}	5.65^{a}	48.39^{a}
4	5.48^{bc}	49.39^{a}	5.61^{b}	48.70^{a}	5.62^{a}	48.49^{a}
5	5.66^{abc}	49.48^{a}	5.58^{b}	48.54^{a}	5.64^{a}	48.01^{a}

Table 3.3. Texture profile analysis of QFC after HVACP treatment in dry air. Same small letter beside each measurement indicated no significant (p>0.05) different within the same treatment voltage

Treatment time (min)	60 kV			80 kV			100 kV		
	Hardness (N)	Cohesiveness (%)	Springiness (%)	Hardness (N)	Cohesiveness (%)	Springiness (%)	Hardness (N)	Cohesiveness (%)	Springiness (%)
0	73.78^{a}	19.91^{a}	63.50^{a}	73.78^{a}	19.91^{a}	63.50^{a}	73.78^{a}	19.91^{a}	63.50^{a}
1	79.14^{a}	19.78^{a}	59.99^{a}	69.96^{a}	20.82^{a}	62.51^{a}	73.51^{a}	19.57^{a}	64.58^{a}
2	72.43^{a}	20.25^{a}	62.00^{a}	69.02^{a}	19.31^{a}	61.43^{a}	72.90^{a}	19.56^{a}	64.38^{a}
3	68.95^{a}	20.40^{a}	61.90^{a}	73.23^{a}	20.50^{a}	62.82^{a}	78.58^{a}	20.42^{a}	64.52^{a}
4	68.58^{a}	19.33^{a}	59.11^{a}	69.32^{a}	20.17^{a}	66.25^{a}	72.19^{a}	18.76^{a}	64.22^{a}
5	77.17^{a}	19.93^{a}	63.01^{a}	68.07^{a}	20.88^{a}	65.77^{a}	62.17^{a}	18.88^{a}	65.74^{a}

Table 3.4. Texture profile analysis of QFC after HVACP treatment in MA65. Same small letter beside each measurement indicated no significant (p>0.05) different within the same treatment voltage

Treatment time (min)	60 kV			80 kV			100 kV		
	Hardness (N)	Cohesiveness (%)	Springiness (%)	Hardness (N)	Cohesiveness (%)	Springiness (%)	Hardness (N)	Cohesiveness (%)	Springiness (%)
0	68.34^{a}	19.91^{a}	64.10^{a}	68.34^{a}	19.91^{a}	64.10^{a}	68.34^{a}	19.91^{a}	64.10^{a}
1	60.10^{a}	18.50^{a}	65.54^{a}	62.88^{a}	19.54^{a}	61.48^{a}	69.42^{a}	19.35^{a}	64.17^{a}
2	54.88^{a}	19.33^{a}	61.40^{a}	61.01^{a}	19.97^{a}	67.41^{a}	65.91^{a}	19.49^{a}	65.28^{a}
3	66.53^{a}	19.91^{a}	63.80^{a}	66.75^{a}	19.37^{a}	62.73^{a}	71.93^{a}	20.46^{a}	68.14^{a}
4	58.42^{a}	19.85^{a}	63.93^{a}	64.48^{a}	19.79^{a}	64.23^{a}	73.69^{a}	20.13^{a}	63.44^{a}
5	66.73^{a}	20.04^{a}	63.69^{a}	63.48^{a}	19.20^{a}	62.53^{a}	66.15^{a}	19.50^{a}	66.60^{a}

Table 3.5. Colorimeter measurement of QFC after HVACP treatment in dry air. Same small letter beside each measurement indicated no significant (p>0.05) different within the same treatment voltage

Treatment time (min)	60 kV				80 kV				100 kV			
	L*	a*	b*	ΔE	L*	a*	b*	ΔE	L*	a*	b*	ΔE
0	92.72^{a}	-0.71^{a}	12.73^{b}		92.72^{a}	-0.71^{a}	12.73^{b}		92.72^{a}	-0.71^{a}	12.73^{b}	
1	92.43^{a}	-0.79^{ab}	13.39^{a}	0.75	92.41^{a}	-0.83^{ab}	13.34^{a}	0.70	92.54^{ab}	-0.92^{b}	13.30^{a}	0.64
2	92.53^{a}	-0.82^{ab}	13.31^{a}	0.63	92.61^{a}	-0.92^{bc}	13.14^{ab}	0.49	92.48^{ab}	-0.90^{b}	13.37^{a}	0.71
3	92.56^{a}	-0.86^{b}	13.04^{ab}	0.39	92.56^{a}	-0.89^{bc}	13.39^{a}	0.71	92.27^{b}	-0.93^{b}	13.22^{ab}	0.71
4	92.60^{a}	-0.90^{b}	13.00^{ab}	0.39	92.48^{a}	-0.95^{bc}	13.27^{a}	0.65	92.25^{b}	-1.08^{c}	13.36^{a}	0.92
5	92.51^{a}	-0.93^{b}	13.22^{a}	0.58	92.54^{a}	-0.97^{c}	13.24^{a}	0.61	92.41^{ab}	-1.15^{c}	13.36^{a}	0.84

Table 3.6. Colorimeter measurement of QFC after HVACP treatment in MA65. Same small letter beside each measurement indicated no significant (p>0.05) different within the same treatment voltage

Treatment time (min)	60 kV				80 kV				100 kV			
	L*	a*	b*	ΔE	L*	a*	b*	ΔE	L*	a*	b*	ΔE
0	93.37^{a}	-0.67^{a}	12.53^{d}		93.37^{a}	-0.67^{a}	12.53^{c}		93.37^{a}	-0.67^{a}	12.53^{d}	
1	92.80^{b}	-0.72^{a}	13.00^{c}	0.75	92.77^{b}	-0.77^{ab}	13.51^{a}	1.16	93.11^{b}	-0.75^{ab}	13.34^{ab}	0.87
2	92.40^{b}	-0.72^{a}	13.58^{a}	1.46	92.69^{b}	-0.83^{bc}	13.34^{ab}	1.08	93.37^{a}	-0.78^{ab}	13.02^{c}	0.51
3	92.72^{b}	-0.77^{ab}	13.44^{ab}	1.13	92.75^{b}	-0.93^{c}	13.05^{b}	0.86	93.18^{ab}	-0.84^{b}	13.16^{abc}	0.69
4	92.60^{b}	-0.76^{ab}	13.44^{ab}	1.20	92.83^{b}	-0.86^{bc}	13.19^{ab}	0.89	93.26^{ab}	-0.83^{b}	13.13^{bc}	0.64
5	92.69^{b}	-0.89^{b}	13.17^{bc}	0.97	93.30^{a}	-0.86^{bc}	13.14^{ab}	0.68	93.09^{b}	-0.85^{b}	13.39^{a}	0.93

Table 3.7. Malondialdehyde content in QFC after HVACP treatment in dry air. Same small letter beside each measurement indicated no significant (p>0.05) different within the same treatment voltage

Treatment time (min)	60 kV	80 kV	100 kV
	MDA (mg/kg)	MDA (mg/kg)	MDA (mg/kg)
0	0.21^{b}	0.21^{b}	0.21^{c}
1	1.20^{a}	0.69^{a}	0.56^{ab}
2	1.19^{a}	0.73^{a}	0.70^{ab}
3	1.12^{a}	0.78^{a}	0.79^{a}
4	1.38^{a}	0.59^{a}	0.60^{ab}
5	1.08^{a}	0.69^{a}	0.53^{b}

Table 3.8. Malondialdehyde content in QFC after HVACP treatment in MA65. Same small letter beside each measurement indicated no significant (p>0.05) different within the same treatment voltage

Treatment time (min)	60 kV	80 kV	100 kV
	MDA (mg/kg)	MDA (mg/kg)	MDA (mg/kg)
0	0.29^{c}	0.29^{d}	0.29^{c}
1	2.31^{ab}	1.19^{ab}	1.18^{a}
2	1.83^{b}	1.38^{a}	1.30^{a}
3	2.55^{a}	1.21^{ab}	1.37^{a}
4	2.40^{ab}	0.81^{c}	1.40^{a}
5	2.07^{ab}	1.06^{bc}	0.73^{b}

Table SI 3.1. Parameter estimates for regression model estimating *E.coli* K-12 population under different treatment time and voltage in dry air

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	7.33	0.21	35.71	<.0001*
Treatment time (min)	-0.51	0.025	-20.45	<.0001*
Voltage	-0.024	0.0024	-9.95	<.0001*

Table SI 3.2. Parameter estimates for regression model estimating *E.coli* K-12 population under different treatment time and voltage in MA65

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	6.95	0.21	33.84	<.0001*
Treatment time (min)	-0.44	0.023	-19.55	<.0001*
Voltage	-0.019	0.0024	-8.03	<.0001*

Table SI 3.3. Parameter estimates for regression model estimating LI population under different treatment time and voltage in dry air

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	6.57	0.11	59.16	<.0001*
Voltage	-0.008	0.0013	-6.63	<.0001*
Treatment time (min)	-0.21	0.012	-16.97	<.0001*

Table SI 3.4. Parameter estimates for regression model estimating LI population under different treatment time and voltage in MA65

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	6.50	0.11	57.94	<.0001*
Voltage	-0.0097	0.00132	-7.34	<.0001*
Treatment time (min)	-0.167	0.013	-13.23	<.0001*

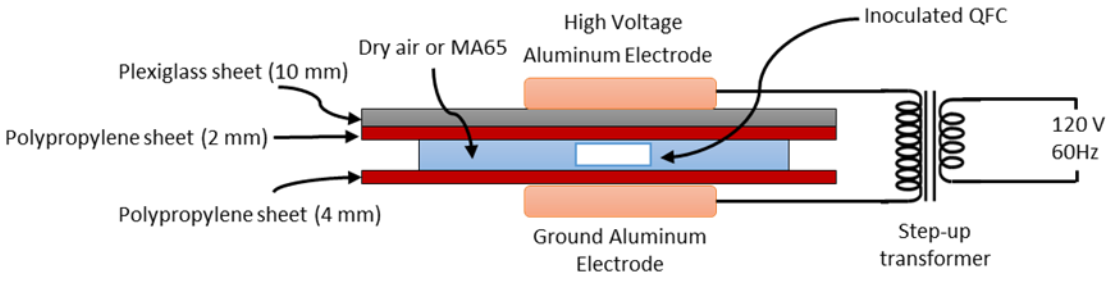

Figure 3.1. Schematic diagram of HVACP treatments

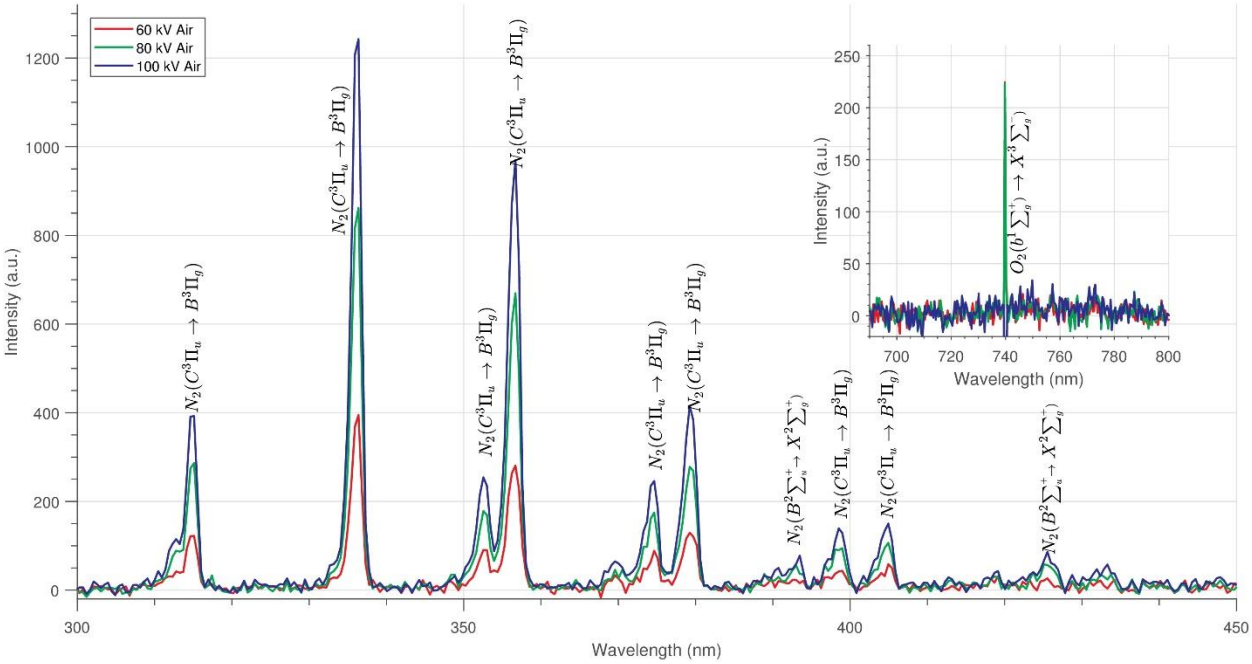

Figure 3.2. Optical emission spectra of direct HVACP treatment of QFC at 60 kV, 80 kV and 100 kV in dry air

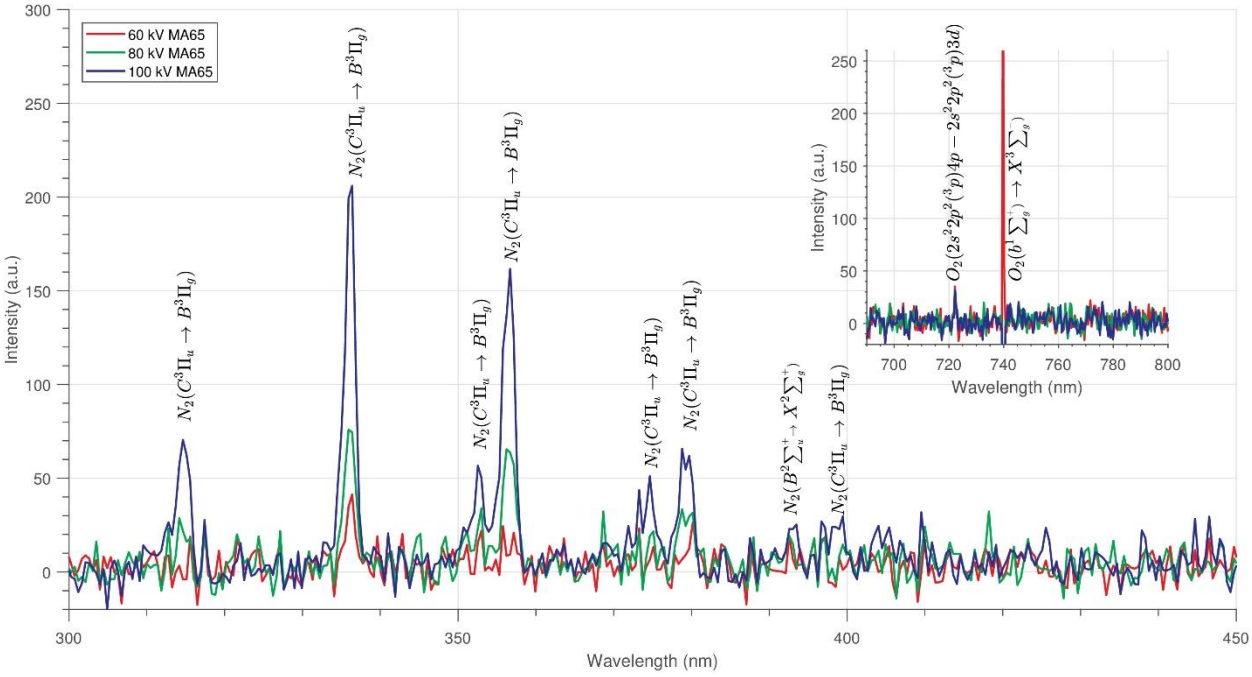

Figure 3.3. Optical emission spectra of direct HVACP treatment of QFC at 60 kV, 80 kV and 100 kV in MA 65

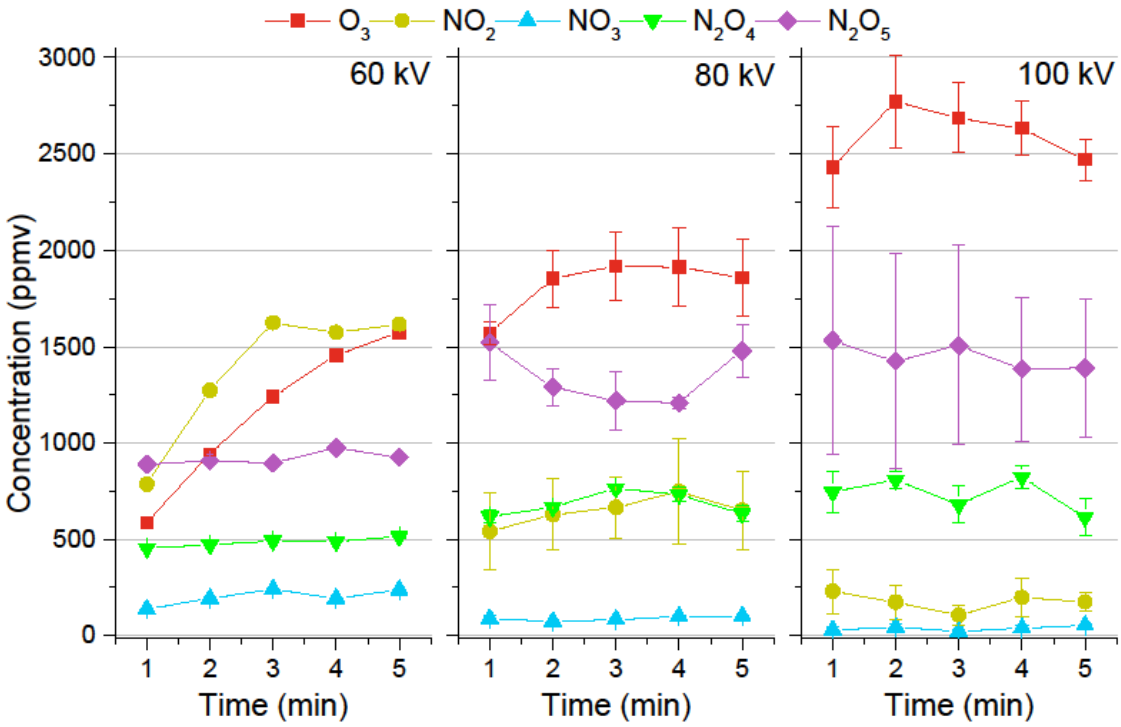

Figure 3.4. Post-discharge composition of the gas for direct HVACP treatment of QFC at 60 kV, 80 kV and 100 kV in dry air

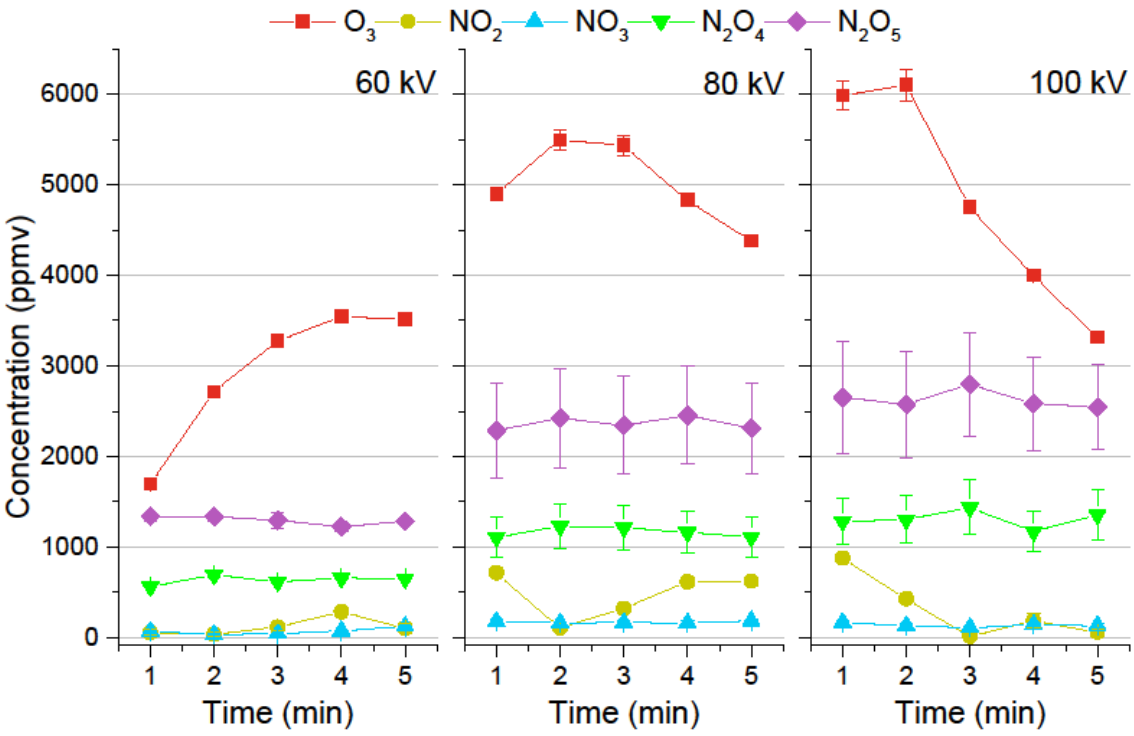

Figure 3.5. Post-discharge composition of the gas for direct HVACP treatment of QFC at 60 kV, 80 kV and 100 kV in MA 65

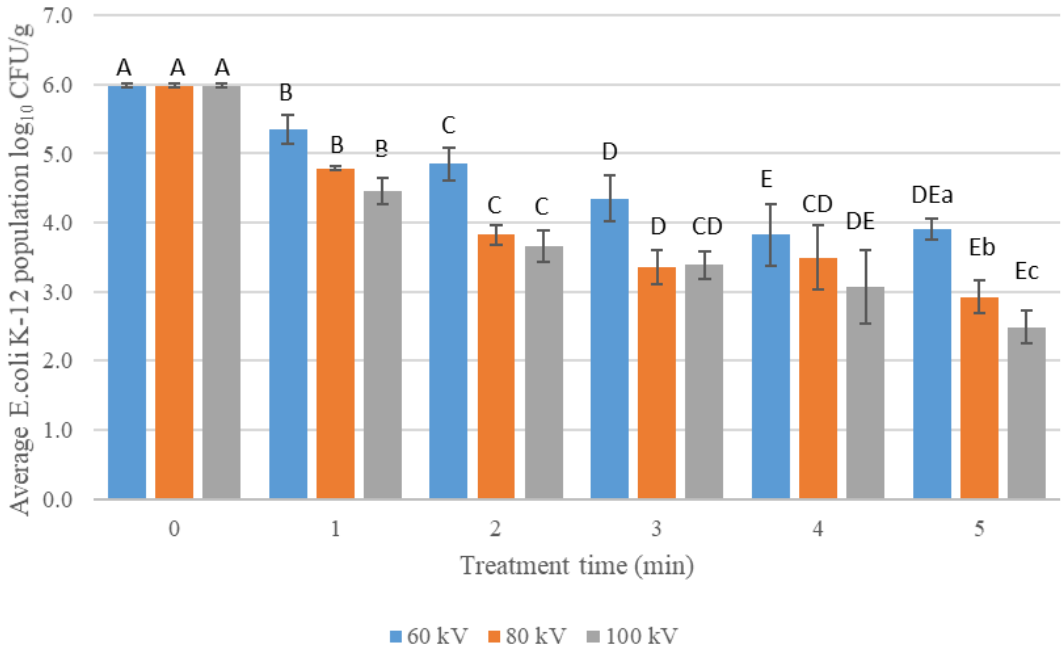

Figure 3.6. Survival of *E.coli* K-12 population (log10 CFU/g) on 10.0 g QFC slice treated with HVACP in dry air at 60, 80 and 100 kV after 24-hour storage at 4 °C recovered on EMB agar. The detection limit of the applied enumeration method was 1.0 log_{10} CFU/g. Same capital letter on top of each bar indicated no significant difference among each bar under the same applied voltage. Same small letter on top of each bar indicated no significant difference among the same treatment time. ($p<0.05$)

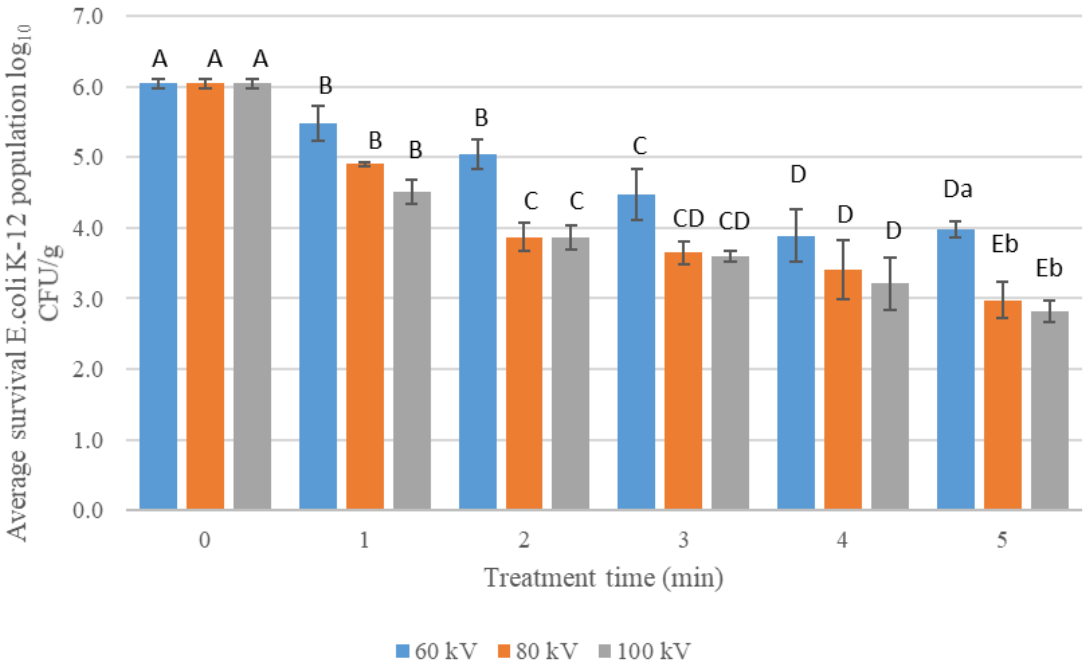

Figure 3.7. Survival of *E.coli* K-12 population (log10 CFU/g) on 10.0 g QFC slice treated with HVACP in dry air at 60, 80 and 100 kV after 24-hour storage at 4 °C recovered by thin agar layer method (TAL). The detection limit of the applied enumeration method was 1.0 log_{10} CFU/g. Same capital letter on top of each bar indicated no significant difference among each bar under the same applied voltage. Same small letter on top of each bar indicated no significant difference among the same treatment time. ($p<0.05$)

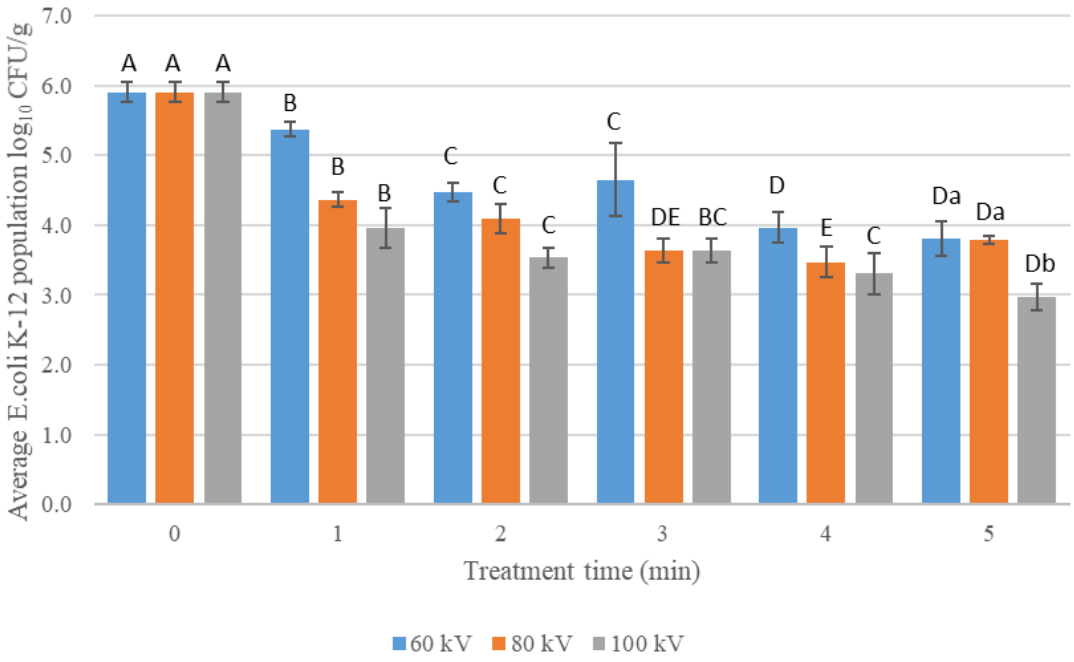

Figure 3.8. Survival of *E.coli* K-12 population (log10 CFU/g) on 10.0 g QFC slice treated with HVACP in MA 65 at 60, 80 and 100 kV after 24-hour storage at 4 °C recovered on EMB agar. The detection limit of the applied enumeration method was 1.0 log_{10} CFU/g. Same capital letter on top of each bar indicated no significant difference among each bar under the same applied voltage. Same small letter on top of each bar indicated no significant difference among the same treatment time. ($p<0.05$)

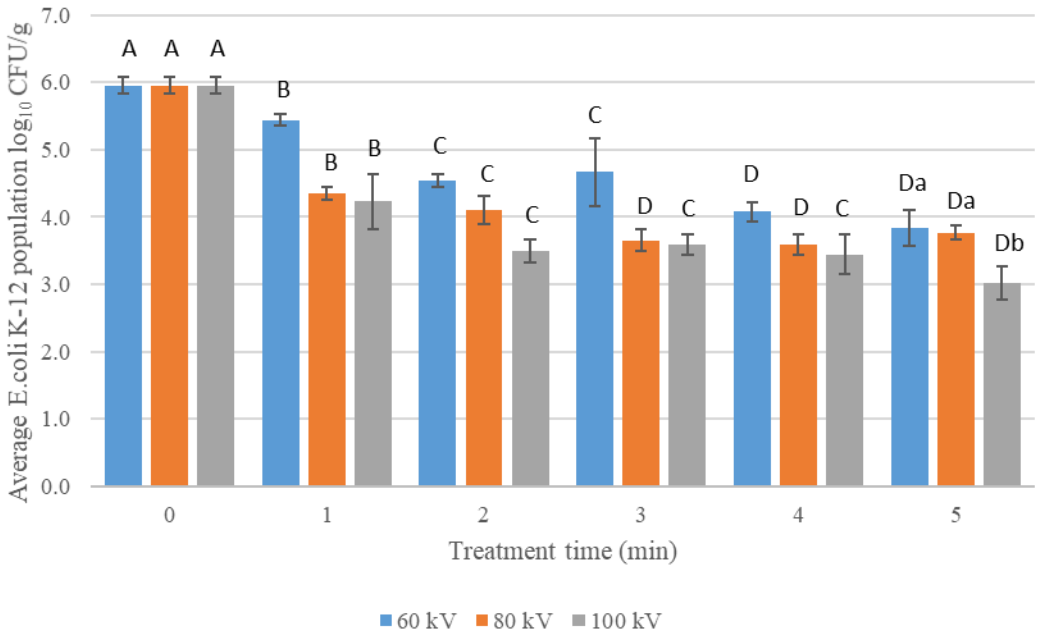

Figure 3.9. Survival of *E.coli* K-12 population (log10 CFU/g) on 10.0 g QFC slice treated with HVACP in MA 65 at 60, 80 and 100 kV after 24-hour storage at 4 °C recovered by thin agar layer method (TAL). The detection limit of the applied enumeration method was 1.0 $\log_{10}$ CFU/g. Same capital letter on top of each bar indicated no significant difference among each bar under the same applied voltage. Same small letter on top of each bar indicated no significant difference among the same treatment time. ($p<0.05$)

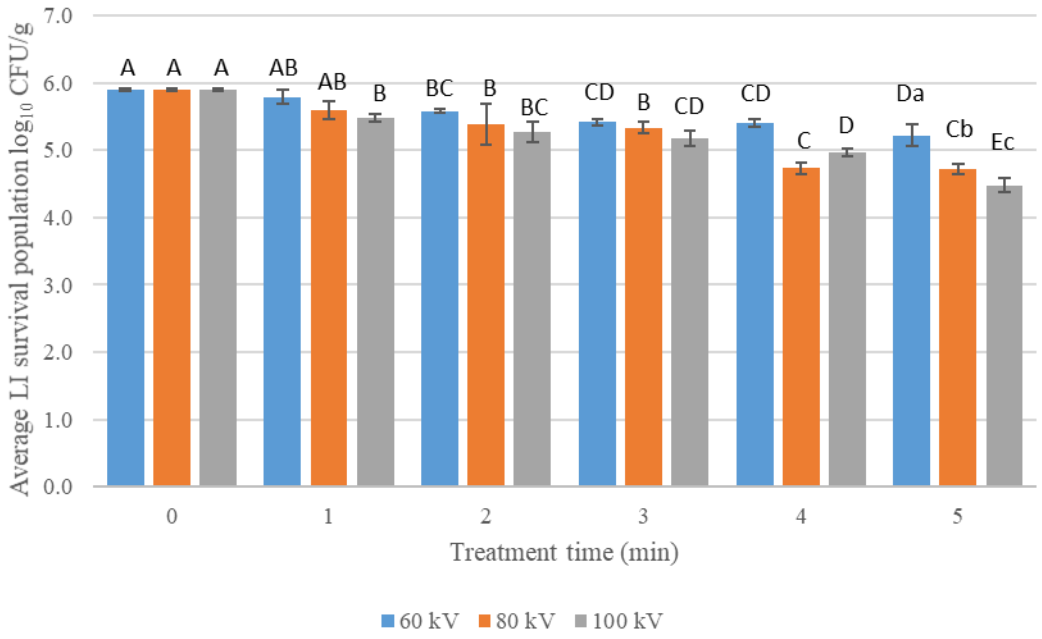

Figure 3.10. Survival of *Listeria innocua* population (log_{10} CFU/g) on 10.0 g QFC slice treated with HVACP in dry air at 60, 80 and 100 kV after 24-hour storage at 4 °C recovered on *Listeria* selective agar (LSA). The detection limit of the applied enumeration method was 1.0 log_{10} CFU/g. Same capital letter on top of each bar indicated no significant difference among each bar under the same applied voltage. Same small letter on top of each bar indicated no significant difference among the same treatment time. ($p<0.05$)

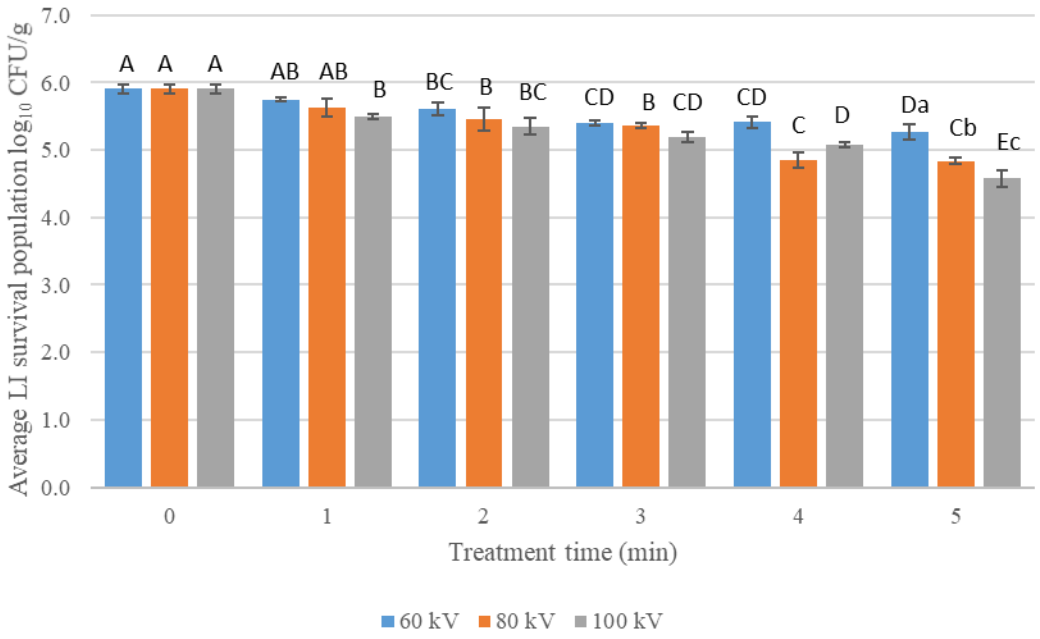

Figure 3.11. Survival of *Listeria innocua* population (log_{10} CFU/g) on 10.0 g QFC slice treated with HVACP in dry air at 60, 80 and 100 kV after 24-hour storage at 4 °C recovered by thin agar layer method (TAL). The detection limit of the applied enumeration method was 1.0 log_{10} CFU/g. Same capital letter on top of each bar indicated no significant difference among each bar under the same applied voltage. Same small letter on top of each bar indicated no significant difference among the same treatment time. ($p<0.05$)

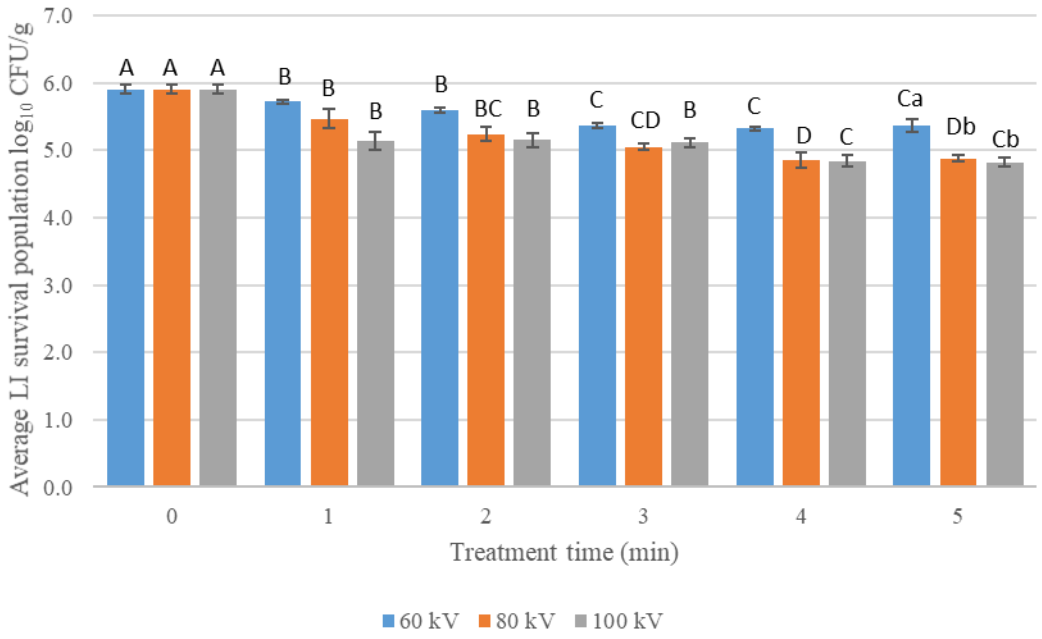

Figure 3.12. Survival of *Listeria innocua* population (log10 CFU/g) on 10.0 g QFC slice treated with HVACP in MA65 at 60, 80 and 100 kV after 24-hour storage at 4 °C recovered on *Listeria* selective agar (LSA). The detection limit of the applied enumeration method was 1.0 $\log_{10}$ CFU/g. Same capital letter on top of each bar indicated no significant difference among each bar under the same applied voltage. Same small letter on top of each bar indicated no significant difference among the same treatment time. ($p<0.05$)

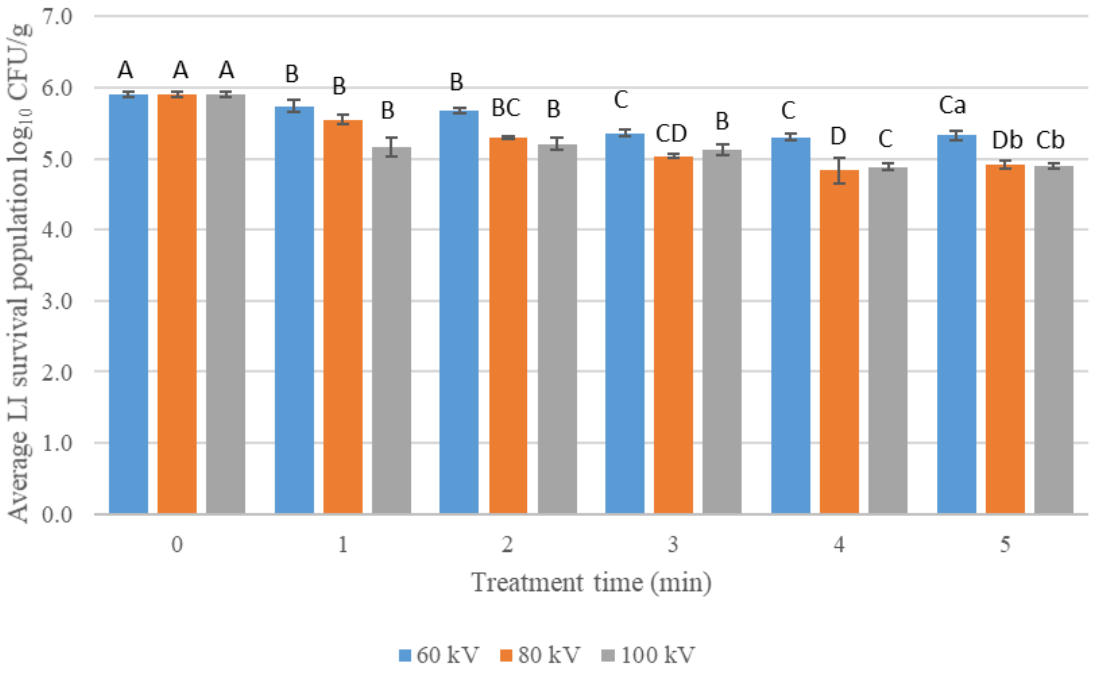

Figure 3.13. Survival of *Listeria innocua* population (log10 CFU/g) on 10.0 g QFC slice treated with HVACP in MA65 at 60, 80 and 100 kV after 24-hour storage at 4 °C recovered by thin agar layer method (TAL). The detection limit of the applied enumeration method was 1.0 $\log_{10}$ CFU/g. Same capital letter on top of each bar indicated no significant difference among each bar under the same applied voltage. Same small letter on top of each bar indicated no significant difference among the same treatment time. ($p<0.05$)

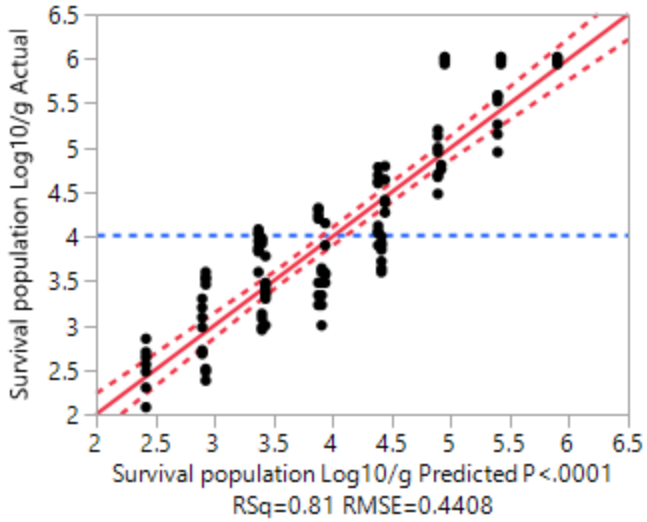

Figure SI 3.1. Actual by predicted plot for regression model estimating *E.coli* K-12 population under different treatment time and voltage in dry air

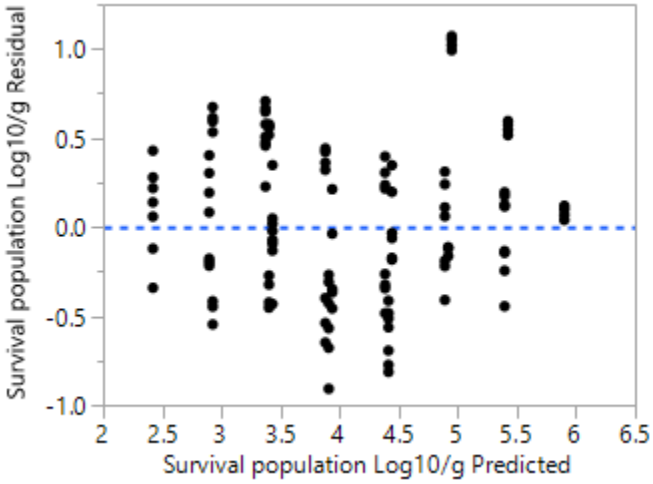

Figure SI 3.2. Residual plot for regression model estimating *E.coli* K-12 population under different treatment time and voltage in dry air

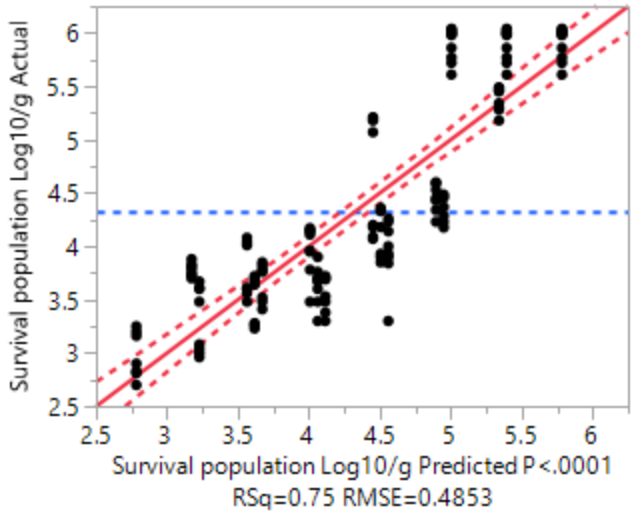

Figure SI 3.3. Actual by predicted plot for regression model estimating *E.coli* K-12 population under different treatment time and voltage in MA65

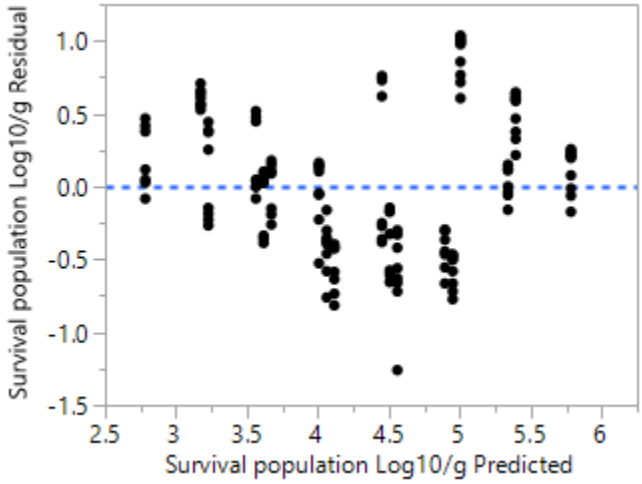

Figure SI 3.4. Residual plot for regression model estimating *E.coli* K-12 population under different treatment time and voltage in MA65

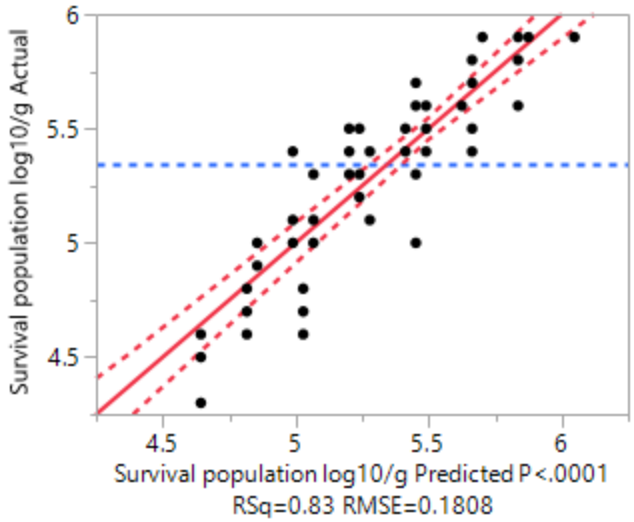

Figure SI 3.5. Actual by predicted plot for regression model estimating LI population under different treatment time and voltage in dry air

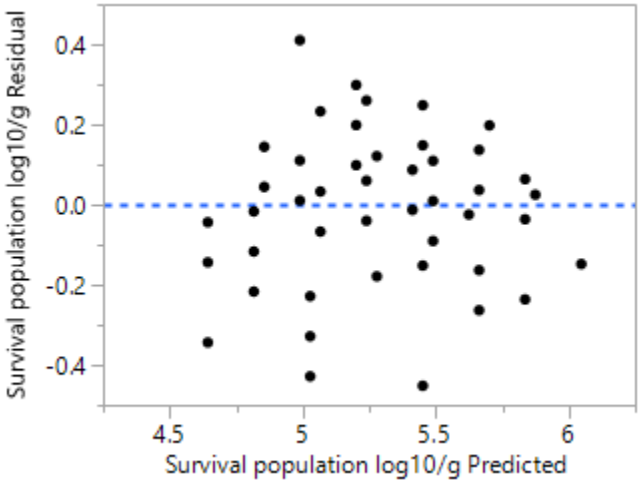

Figure SI 3.6. Residual plot for regression model estimating LI population under different treatment time and voltage in dry air

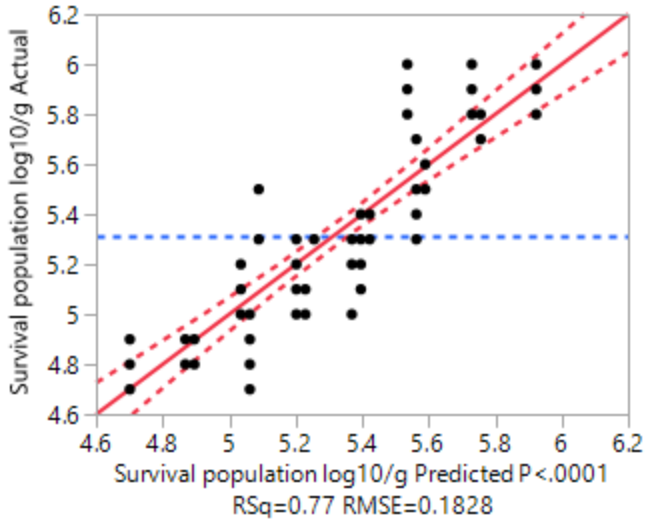

Figure SI 3.7. Actual by predicted plot for regression model estimating LI population under different treatment time and voltage in MA65

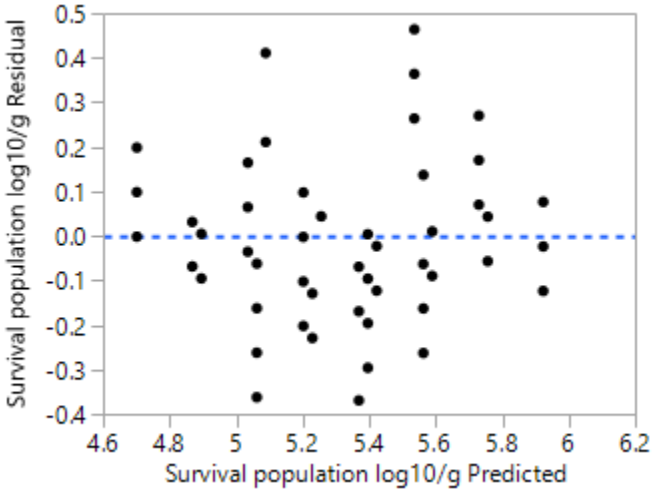

Figure SI 3.8. Residual plot for regression model estimating LI population under different treatment time and voltage in MA65

CHAPTER 4: HIGH VOLTAGE ATMOSPHERIC COLD PLASMA INACTIVATION OF *LISTERIA INNOCUA* ON QUESO FRESCO CHEESE: PART 1, EVALUATION OF TREATMENT CONDITIONS, MICROBIAL INACTIVATION AND QUALITY OF PLASMA TREATED CHEESE

A manuscript prepared for submission to Innovative food science and emerging technologies

Zifan Wan[a], NN Misra[b], Kevin M. Keener[a,b]*

[a] Food Science and Human Nutrition Department

[b] Center for Crops Utilization Research

*Corresponding Author

Iowa State University

Abstract

As a novel, non-thermal technology, high voltage atmospheric cold plasma (HVACP) has shown great potential in microbial inactivation while retaining the quality of food products. In this study, *Listeria innocua* (LI) inoculated crumbled queso fresco cheese (QFC) samples were treated with HVACP under direct mode of exposure in various gas environments for up to 10 min at 100 kV followed by 24 h refrigerated storage. A 0.8 $\log_{10}$ CFU/g and 2.6 $\log_{10}$ CFU/g LI reduction was observed after four and six minutes dry air HVACP treatment, respectively, while still retaining the quality of the cheese. Higher LI inactivation efficiency of HVACP was found with increased treatment time and under the gas environment in the presence of oxygen. Moreover, the presence of oxygen in dry air and moist air resulted in higher lipid oxidation compared to the modified gas (MA50: 50% N_2 and 50% CO_2) in which oxygen was not present. After six minutes direct HVACP treatment, dry air resulted in 2.3 mg/kg MDA formation compared to 1.01 mg/kg after MA50 treatment. However, insufficient LI reduction was observed after MA50 HVACP treatment with a 0.3 $\log_{10}$ CFU/g and 0.8 $\log_{10}$ CFU/g reduction after four and six minutes treatment, respectively. In brief, this study demonstrates the significance of

direct dry air HVACP at 100 kV in microbial inactivation with minor effects on cheese quality, thus making it promising for applications in fresh cheese production to ensure the safety of the product.

Highlights

- Significant inactivation of *Listeria innocua* was achieved by HVACP treatment in dry air
- Gas blend with oxygen and higher nitrogen content resulted in a more efficient inactivation of *Listeria innocua*
- Minimal effects were observed in the pH, lipid oxidation and moisture of queso fresco after cold plasma treatment

Key words

Atmospheric cold plasma, cheese, safety

1. Introduction

In the United States, there was an estimated 17.4% Hispanic population in 2015 and it is projected to increase to 28.6% by 2060 (U.S. Census Bureau, 2015). With the increase in Hispanic population, a potential growth in Hispanic-style cheese market is expected as well (NASS, 2005). Queso fresco cheese (QFC) is a popular Mexican-style soft cheese with a high moisture, low salt content and near neutral pH providing an ideal substrate for the growth of spoilage and pathogenic microorganisms (Hnosko et al., 2012). *Listeria monocytogenes* (LM), a gram-positive pathogen, is especially a concern for queso fresco in which several LM outbreaks resulted from contaminated queso fresco (CDC, 2018). Thus, people with weakened immune system are suggested not to consume fresh cheese like queso fresco due to the risk of listeriosis (CDC, 2016). With the ability to grow under refrigerated

temperature, LM is especially a concern for ready-to-eat products such as fresh cheese, ready-to-eat meats, etc. (Farber & Peterkin, 1991).

As a fresh cheese, in the U.S., queso fresco is required to be made from pasteurized milk (CFR, 2011). During pasteurization, at least 5-log reduction of the pathogen *Coxiella burnetii* is achieved to ensure the elimination of pathogens in milk (Cerf & Condron, 2006). Ideally, with proper pasteurization and good manufacturing practices, there should be no pathogen contamination in queso fresco. However, there is still a risk of post-pasteurization contamination during queso fresco production. As an ideal substrate, after being contaminated, queso fresco allows the survival and proliferation of spoilage and pathogenic microorganisms during storage and distribution thus leading to potential foodborne illness. To eliminate post-manufacturing contaminants, efforts have been taken on identifying a potent method/technology to eliminate pathogens at the same time retaining the quality of the cheese. Thus, non-thermal technologies or methods have been evaluated, such as high pressure processing (Hnosko et al., 2012; Tomasula et al., 2014) and application of antimicrobial agents (e.g. nisin, bacteriophage, etc.) (Gadotti et al., 2014; Soni et al., 2012). However, consumers are seeking a clean label which would not include the addition of antimicrobial agents (Asioli et al., 2017). High pressure processing (HPP) showed efficient inactivation of *Listeria innocua* in queso fresco, achieving greater than 5 $\log_{10}$ CFU/g at 500, 550, or 600 MPa for up to 15 minutes, but led to inferior quality of QFC (Hnosko et al., 2012).

Cold plasma is a relatively new non-thermal technology considered for application in food processing. It has been used in industrial processes such as electronics cleaning, bonding of plastics or binding of dye to textile fibers, but its potential remains untapped in the food

industry. Plasma consists of highly reactive gas species including electrons, photons, positive and negative ions, free radicals, metastable molecules and atoms in a net neutral charge (Misra, Moiseev, et al., 2014). These reactive gas species, such as O_3, $^1O_2$, NO, have demonstrated effective bactericidal effects (Han et al., 2016; Liao et al., 2017). Atmospheric cold plasma (ACP) is in particular attractive for commercial application in food industries due to its adaptability to high-speed manufacturing and capability of implementation under normal operating conditions (Misra, Keener, et al., 2014). High voltage atmospheric cold plasma (HVACP) is an advanced ACP, which utilizes high voltage of 30 kV or more to create plasma in a few seconds to a few minutes, allowing more efficient bacterial inactivation (Xu et al., 2017; Ziuzina et al., 2014).

In a previous study (Wan et al., 2019), a 1.6 $\log_{10}$ CFU/g *Listeria* reduction was observed in queso fresco after five minutes direct HVACP treatment of a 4*2*1 cm^3 cheese block at 100 kV, followed by 24-hour refrigerated storage. To enhance inactivation efficiency of HVACP, surface area of the cheese was increased to allow larger exposure area for reactive gas species to react during HVACP treatment. Thus, crumbled queso fresco was employed for this study. In this study *Listeria innocua* was selected as a non-pathogenic surrogate for LM (Friedly et al., 2008; Hnosko et al., 2012). Crumbled queso fresco was inoculated with LI and then treated with a direct HVACP treatment at 100 kV for up to 10 minutes in air and modified gas MA50 (50% CO_2 and 50% N_2). Two gas blends with two different relative humidity were utilized to examine the effect of gas composition and relative humidity on *Listeria* inactivation in queso fresco by HVACP treatment. pH, moisture content and lipid oxidation of queso fresco was also assessed to perceive the effect of plasma on cheese quality.

2. Materials and Methods

2.1 Bacterial strain and inoculum preparation

The strain of *Listeria innocua* (ATCC® 33090™) was obtained from the microbiology stock culture of the Department of Food Science and Human Nutrition, Iowa State University. The stock cultures were stored with 50% glycerol in a – 80 °C freezer. To prepare the fresh working culture, 0.1 ml frozen culture was inoculated in 50 ml Brain Heart Infusion (BHI) broth and incubated at 37 °C for 24 h. The culture was spread plated on Tryptic soy agar (TSA) and incubated at 37 °C for 48 h. After incubation, one isolated colony on TSA was inoculated into 50 ml BHI broth and was grown under shaking at 160 rpm on an orbital shaker at 37 °C. After 18 h of incubation, bacteria cells were harvested by centrifugation at 10,000 rpm for 10 min at 4 °C, and washed twice in sterile phosphate buffered solution (PBS). Lastly, the washed cells were suspended in PBS to a final cell concentration of approximately 8 $\log_{10}$ CFU/ml, and was used as the working inoculum. The concentration of the working inoculum was confirmed by plating serial dilutions on TSA, followed by incubation at 37 °C for 48 h.

2.2 Sample preparation and inoculation

For this study, queso fresco cheese (QFC) was purchased from a local grocery store. 10 g QFC was placed in a sterile Stomacher bag and crumbled by the Stomacher at 230 rpm for 15 s followed by another 15 s after inoculation of *Listeria innocua* inoculum resulting in approximately 6 $\log_{10}$ CFU/g. Inoculated QFC samples were allowed to dry under refrigeration temperature for 1 h to allow cell attachment.

2.3 HVACP treatment

A schematic diagram of the plasma set-up used in this study is presented in Figure 4.1. 10 g inoculated crumbled QFC sample was placed inside a polypropylene ArtBin® box (168 x 121 x 28 mm). The box was then pillow sealed with a high barrier film leaving an opening for gas flushing. The distance between two circular aluminum electrodes (outer diameter = 152 mm) was 28 mm which was the height of the polypropylene box.

The pillow pack (box in bag) as a whole was flushed with dry air, moist air, dry MA50 (50% CO_2, 50% N_2) or moist MA50 and was then completely sealed by an impulse sealer. For humidification, dry air or dry MA50 gas went through a bubbling system (1 L bottle) containing 500 ml deionized water. The sealed pack samples were treated under direct mode of exposure 100 kV for up to ten minutes. Control samples were also packed under the same condition but without HVACP treatment. Treated and control samples were stored at 4 °C for 24 h prior to microbial recovery and quality analysis.

2.4 Media preparation and enumeration

To account for the injured cells, *Listeria* selective agar (Oxoid CM856) and thin agar layer (TAL) method was employed for microbial enumeration. Thin agar layer method is used to recover injured *Listeria* cells as well as differentiate the target species from mixed population. A thin layer of Tryptic soy agar (TSA) was laid on top of *Listeria* selective agar. In TAL method, sample aliquot was plated on top of the TSA layer, which allowed injured bacteria cells to recover. During incubation, selective agents diffused from the selective agar layer to the top thin TSA layer and allowed for selective growth of *Listeria innocua* (Kang & Fung, 1999; Wu et al., 2001).

After 24 h storage at 4 °C, HVACP treated or control QFC sample was removed from the package and placed into a sterile filtered Stomacher bag with the addition of 90 ml sterile 2% sodium citrate solution, and was then stomached at 230 rpm for 45 s. After stomaching, the resulting suspension was serial diluted with 9 ml 0.1% peptone water. For each dilution, aliquots were spread plated on by TAL method and *Listeria* selective agar. Plates were incubated at 37 °C for 48 h before counting. The number of injured cells were calculated by the differences between recovered cells on these two media (*Listeria* selective and TAL). The limit of detection for bacteria recovery on the samples was 1.0 $\log_{10}$ CFU/g.

2.5 pH and moisture content measurement

To measure the pH of treated and control QFC, a 1.0 g sample was homogenized with 9.0 ml HPLC grade water for 30 seconds at 16,000 rpm (Lee et al., 2012). The pH of the sample was measured by Orion Dual Star pH/SE meter. The pH meter was calibrated with the standard buffer solutions pH 4.00, 7.00, and 10.00 at room temperature prior to measurements. The measurement of moisture content followed AOAC method 948.12 (AOAC, 1995), in which 3 g QFC sample was weighed and oven dried at 102 °C.

2.6 Lipid peroxidation

Lipid peroxidation test is performed based upon the method modified from the protocol described in Jung et al. (2015). In brief, 3 g of QFC sample was homogenized with 9 ml HPLC grade water and 50 µl 7.2% BHT in ethanol for 30 s at 16,000 rpm. The homogenate was centrifuged at 10,000 g for 15 min at 4 °C. The supernatants were then used for malondialdehyde (MDA) measurement. 2.0 ml of 20 mM thiobarbituric acid (TBA) in 15% trichloroacetic (TCA) reagent was added into 1.0 ml supernatants in a 15 ml tube. After mixing, the tubes were incubated in a 90 °C water bath for 30 min. After 30 min, the tubes

were cooled in ice for 10 min. After cooling, the test tubes were centrifuged at 3,000 rpm for 10 min at 4 °C and were allowed to stable at room temperature for 30 min prior to absorbance spectroscopy measurement at 532 nm.

2.7 Optical emission spectroscopy

Optical emission spectroscopy was employed to characterize reactive gas species generated inside the package during HVACP treatment. The sealed packages with QFC samples were used for optical emission spectra collection. Ocean Optics spectrometer (Ocean Optics, Inc. Florida, USA) was used to record the emission spectra of plasma generated during treatment of QFC by HVACP. An Ocean Optics optical fiber, with a core diameter of 1000 µm, was utilized to deliver the light from the plasma. In order to maximize the light captured from the plasma entering the optical fiber, collimating lenses with 5 mm diameter was adopted. The collimating lenses was placed 15 cm away from the edge of the box containing sample. The emission spectra were collected and saved every 30 seconds.

2.8 Statistical analysis

Statistical analysis was performed by JMP statistical package (SAS Institute Inc., Cary, NC, USA). Analysis of variance (ANOVA) was used and the data was represented as mean value ± standard deviation. Tukey's multiple sample comparison tests was done to deliver the significance of difference between treatments. Statistical significance was indicated at a confidence level of 95% ($p \leq 0.05$).

3. Results and Discussion

3.1 Optical emission spectroscopy

The optical emission spectra of the dry air, moist air, dry MA50 and moist MA50 at 100 kV are presented in Figure 4.2. Under all the four gas environments, the majority of the peaks

were observed near UV region (300 - 400 nm) which represent mostly excited nitrogen species including nitrogen second positive system (SPS) N_2(C-B) and first negative system N_2^+(B-X) (Misra, Pankaj, et al., 2014; Sarangapani et al., 2016). At 720 nm and 740 nm wavelength, two peaks from excited molecular oxygen were observed in MA50 plasma (Ingvar, 1990). The peaks from excited nitrogen species showed higher emission intensity in dry air and moist air compared to dry MA50 and moist MA50. This was due to the higher N_2 content in dry air and moist air as compared to the MA50 gas blend. With higher N_2 and the presence of oxygen in air, excited oxygen species were not captured by the optical emission spectroscopy, which is likely due to the quenching effect of the excited oxygen in air plasma (Walsh et al., 2010). Atomic oxygen can undergo self-recombination or react with ozone to form molecular oxygen (Fridman, 2008):

$$O + O + M \rightarrow O_2 + M \quad (1)$$

$$O + O_3 \rightarrow O_2 + O_2 \quad (2)$$

Moreover, excited atomic or molecular oxygen can further react with excited nitrogen for the synthesis of N_xO_y (e.g. NO, NO_2) species (Fridman, 2008):

$$N + O_2 \rightarrow NO + O \quad (3)$$

$$O + N_2 \rightarrow NO + N \quad (4)$$

$$NO + O + M \rightarrow NO_2 + M, \text{ where } M = N_2/O_2/NO/NO_2 \quad (5)$$

These N_xO_y species are found to be highly toxic, resulting in DNA damage of the cell thus leading to cell death (Davies et al., 2011). Lastly, as shown in Figure 4.2, no plasma emission peaks were observed under 300 nm of the UV region. Hence, the antibacterial action of the HVACP was considered to be dominated by the reactive oxygen and nitrogen species, with minimal effect from UV radiation (Laroussi, 2002).

3.2 *Listeria innocua* (LI) inactivation

Figures 4.3 and 4.4 provide the data for LI reductions after HVACP treatment at 100 kV in dry air (<5% RH) and moist air (99.9% RH) recovered on *Listeria* selective agar (LSA) and by thin agar layer method (TAL), respectively. Thin agar layer method is used to differentiate LI cells from background microorganisms, by allowing the growth of injured LI cells which cannot be recovered on LSA (Kang & Fung, 1999). Increasing in treatment was found to significantly ($p<0.05$) increase the efficiency of air plasma treatment on LI inactivation. Based upon the recovery on LSA, after 4, 6, 8, and 10 minutes of HVACP treatment at 100 kV in dry air, a 0.8 $\log_{10}$ CFU/g, 2.6 $\log_{10}$ CFU/g, 3.5 $\log_{10}$ CFU/g, and 4.9 $\log_{10}$ CFU/g LI reduction were noted respectively. The D-value for LI was found to be 1.99 min and 2.32 min at 100 kV in dry air and moist air, respectively. A linear regression was created to estimate the LI population under different treatment time at 100 kV in dry air (6) and moist air (7), in which LI population decreases with increasing treatment time {T: treatment time (> 0 min)}. Table SI 4.1 and 4.2 represent the parameter estimates for linear regression model in dry air and moist air, respectively.

$$\text{LI population } (\log_{10} \text{CFU/g}) = -0.47*T + 6.25 \quad (R^2=0.92) \tag{6}$$

$$\text{LI population } (\log_{10} \text{CFU/g}) = -0.41*T + 6.24 \quad (R^2=0.87) \tag{7}$$

For moist air HVACP treatment, after 4, 6, 8, and 10 min treatment, a reduction of 0.8 $\log_{10}$ CFU/g, 1.9 $\log_{10}$ CFU/g, 2.9 $\log_{10}$ CFU/g, and 4.7 $\log_{10}$ CFU/g, respectively were observed via recovery on LSA. The results demonstrate that after six and eight minutes HVACP treatment, dry air plasma led to significantly higher LI reduction compared to moist air plasma ($p<0.05$). After six minutes of treatment, a 2.6 $\log_{10}$ CFU/g LI reduction was

achieved in dry air, whereas a 1.9 $\log_{10}$ CFU/g LI reduction was recorded in moist air. Moreover, ten minutes of HVACP treatment in moist air resulted a 1.0 $\log_{10}$ CFU/g injured LI cells compared to 0.4 $\log_{10}$ CFU/g injured LI cells in dry air treatment. This result does not concur with the previous study in which authors have observed an enhanced inactivation of *Bacillus atrophaeus* spores under increased relative humidity by HVACP treatment (Patil et al., 2014). Likely, for the inactivation of LI in QFC, the plasma ionization of water vapor leads to peroxides formation which are less effective than ROS generated in dry air. Results of LI reductions after dry MA50 and moist MA50 HVACP treatment recovered on LSA and via TAL method are presented in Figure 4.5 and 4.6, respectively. Ten minutes of HVACP treatment at 100 kV was found to result in 1.7 $\log_{10}$ CFU/g and 1.0 $\log_{10}$ CFU/g LI reduction, as recovered on LSA, in dry MA50 (<5% RH) and moist MA50 (99.9% RH), respectively. The D-value for LI was found to be 6.31 min and 10.07 min at 100 kV in dry MA50 and moist MA50, respectively. A polynomial regression was utilized to estimate the LI population under different treatment time at 100 kV in dry MA50 (8), while a linear regression was used to estimate the LI population under different treatment time at 100 kV in moist MA50 (9). Similar to air treatment, LI population decreases with increasing treatment time in MA50 HVACP treatment {T: treatment time (> 0 min)}. Parameter estimates for regression models in dry MA50 and moist MA50 are presented in Table SI 4.3 and 4.4, respectively.

$$\text{LI population } (\log_{10} \text{CFU/g}) = 6.60 - 0.21*T - 0.03*(T-4.86)^2 \quad (R^2=0.90) \tag{8}$$

$$\text{LI population } (\log_{10} \text{CFU/g}) = -0.10*T + 5.88 \quad (R^2=0.87) \tag{9}$$

Dry air and moist air HVACP treatment showed a significantly higher LI reduction in crumbled QFC than dry MA50 and moist MA50 treatment, which is likely due to the

presence of oxygen and higher nitrogen content that results in higher production of ROS and RNS. ROS and RNS generated during HVACP treatment (e.g. O, O_2*, O_3, $OH^{\bullet}$, NO, NO_2, NO_3, N_2O_4) can lead to an intensive oxidative stress to cell membrane as well as intracellular components (e.g. DNA and protein), thus resulting in lethality of the cell (Liao et al., 2017). Cell membrane lipid, which contains mostly unsaturated fatty acid, is particularly vulnerable to ROS (e.g. $OH^{\bullet}$ and $^1O_2$) attack leading to the formation of lipid hydroperoxide (ROOH) (Abramzon et al., 2006; Gavahian et al., 2018), thus resulting in cell membrane damage. Moreover, N_xO_y can further lead to the formation of HNO_x which also may have a significant role in microbial inactivation (Gentile & Kushner, 1995; Moiseev et al., 2014). Overall, the difference between the efficacy of moist MA50 and dry MA50 plasma treatment was not considerably different.

3.3 Lipid peroxidation

Table 4.1 summarizes the measurement for malondialdehyde (MDA), moisture content and pH for HVACP treatments of crumbled commercial QFC samples in air (dry and moist). Under HVACP treatments, both dry and moist air led to the formation of MDA in crumbled QFC samples. For comparison, 2.00 mg/kg MDA was used as the benchmark, which is the acceptable limit of MDA for fresh meat (Greene & Cumuze, 1982; Sánchez-Escalante et al., 2003). HVACP treatment for up to ten minutes in moist air showed no significant changes in MDA (an average of 2.14 mg/kg) in treated QFC samples. However, ten minutes HVACP treatment in dry air resulted in significantly ($p<0.05$) higher MDA (2.79 mg/kg) compared to samples treated for eight minutes or less. Furthermore, an increase in the relative humidity of gases reduced the amount of MDA formed in cheese ($p<0.05$), in which dry air induced the most MDA formation followed by moist air, dry MA50 and moist MA50. Reduced lipid

oxidation under increased relative humidity is likely due to the dilution effect of reactive gas species under high relative humidity. Table 4.2 shows the measurement for malondialdehyde (MDA), moisture content and pH for MA50 (dry and moist) HVACP treatment on crumbled QFC samples. MA50 Plasma led to the formation of MDA in QFC, but the amount of MDA produced was below 2.00 mg/kg. Lower MDA formation in MA50 plasma compared to air plasma likely resulted from lower concentration of reactive oxygen species (ROS) which would result in oxidation of lipids in QFC (Gavahian et al., 2018). Similar results were observed by Kim et al. (2013) in which higher lipid oxidation of pork loin was found using a gas mixture of helium and 0.3% O_2 compared to 99.999% helium gas.

3.4 pH and moisture measurement

HVACP treatment for up to ten minutes had minimal impact on moisture content and pH. The minimal impact of pH on QFC was attributed to the buffering capacity of cheese proteins (Olthuis et al., 1994; Salaün et al., 2005). After ten minutes of treatment, both dry air and moist air treatment led to insignificant effect on moisture content of QFC ($p<0.05$). Although the insignificance on moisture content was observed, treatment times beyond six minutes were found to result in surface hardening and texture degradation of QFC. Figure 4.7 shows the representative images for samples treated in dry air for 0 (control), 6 (slight surface hardening), 8 minutes (surface hardening and texture degradation) and 10 minutes (severe surface hardening and texture degradation). In air, pH for control samples ranged between 5.7 and 5.8, whereas for plasma treated samples, pH ranged between 5.3 and 5.5. The slight decrease of pH after air plasma treatment is likely due to the formation of nitric acid (HNO_3) and nitrous acid (HNO_2) by reaction with NO and NO_2 (Misra et al., 2015). The reactions can be summarized as follows:

$$2NO + O_2 \rightarrow 2NO_2$$

$$H_2O + e \rightarrow OH + H + e$$

$$NO_2 + OH \rightarrow HNO_3$$

$$NO + OH \rightarrow HNO_2$$

The diffusion and solubilization of these reactive gas species, resulting in the formation of acids at very low concentrations, was likely to cause the decrease of pH. However, it should be noted here that this increase in acidity after air plasma treatment is unlikely to inactivate *Listeria innocua*, which can survive at a pH of 4.3 (Farber & Peterkin, 1991; Le Marc et al., 2002).

Ten minutes of HVACP treatment in MA50 was found to have minimal impacts on moisture loss and pH as well. After ten minutes of treatment, no significant effect in moisture content was observed under moist MA50 plasma ($p<0.05$), while negligible effect was found under dry MA50 treatment, in which a 48.4% moisture content was observed in dry MA50 treated samples, while 49.6% for untreated samples in dry MA50. However, similar as in air plasma treatment, eight and ten minutes of HVACP treatment in MA50 gas blend resulted in surface hardening and texture degradation. In MA50, the pH for control samples and plasma treated samples ranged between 5.6 and 5.7. This relatively smaller change of pH after MA50 treatment compared to air is most likely resulted from the lower reactive oxygen species which are responsible for the formation of nitric acid and nitrous acid leading to a decrease of pH.

4. Conclusion

This study has demonstrated the significance of dry air HVACP treatment in *Listeria innocua* inactivation for queso fresco. Increasing treatment time and the presence of oxygen in the gas blend resulted in a more efficient inactivation of *Listeria innocua.* A 0.8 $\log_{10}$ CFU/g and 2.6 $\log_{10}$ CFU/g LI reduction was observed after four and six minutes dry air HVACP treatment, respectively, while retains acceptable quality of the cheese. The higher inactivation efficacy of air plasma compared to MA50 is likely due to the increased reactive oxygen species (ROS) and reactive nitrogen species (RNS) which lead to oxidation of cell membrane and intracellular components resulting in cell deatah. Minimal changes were observed in the pH and moisture content of queso fresco after HVACP treatment. As expected, higher lipid oxidation was observed under air plasma due to the higher amount of reactive oxygen species. Even though, longer treatment time resulted in higher *Listeria* inactivation, surface hardening and texture degradation was observed after 8 and 10 min treatments. Thus, in the next phase of this project, queso fresco was treated for 4 min and 6 min in dry air at 100 kV for evaluation of the microbial population and quality of plasma treated cheese over an extended refrigerated storage. Overall, this study has demonstrated the potential of HVACP for application in fresh cheese processing to prevent post-manufacturing contamination.

Acknowledgement

Research funding support provided by the National Dairy Council and Innovation Center for US Dairy.

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Table 4.1. Lipid peroxidation (MDA), moisture content and pH measurement of crumbled QFC treated with HVACP in direct mode of exposure from 0 to 10 minutes treatment in dry air and moist air after 24-hour storage at 4 °C. Same small letter beside each measurement indicated no significant (p>0.05) different within the same treatment gas.

Gas	Treatment time (min)	MDA (mg/kg)	Moisture content % (wet basis)	pH
Dry air	0	0.48^{a}	49.2^{a}	5.7^{a}
Dry air	4	2.03^{b}	49.6^{a}	5.5^{b}
Dry air	6	2.30^{b}	49.1^{a}	5.5^{bc}
Dry air	8	2.27^{b}	48.2^{a}	5.4^{c}
Dry air	10	2.79^{c}	48.0^{a}	5.3^{d}
Moist air	0	0.48^{a}	49.8^{a}	5.8^{a}
Moist air	4	2.25^{b}	49.6^{a}	5.4^{b}
Moist air	6	1.97^{b}	49.4^{a}	5.4^{bc}
Moist air	8	2.18^{b}	48.8^{a}	5.4^{bc}
Moist air	10	2.15^{b}	48.9^{a}	5.4^{c}

Table 4.2. Lipid peroxidation (MDA), moisture content and pH measurement of crumbled QFC treated with HVACP in direct mode of exposure from 0 to 10 minutes treatment in dry MA50 and moist MA50 after 24-hour storage at 4 °C. Same small letter beside each measurement indicated no significant (p>0.05) different within the same treatment gas.

Gas	Treatment time (min)	MDA (mg/kg)	Moisture content % (wet basis)	pH
Dry MA50	0	0.54^{a}	49.6^{a}	5.7^{a}
Dry MA50	4	1.09^{b}	49.7^{a}	5.7^{ab}
Dry MA50	6	1.01^{b}	48.9^{ab}	5.6^{b}
Dry MA50	8	1.15^{b}	48.7^{ab}	5.6^{b}
Dry MA50	10	1.03^{b}	48.4^{b}	5.7^{ab}
Moist MA50	0	0.39^{a}	50.2^{a}	5.6^{a}
Moist MA50	4	0.65^{b}	50.7^{a}	5.7^{a}
Moist MA50	6	0.54^{b}	50.3^{a}	5.6^{a}
Moist MA50	8	0.61^{b}	50.1^{a}	5.7^{a}
Moist MA50	10	0.59^{b}	49.5^{a}	5.7^{a}

Table SI 4.1. Parameter estimates for regression model estimating LI population under different treatment time in dry air at 100 kV

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	6.25	0.20	30.67	<.0001*
Treatment time (min)	-0.47	0.035	-13.34	<.0001*

Table SI 4.2. Parameter estimates for regression model estimating LI population under different treatment time in moist air at 100 kV

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	6.24	0.25	25.37	<.0001*
Treatment time (min)	-0.41	0.041	-10.05	<.0001*

Table SI 4.3. Parameter estimates for regression model estimating LI population under different treatment time in dry MA50 at 100 kV

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	6.60	0.166	39.75	<.0001*
Treatment time (min)	-0.21	0.021	-9.84	<.0001*
(Treatment time (min)-4.86)^2	-0.03	0.007	-4.27	0.0013*

Table SI 4.4. Parameter estimates for regression model estimating LI population under different treatment time in moist MA50 at 100 kV

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	5.88	0.06	95.63	<.0001*
Treatment time (min)	-0.10	0.011	-9.60	<.0001*

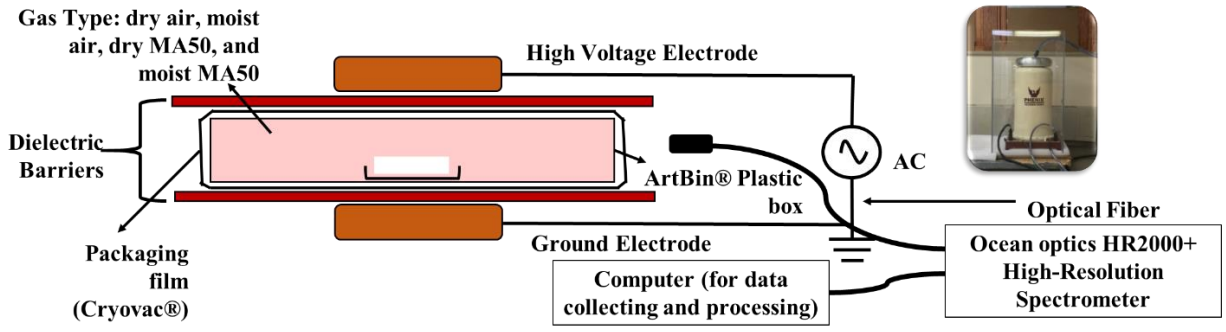

Figure 4.1. Schematic of plasma set-up

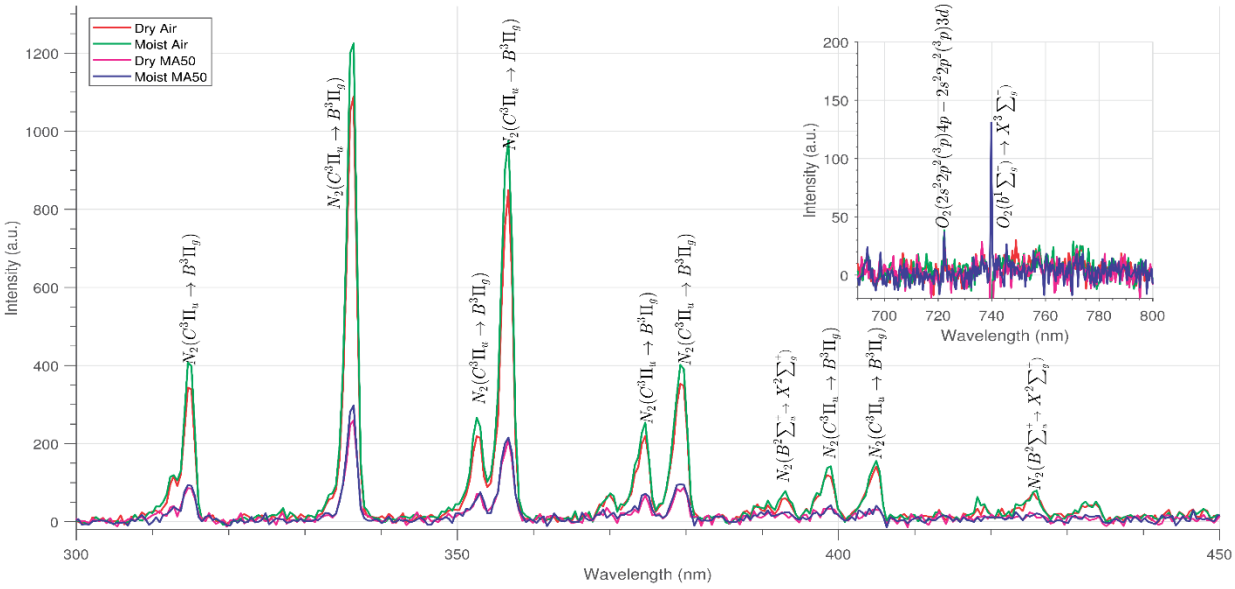

Figure 4.2. Optical emission spectra of direct HVACP treatment of QFC in dry air, moist air, dry MA50 and moist MA50 at 100 kV

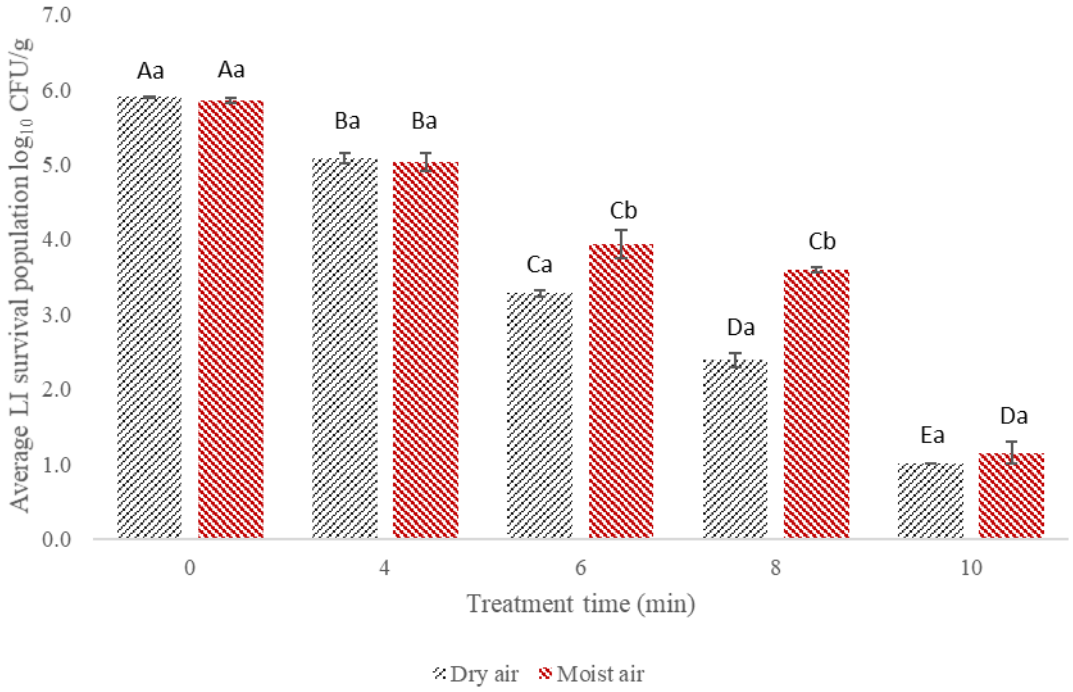

Figure 4.3. *Listeria innocua* population (log_{10} CFU/g) for 10.0 g crumbled queso fresco cheese (QFC) treated with HVACP in dry air and moist air at 100 kV at 0 (control), 4, 6, 8 and 10 minutes treatment after 24-hour storage at 4 °C recovered on *Listeria* selective agar (LSA). The detection limit of the applied enumeration method was 1.0 log_{10} CFU/g. Same capital letter on top of each bar indicated no significant difference among each bar in the same gas blend. Same small letter on top of each bar indicated no significant difference among the same treatment time. ($p<0.05$).

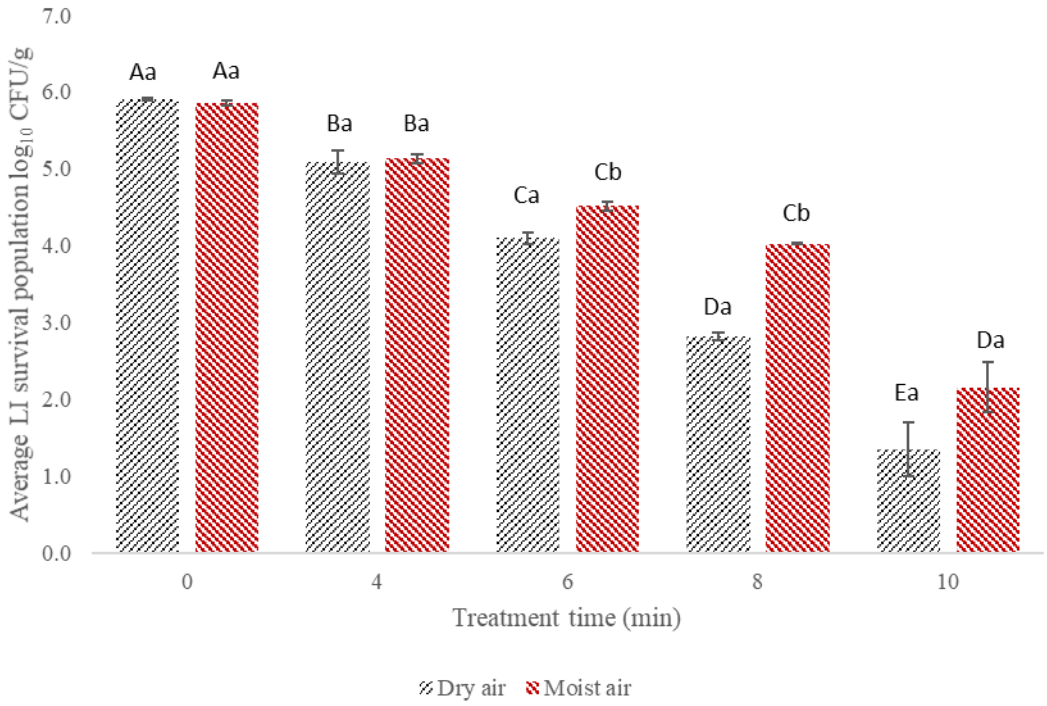

Figure 4.4. *Listeria innocua* population (log_{10} CFU/g) for 10.0 g crumbled queso fresco cheese (QFC) treated with HVACP (Direct) in dry air and moist air at 100 kV at 0 (control), 4, 6, 8 and 10 minutes treatment after 24-hour storage at 4 °C recovered using thin agar layer method (TAL). The detection limit of the applied enumeration method was 1.0 log_{10} CFU/g. Same capital letter on top of each bar indicated no significant difference among each bar in the same gas blend. Same small letter on top of each bar indicated no significant difference among the same treatment time. ($p<0.05$).

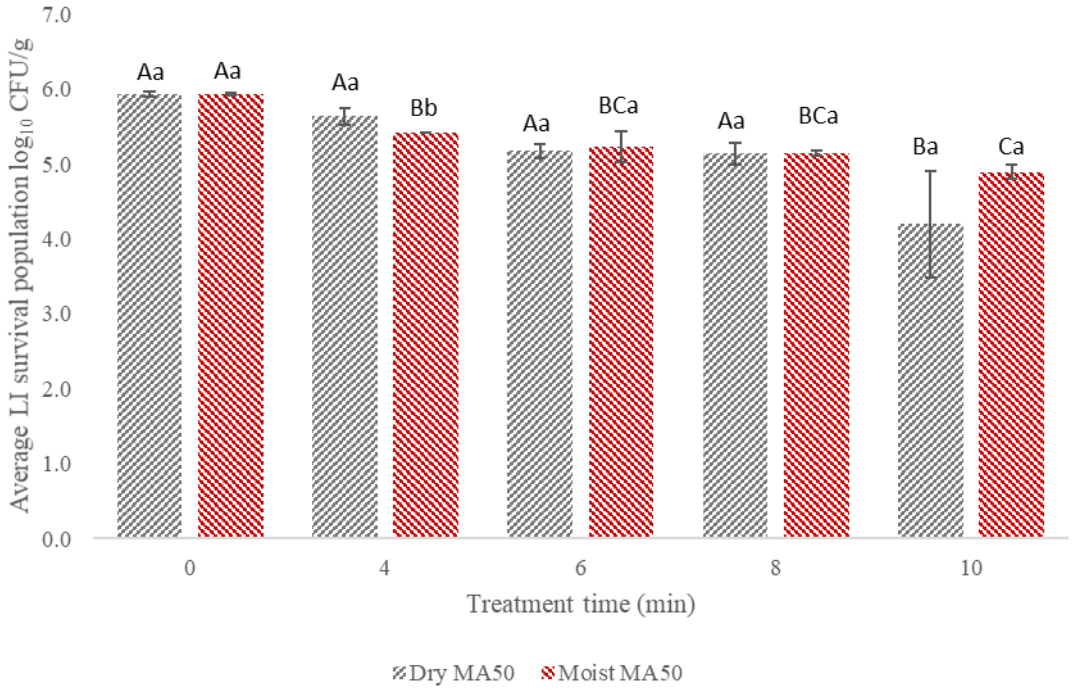

Figure 4.5. *Listeria innocua* population (log_{10} CFU/g) for 10.0 g crumbled queso fresco cheese (QFC) treated with HVACP (Direct) in dry MA50 and moist MA50 at 100 kV at 0 (control), 4, 6, 8 and 10 minutes treatment after 24-hour storage at 4 °C recovered on *Listeria* selective agar (LSA). The detection limit of the applied enumeration method was 1.0 log_{10} CFU/g. Same capital letter on top of each bar indicated no significant difference among each bar in the same gas blend. Same small letter on top of each bar indicated no significant difference among the same treatment time. ($p<0.05$).

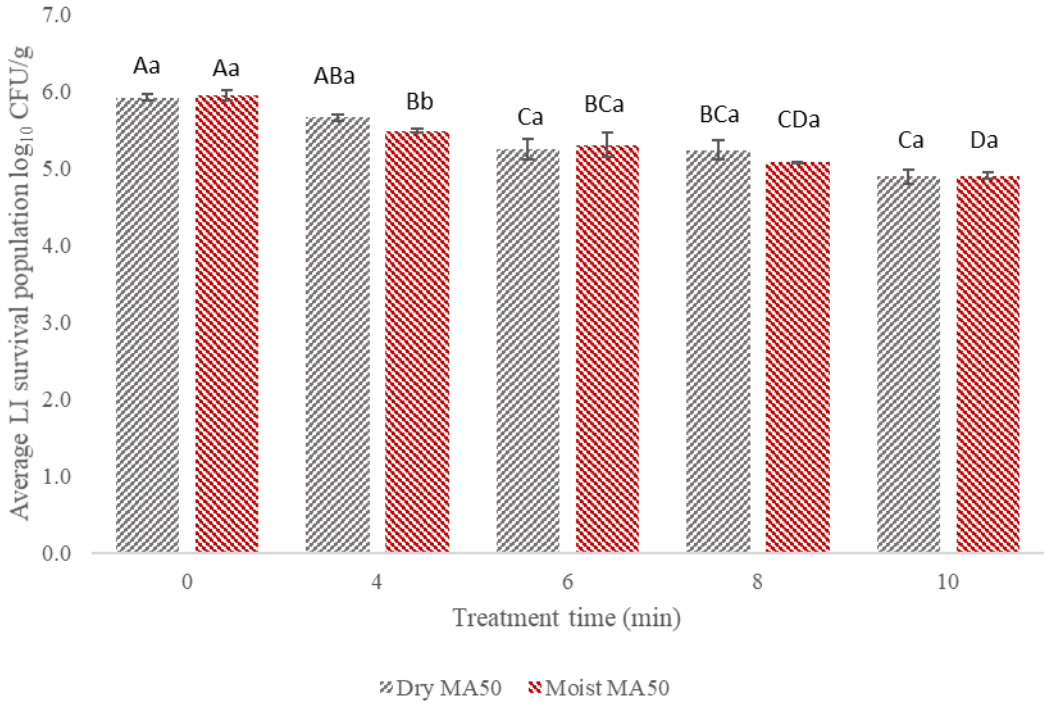

Figure 4.6. *Listeria innocua* population (log_{10} CFU/g) for 10.0 g crumbled queso fresco cheese (QFC) treated with HVACP (Direct) in dry MA50 and moist MA50 at 100 kV at 0 (control), 4, 6, 8 and 10 minutes treatment after 24-hour storage at 4 °C recovered using thin agar layer method (TAL). The detection limit of the applied enumeration method was 1.0 log_{10} CFU/g. Same capital letter on top of each bar indicated no significant difference among each bar in the same gas blend. Same small letter on top of each bar indicated no significant difference among the same treatment time. ($p<0.05$).

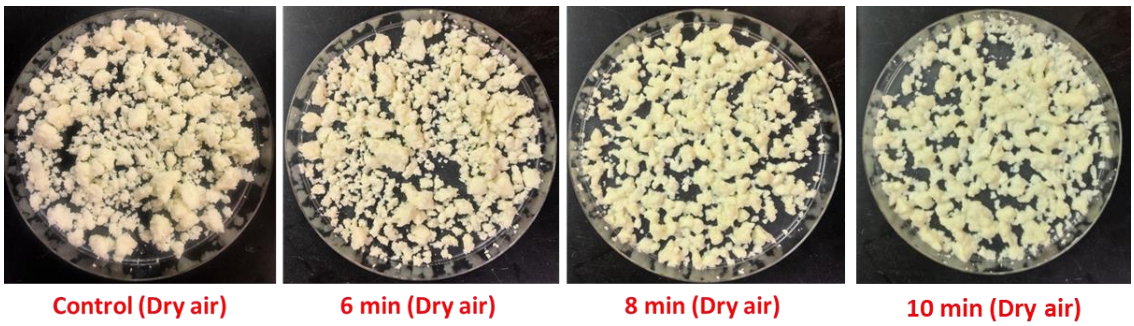

Figure 4.7. Representative images of 10.0 g crumbled queso fresco treated with HVACP (direct) in dry air at 100 kV at 0 (control), 6, 8 and 10 minutes after 24-hour storage at 4°C.

CHAPTER 5: HIGH VOLTAGE ATMOSPHERIC COLD PLASMA INACTIVATION OF *LISTERIA INNOCUA* ON QUESO FRESCO CHEESE: PART 2, EVALUATION OF MICROBIAL POPULATION AND QUALITY OF PLASMA TREATED CHEESE OVER 28 DAYS OF STORAGE

A manuscript prepared for submission to Innovative food science and emerging technologies

Zifan Wan[a], NN Misra[b], Kevin M. Keener[a,b]*

[a] Food Science and Human Nutrition Department

[b] Center for Crops Utilization Research

*Corresponding Author

Iowa State University

Abstract

High voltage atmospheric cold plasma (HVACP), a novel non-thermal technology, has demonstrated significance in microbial inactivation with negligible effect on product quality. This study evaluated the capability of HVACP treatment in inhibition of *Listeria innocua* and background microflora in queso fresco cheese with an extended 28-day storage under refrigerated condition. At the end of the storage period, a 1.4 $\log_{10}$ CFU/g and 1.0 $\log_{10}$ CFU/g *Listeria innocua* population was observed after four and six minutes treatment, respectively, while around 7.4 $\log_{10}$ CFU/g *Listeria* population was found in untreated inoculated samples. Similarly, an inhibition of background microflora growth was also observed after direct HVACP treatment. Around 1.0 $\log_{10}$ CFU/g survival of mesophilic, psychrotrophic and enterobacteriaceae population was found in treated cheese after 28 days of storage, while greater than 6.0 $\log_{10}$ CFU/g was noted in untreated samples. After HVACP treatment, a slight decrease in pH and increase lipid oxidation was observed due to the interaction with reactive gas species. Moreover, during storage, decreasing of moisture was found with increasing storage period. A maximum of 7.19 % loss in samples after six minutes HVACP treatment was seen, whereas a

maximum of 6.72 % loss was observed in control samples. This study demonstrates the capability of HVACP for application in pathogen control in fresh cheese like queso fresco and it may provide extended shelf-life for perishable products.

Highlights

- HVACP treatment inhibited the growth of *Listeria innocua* and background microflora over 28 days of storage at 4 °C.
- HVACP treatment resulted in slight changes in pH and lipid oxidation of queso fresco.
- In-package HVACP treatment allows extended exposure of reactive gas species with queso fresco.

Key words

Atmospheric cold plasma, cheese, shelf-life

1. Introduction

Queso fresco cheese (QFC) is a type of Mexican style fresh cheeses which is common part of the Hispanic diet and is becoming more popular in the U.S. market with the increasing Hispanic population (U.S. Census Bureau, 2015). As a fresh cheese, queso fresco has a high moisture (typically ranging from 46% to 57%), low salt (1% to 3%) and near neutral pH (Hwang & Gunasekaran, 2001). Traditionally, queso fresco is made from raw milk, which results in a high risk of pathogens including *Listeria monocytogenes* and *Salmonella typhimurium* leading to concerns for foodborne illness (Altekruse et al., 1998; MacDonald et al., 2005; Villar et al., 1999). Thus, in the United States, regulations require the use of pasteurized milk for industrial manufacture of queso fresco to ensure the safety of this product (CFR, 2011). However, even though pasteurization of milk prior to cheese making

can effectively eliminate pathogens from milk, there is still a risk of contamination post pasteurization during the production of fresh cheese. Thus, there is a critical need for a technology to remove post-pasteurization pathogenic contaminants from fresh cheese without compromising cheeses' quality.

High voltage atmospheric cold plasma (HVACP) is a novel non-thermal technology, which show potential for elimination of pathogens and reduction of spoilage microorganisms in queso fresco post pasteurization. Plasma is an excited gas consisting of electrons, photons, photons, positive and negative ions, free radicals, metastable molecules and atoms in a net of neutral charge (Sarangapani et al., 2016), while these reactive gas species such as O_3, $^1O_2$, NO, are recognized as the key for microbial inactivation by cold plasma treatment (Han et al., 2016; Liao et al., 2017). Research has demonstrated the effectiveness of cold plasma treatment in microbial inactivation while maintaining products' quality (Pankaj et al., 2018; Pignata et al., 2017). As an advanced cold plasma treatment, in-package HVACP utilizes voltage greater than 30 kV allowing the generation of reactive gas species inside the package in a short time and enabling the feasibility for industrial scale-up (Misra, Patil, et al., 2014; Ziuzina et al., 2016). In the previous study, direct HVACP has exhibited the efficiency in inactivation of *Listeria innocua*, a non-pathogenic surrogate for *Listeria monocytogenes* (Hnosko et al., 2012; Noriega et al., 2011), in queso fresco 24-h post treatment (Wan et al., 2019). However, it is unknown that HVACP would be able to result in continuous inhibition of *Listeria* on queso fresco over an extended refrigerated storage. In Part 1 of this work, the optimized treatment conditions were identified as four and six min direct HVACP treatment in dry air at 100 kV, which led to significant *Listeria innocua* inactivation without quality degradation of the cheese. Thus, in this study, queso fresco was treated for four and six min

in dry air plasma followed by a 28 days refrigerated storage and *Listeria innocua* population was examined during the 28 days for treated and control QFC samples.

Besides the effectiveness in pathogen control, HVACP has demonstrated the ability to inactivate spoilage microorganisms, resulting in extended shelf-life of fresh produces such as strawberry (Misra, Moiseev, et al., 2014; Misra, Patil, et al., 2014). As a fresh cheese, queso fresco has a limited shelf-life and the primary limiting factor for shelf-life of fresh cheese is microbial spoilage rather than chemical deteriorations (e.g. oxidative deterioration) (Mortensen et al., 2010; Tunick, 2006). Hence, in this study, plasma treated queso fresco samples were stored for up to 28 days under refrigerated condition along with the control untreated samples. Background microflora were evaluated during the 28 days of storage along with quality measurement including pH, moisture and lipid oxidation. The successful inhibition of background microflora and *Listeria* by HVACP treatment would allow the application of HVACP in elimination of post-manufacture pathogen contamination and extending the shelf-life of fresh cheese, such as queso fresco.

2. Materials and Methods

2.1 Bacterial strain and inoculum preparation

Listeria innocua (ATCC® 33090™) strain was obtained from the microbiology stock culture of the Department of Food Science and Human Nutrition, Iowa State University. Stock cultures were stored with 50% glycerol at – 80 °C. Fresh working culture was prepared by inoculating 0.1 ml of the stock culture in 50 ml Brain Heart Infusion (BHI) broth and incubated at 37 °C for 24 h. The culture was then spread plated on Tryptic soy agar (TSA) and incubated at 37 °C for 48 h. After incubation, one isolated *Listeria innocua* colony on TSA was transferred into 50 ml BHI broth and then incubated at 37 °C for 18 h under

shaking at 160 rpm on an orbital shaker. After incubation, bacteria cells were harvested by centrifugation at 10,000 rpm for 10 min at 4 °C, and washed twice in sterile phosphate buffered solution (PBS). The washed cells were finally suspended in PBS to a final cell concentration of approximately 8 $\log_{10}$ CFU/ml, which was used as the working inoculum. The concentration of the working inoculum was confirmed by plating serial dilutions on TSA, followed by incubation at 37 °C for 48 h.

2.2 Sample preparation and inoculation

In this study, queso fresco cheese (QFC) was purchased from a local grocery store. To evaluate the effect of HVACP treatment in *Listeria innocua* population on QFC over an extended storage, QFC was placed in a sterile Stomacher bag and crumbled by the Stomacher followed by inoculation of *Listeria innocua* inoculum resulting in an approximately 6 $\log_{10}$ CFU/g population. Inoculated QFC samples were allowed to dry under refrigerated temperature for 1 h allowing the attachment of *Listeria* cells. For the examination of background microflora population on plasma treated QFC, crumbled QFC samples without microbial inoculation were utilized for HVACP treatment.

2.3 HVACP treatment

A schematic diagram of the set-up used in this study is presented in Figure 5.1. The distance between two circular aluminum electrodes (outer diameter = 152 mm) was 28 mm which was the height of the polypropylene box. 10 g of QFC sample was placed inside a polypropylene ArtBin® box (168 x 121 x 28 mm). The box was sealed with a high barrier film leaving an opening for gas flushing.

The pillow pack (box in bag) as a whole was flushed with dry air and was then completely sealed by an impulse sealer. The sealed pack samples were treated under direct mode of

exposure 100 kV for four and six minutes. Control samples were also packed under the same condition but without HVACP treatment. Treated and control samples were stored at 4 °C for up to 28 days for microbial and quality analysis.

2.4 Media preparation and enumeration

For background microbial population, mesophile, psychrotroph and Enterobacteriaceae were determined. 10 g of sample was removed from the package and placed into a sterile filtered stomacher bag with addition of 90 ml sterile 2% sodium citrate solution, and was then stomached at 230 rpm for 45 s. After stomaching, the resulting suspension was serial diluted with 9 ml 0.1% peptone water. Mesophilic organisms were determined by spread-plate in Tryptic soy agar (TSA) with incubation at 35 °C for up to 2 days. The psychrophilic organisms were quantified by spread-plate on TSA with incubation at 7 °C for 10 days. Total Enterobacteriaceae counts were determined by pour plate of 20 ml TSA and overlaying with 7 ml violet red bile glucose agar (VRBD) followed by incubation at 35 °C for 24 h.

To examine *Listeria innocua* population, *Listeria* selective agar (Oxoid CM856) and thin agar layer (TAL) method was employed for microbial enumeration in order to account for the injured cells. TAL method is utilized to recover the injured *Listeria* cells at the same time differentiate the *Listeria* from the mixed microbial population in QFC. To prepare TAL agar plate, a thin layer of TSA was laid on top of *Listeria* selective agar (Kang & Fung, 1999). After the thin TSA layer solidified, sample aliquot was plated on top of the TSA layer allowing the injured *Listeria* cells to recover. During incubation, selective agents diffused from the selective agar layer to the top thin TSA layer, thus allowed for selective growth of *Listeria innocua* (Kang & Fung, 1999; Wu et al., 2001). The limit of detection for microbial recovery on the samples was 1.0 $\log_{10}$ CFU/g.

2.5 pH, nitrate, nitrite, peroxide and moisture content measurement

To measure the pH, nitrate, nitrite and peroxide content of QFC, 1.0 g QFC sample was homogenized with 9.0 ml HPLC grade water for 30 seconds at 16,000 rpm (Lee et al., 2012). The pH of the sample was measured by Orion Dual Star pH/SE meter. Before measurements, the pH meter was calibrated with the standard buffer solutions pH 4.00, 7.00, and 10.00 at room temperature. Nitrate, nitrite and peroxide contents were measured by MQuant® test strips (Sigma-Aldrich®, MO, USA). Moisture content of QFC was measured by weighing 3 g QFC sample and oven drying at 102 °C following AOAC method 948.12 (AOAC, 1995).

2.6 Lipid peroxidation

Lipid oxidation on QFC was analyzed by thiobarbituric acid method following the protocol modified from method described in Jung et. al. (2015). 3 g of QFC sample was homogenized with 9 ml HPLC grade water with the addition of 50 µl 7.2% BHT in ethanol for 30 s at 16,000 rpm. The homogenate was then centrifuged at 10,000 g for 15 min at 4 °C. 1.0 ml supernatants was put into a 15 ml centrifuge tube and mixed with 2.0 ml 20 mM thiobarbituric acid (TBA) in 15% trichloroacetic (TCA) reagent. After mixing, the tubes were placed into a 90 °C water bath for 30 min and was then cooled in ice for 10 min. After cooling, the test tubes were centrifuged at 3,000 rpm for 10 min at 4 °C and then stabilized at room temperature for 30 min prior to absorbance spectroscopy measurement at 532 nm.

2.7 Optical absorption spectroscopy

To understand the plasma composition post-discharge with an extend storage, direct measurements of the concentrations of active reactive oxygen and reactive nitrogen species (RONS) inside the package were conducted using optical absorption spectroscopy for up to 24-hr post plasma discharge. The UV-Vis absorption spectroscopy allows the measurement

of the concentration of the post-discharge long-lived reactive species inside the package. To measure the incident and transmitted spectral intensity I_0 (λ=200-800 nm) and I_T (λ=200-800 nm) of a beam from a UV-Vis tungsten-deuterium-halogen light source (BDS 130A, BW Tek, Delaware, USA) crossing the post-discharge gas inside the package, an imaging spectrograph, equipped with a charge coupled device (CCD) camera (HR 2000+, Ocean Optics, Inc., Florida, USA) was employed. The relationship between the intensities and the RONS concentration is calculated by the Lambert-Beer law (1):

$$I_T(\lambda) = I_0(\lambda)e^{-\sum_i \sigma_i(\lambda)C_i L} \quad (1)$$

where, L is the path length (cm), $\sigma_i(\lambda)$ is the wavelength dependent absorption cross-section of each species (cm^2/molecule), and C_i is the number density of each species (cm^{-3}). The open path-length was set at 2.1 cm. Numerical deconvolution was utilized to determine the concentrations of RONS including O_3, NO_2, NO_3, N_2O_4, and N_2O_5.

2.8 Statistical analysis

Statistical analysis was conducted by JMP statistical package (SAS Institute Inc., Cary, NC, USA). Analysis of variance (ANOVA) was used and the data was represented as mean value ± standard deviation. Tukey's multiple sample comparison tests was performed to assess the significance of difference between treatments. Statistical significance was indicated at a confidence level of 95% ($p \leq 0.05$).

3. Results and Discussion

3.1 Optical absorption spectroscopy

To understand the plasma-sample interaction post plasma-discharge, optical absorption spectroscopy measurement 4-h post plasma-discharge was employed to monitor the concentration of O_3, NO_2, NO_3, N_2O_4, and N_2O_5. Figure 5.2 and 5.3 represent the post-

discharge plasma composition after 4 min and 6 min direct HVACP treatment at 100 kV for up to 24-hr. Immediately after turning-off the plasma device, a ca. 3,500 ppmv concentration of O_3 was observed after six-min HVACP treatment compared to ca. 3,000 ppmv after four min treatment. After turning-off the plasma generator, ozone concentration starts to decrease and reach the minimal ~30 min post plasma-discharge, while NO_2 and N_2O_5 content increases to the maximum of approximately 3,000 ppmv and 1,500 ppmv respectively. This is likely resulted from the recombination of reactive nitrogen species with reactive oxygen species. The atomic oxygen could oxidize with the nitrogen monoxide (NO) to form NO_2 via a three-body reaction (Fridman, 2008):

$$NO + O + M \rightarrow NO_2 + M, \text{ where } M = N_2/O_2/NO/NO_2 \quad (2)$$

Ozone (O_3) could also reactive with NO to form NO_2 (Fridman, 2008):

$$O_3 + NO \rightarrow O_2 + NO_2 \quad (3)$$

The N_2O_5 forms via a reversible reaction from NO_3 and NO recombination (Hjorth et al., 1992):

$$NO_3 + NO + M \leftrightarrow N_2O_5 + M, \text{ where } M = N_2/O_2 \quad (4)$$

The decrease of ozone content could be ascribed to the reaction with NO for the formation of NO_2 (reaction (3)), while the increasing of NO_2 and N_2O_5 is resulted from reactions (3) and (4). The exact mechanism for the interaction between reactive gas species and the microorganisms are still not clear. However, many studies have attributed the efficiency of microbial inactivation to the presence of reactive oxygen and reactive nitrogen species, such as NO, NO_2, O_3, OH by disruption of cell membrane or cellular components through oxidative stress, DNA damage, protein degradation, and inducing apoptosis of microbial cells (Liao et al., 2017).

This plasma composition results post plasma-discharge reveal the importance of the 24 hr storage post HVACP treatment which allow an extended exposure of reactive gas species with the sample leading to higher microbial inactivation. As shown in Figures 5.2 and 5.3, after 24-hr, the concentration of measured reactive gas species dropped to zero leaving no chemical residuals in the package. Employing HVACP as the last step for fresh cheese production inside the package material allows the extended exposures between reactive gas species with the product during storage and distribution rendering the safety and extending the shelf-life of the products.

3.2 *Listeria innocua* (LI) population after four and six minutes HVACP treatment with up to 28 days storage

Direct HVACP in dry air at 100 kV effectively reduced the *Listeria innocua* population on queso fresco cheese (QFC), and persistently inhibited the growth of LI throughout the 28 days storage of the study. Figure 5.4 and 5.5 demonstrate the data for LI reductions after four minutes of dry air direct HVACP treatment for up to 28-day of storage at 4 °C recovered on *Listeria* selective agar (LSA) and via thin agar layer (TAL) method, respectively. Immediately after four minutes direct HVACP treatment in dry air, 5.8 $\log_{10}$ CFU/g LI population was observed, while a LI population of 4.7 $\log_{10}$ CFU/g was found 24-hr post HVACP treatment stored at 4 °C, recovered on LSA. Based upon the optical absorption analysis discussed in the previous section, the additional 24-hr exposure post plasma treatment allows enhanced microbial inactivation by HVACP through extended interaction between the reactive gas species (e.g. O_3, NO_2 and N_2O_5) and microbial cells. These reactive gas species have been proven for microbial inactivation possibly by DNA damage (Boudam et al., 2006; Winter et al., 2011), lipid oxidation of the cell membranes or intracellular

components (Alkawareek et al., 2014; Montie et al., 2000), and modulation of protein structure (Digel et al., 2005; Dobrynin et al., 2009). Moreover, it is worth noting that these reactive gas species (RGS) generally exist for 24-hr or less after which the RGS in the package revert back into the original inert gas composition thereby leaving the package safe and free of chemical residues (Misra et al., 2013).

For the four-minute HVACP treated QFC, LI population continued to decrease with increased storage period at refrigerated temperature. After 6 days storage at 4 °C, treated QFC exhibited a LI population of 2.8 $\log_{10}$ CFU/g and 3.5 $\log_{10}$ CFU/g recovered on LSA and TAL, respectively, with a population of 6.3 $\log_{10}$ CFU/g in untreated samples. After 15 days of storage, a population of 1.8 $\log_{10}$ CFU/g and 2.7 $\log_{10}$ CFU/g recovered on LSA and TAL, respectively, was observed in treated QFC, while a population of 6.5 $\log_{10}$ CFU/g in untreated QFC. At 28 days of storage, 1.4 $\log_{10}$ CFU/g and 2.2 $\log_{10}$ CFU/g LI population recovered on LSA and TAL, respectively, was observed in treated QFC, while 7.4 $\log_{10}$ CFU/g in untreated QFC. After 28 days of storage, a 7.4 $\log_{10}$ CFU/g LI population was observed compared to the initial inoculation of 6.0 $\log_{10}$ CFU/g. This extended inhibition of LI by HVACP treatment is likely due to the injury of LI cells after HVACP treatment, which prohibit the growth and recovery of LI cells during the 28 days of storage at 4 °C. Song et al. (2009) have also reported an inhibition of *Listeria monocytogenes* growth in sliced cheese (60x6x2 mm) after DBD plasma treatment at 13.56 MHz in Helium with three weeks storage at 10 °C.

Similar to four minutes direct HVACP treatment at 100 kV, six minutes direct HVACP treatment at 100 kV exhibited the inhibition of LI growth and recovery during 28 days of storage under refrigerated condition as well. Figure 5.6 and 5.7 show the data for LI

reductions after six minutes of dry air direct HVACP treatment for up to 28 days of storage at 4 °C, recovered on *Listeria* selective agar (LSA) and by thin agar layer method (TAL), respectively. After six minutes of direct dry air HVACP treatment, a 5.0 log_{10} CFU/g and 3.4 log_{10} CFU/g LI population, recovered on LSA, was observed after 0 hour and 24 hours of storage at 4 °C, respectively, while a population of 5.9 log_{10} CFU/g in untreated QFC samples. After 6 days of storage, 1.3 log_{10} CFU/g and 1.7 log_{10} CFU/g LI population, recovered on LSA and TAL, respectively, was observed on treated QFC, while 5.9 log_{10} CFU/g in untreated QFC samples. At 10 days of storage and beyond, with 1.0 Log_{10} CFU/g detection limit, the treated QFC showed less than 1.0 log_{10} CFU/g LI population, recovered on both LSA and TAL, while 6.8 log_{10} CFU/g in untreated QFC. For LI inoculated untreated QFC, with increasing storage time, LI population continued to rise. After 28 days of storage, a 7.5 log_{10} CFU/g LI population was observed compared to the initial inoculation level of 5.9 log_{10} CFU/g.

Ziuzina et al. (2014) also reported the ability of HVACP for reduction of inoculated pathogens on fresh produce and reported the reduction of *Salmonella, E.coli* O157:H7 Sand *Listeria monocytogenes* population to undetectable levels on tomato from the initial populations of 3.1, 6.3, 6.7 log_{10} CFU/sample. This efficient reduction and inhibition of *Listeria innocua* after in-package HVACP treatment suggests the elimination of a possible post-process contamination of fresh cheese like QFC, thus allows the application of HVACP to ensure the safety of the products.

3.3 Background microflora population after four and six minutes HVACP treatment with up to 28 days storage

Besides the effective inactivation and inhibition of LI, direct HVACP in dry air also demonstrated its efficacy in reduction of background microflora on QFC throughout the 28 days storage under refrigerated condition. During storage at 4 °C, untreated control samples demonstrated a steady microbial growth, while the growth of background microflora on HVACP treated QFC was suppressed. Similar observation was found by Lacombe et al. (2015) in which they also observed the inhibition of spoilage microorganisms on blueberry after cold plasma treatment followed by 7 days storage at 4 °C.

Four minutes HVACP treatment shown inhibition of background microflora in QFC. Figure 5.8, 5.9 and 5.10 demonstrate the data for mesophilic microorganisms, psychrotrophic microorganisms, and total enterobacteriaceae, respectively, after four minutes of dry air direct HVACP treatment for up to 28 days storage at 4 °C. After HVACP treatment, a 1.7 $\log_{10}$ CFU/g and 1.3 $\log_{10}$ CFU/g mesophilic microorganism population was observed at day 0 and day 1, respectively, while 2.2 $\log_{10}$ CFU/g and 3.0 $\log_{10}$ CFU/g was found in control samples at day 0 and day 1, respectively. While, at 28 days of storage, 1.2 $\log_{10}$ CFU/g of mesophilic population was observed in treated QFC compared to 6.2 $\log_{10}$ CFU/g in control samples.

For psychrotrophic microorganisms, after four minutes of HVACP treatments, 2.1 $\log_{10}$ CFU/g psychrotrophic population was observed immediately after plasma treatment compared to 1.0 $\log_{10}$ CFU/g population in QFC stored for 24-hr at 4 °C following plasma treatment. 2.4 $\log_{10}$ CFU/g and 3.1 $\log_{10}$ CFU/g was found for control samples at day 0 and day 1, respectively. At and after 5 days of storage at 4°C, psychrotrophic microorganisms revealed a population of 1.0 $\log_{10}$ CFU/g until the end of the 28 days of storage. However, for control samples, psychrotrophic population continued to grow during the storage at 4 °C.

At day 15, 6.1 log_{10} CFU/g population was observed, while at day 28, 6.6 log_{10} CFU/g population was found in control QFC.

Moreover, for enterobacteriaceae population, after four minutes of HVACP treatments, 1.2 log_{10} CFU/g and 1.0 log_{10} CFU/g population was observed at day 0 and day 1, while 1.6 log_{10} CFU/g and 2.4 log_{10} CFU/g for control samples at day 0 and day 1, respectively. At and after 5 days of storage at 4 °C, total enterobacteriaceae exhibited a population ranging between 1.0 log_{10} CFU/g and 1.5 log_{10} CFU/g until the end of the 28 days of storage. On the contrary, in control samples, enterobacteriaceae population continued to grow along the storage at 4 °C, for which at day 28, a 6.3 log_{10} CFU/g population was observed in control QFC.

Figures 5.11 and 5.12 present the population of mesophilic microorganisms and psychrotrophic microorganisms, respectively, after six minutes of dry air direct HVACP treatment for up to 28 days storage at 4 °C. Same as the four minutes direct HVACP treatment, six minutes HVACP treatment in air also resulted in the inhibition of background microflora. At 28 days of storage, 1.5 log_{10} CFU/g mesophilic population was observed in treated QFC compared to 7.9 log_{10} CFU/g in control samples. For psychrotrophic microorganisms, at and after 5 days of storage at 4 °C, psychrotrophic microorganism showed a population of 1.0 log_{10} CFU/g until the end of the 28 days of storage. For control samples however, psychrotrophic population continued to grow along the storage at 4 °C. At Day 15, 6.5 log_{10} CFU/g population was observed in control QFC, while at day 28, 7.9 log_{10} CFU/g population was noted. Enterobacteriaceae population was not detected in the QFC samples, purchased from the local grocery store, for the six minutes HVACP treatment storage study. However, from the results of four minutes of HVACP treatment storage study,

it clearly demonstrated the capability of HVACP treatment to inhibit enterobacteriaceae growth.

Other studies have also reported the capacity of HVACP in inactivation of spoilage microorganisms (Misra, Patil, et al., 2014; Patange et al., 2017; Suhem et al., 2013). The suppression of background microflora by HVACP treatment enables the shelf-life extension for fresh cheese like QFC, which commonly has a limited shelf-life due to microbial spoilage (Mortensen et al., 2010; Tunick, 2006).

3.4 Lipid peroxidation, pH and moisture content of QFC after four and six minutes HVACP treatment

Table 5.1 shows the data for malondialdehyde (MDA) content, moisture content and pH for QFC after four minutes HVACP treatment in dry air for up to 28-day storage at 4 °C. For moisture content, with increasing storage period a decrease in moisture content was noted. However, there was no significant difference ($p<0.05$) between control and treated samples within 28 days storage at 4 °C. Minimal influences in pH were observed within 28 days, in which pH ranged between 5.53 and 5.82 for treated QFC, while it lay between 5.50 and 6.00 for control samples. There was a slight decrease of pH in treated QFC sample 24-h post HVACP treatment for both four and six minutes treatment. This can be explained by the acid-forming reactions induced by HVACP treatment leading the slightly decrease of pH (Misra, 2015):

$$H_2O + e \rightarrow OH + H + e \quad (5)$$

$$NO_2 + OH \rightarrow HNO_3 \quad (6)$$

$$NO + OH \rightarrow HNO_2 \quad (7)$$

As for MDA content, HVACP treatment in dry air for four minutes led to higher MDA formation in treated QFC than untreated QFC. Both four and six minutes direct HVACP induced lipid oxidation of QFC, which is also observed in pork butt and beef loin (Jayasena et al., 2015), and Cheddar cheese (Yong et al., 2015) after cold plasma treatment. This increased lipid oxidation is likely resulted from the reaction with reactive oxygen species (e.g. O_3) and radicals (e.g. OH•) formed by HVACP treatment (Gavahian et al., 2018). For this storage study, increasing storage time resulted in a minor increase in MDA content in control samples, with the MDA content peaking to 0.30 mg/kg on 28th day. There were no gradual increases in MDA content in treated QFC along the 28 days of storage. The highest MDA content recorded was 1.37 mg/kg after four minutes of HVACP treatment on day 0. Table 5.2 summarizes the data for MDA content, moisture content and pH for QFC after six minutes of HVACP treatment in dry air followed by storage for 28 days at 4 °C. For moisture content, increasing storage period led to decrease in moisture content. However, there was no significant difference ($p<0.05$) between control and treated samples within 28 days of storage at 4 °C. Minimal influences in pH were observed over the 28 days of storage, in which pH ranged between 5.26 and 5.72 for treated QFC, and it lay between 5.46 and 6.04 for control samples. Similar to four minutes HVACP treatment, six minutes direct HVACP treatment in dry air also led to a higher lipid oxidation in treated QFC compared to control samples, while the increasing storage time resulted in minimal effect on MDA content, with the highest MDA content reaching 2.86 mg/kg at 15th day in treated QFC.

3.5 Nitrite, nitrate and peroxide content of QFC after four and six minutes HVACP treatment

Tables 5.3 and 5.4 summarize the nitrite, nitrate and peroxide content of QFC after four and six minutes HVACP treatment in dry air, respectively, for up to 28 days storage at 4 °C.

After four minutes HVACP treatment, a maximum of 100 ppm, 12.5 ppm and 275 ppm was observed in treated QFC for peroxide, nitrite and nitrate, respectively, while none was detected in untreated QFC during the storage. Six minutes HVACP treated samples demonstrated a maximum of 100 ppm, 20 ppm and 500 ppm content for peroxide, nitrite and nitrate, respectively, during the 28-day storage, and none was detected in untreated QFC as well. As discussed earlier, HVACP treatment leads to the generation of reactive gas species including ROS and RNS, which can interact with each other leading to a complex chemistry in the gas phase. Besides of the reactions in the gas phase, these reactive gas species (e.g. ROS and RNS) can diffuse and interact with the water molecules in the substrates forming reactive species (e.g. NO_3^-, NO_2^-, O_3^-, H^+, e^-) in the aqueous phase (Samukawa et al., 2012). N_2O_4 (8) or NO_2 (9) can diffuse and interact with water to yield nitrate (NO_3^-) and nitrite (NO_2^-) (Ignarro et al., 1993; Thirumdas et al., 2018). NO diffuses into the aqueous phase containing O_2 resulting in the formation of nitrite (NO_2^-) (10) (Ignarro et al., 1993). Water molecules can collide with electron and be split into OH• and H• (11), while OH• can lead to the formation of H_2O_2 (12) (Thirumdas et al., 2018). In the aqueous phase, NO_2^- can further react with H^+ (13) or H_2O_2 (14) to form NO_3^- (Thirumdas et al., 2018). This reaction between NO_2^- and H_2O_2 is likely contributing to the decrease of nitrite and peroxide during the storage, while increased NO_3^- content was observed.

$$N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+ \quad (8)$$

$$2NO_2 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+ \quad (9)$$

$$4NO + O_2 + 2H_2O \rightarrow NO_2^- + 4H^+ \quad (10)$$

$$H_2O + e \rightarrow OH\bullet + H\bullet + e^- \quad (11)$$

$$OH\bullet + OH\bullet \rightarrow H_2O_2 \quad (12)$$

$$3NO_2^- + 3H^+ \rightarrow 2NO + NO_3^- + H_3O^+ \quad (13)$$

$$NO_2^- + H_2O_2 + H^+ \rightarrow NO_3^- + H_2O + H^+ \quad (14)$$

Nitrite, nitrate and peroxide is recognized being effective in antimicrobial activity. Christiansen et al. (1973) and Majou and Christieans (2018) have demonstrated effectiveness of nitrite and nitrate in microbial inhibition including pathogens (e.g. *Clostridium botulinum*) in cured meats. Nitrite, peroxynitrite (15) and peroxide lead to the oxidative stress of bacteria resulting in inhibition of microbial growth (Majou & Christieans, 2018). While nitrate contributes to an increased production of acetate in some bacteria (e.g. *Staphylococcus aureus* and *Clostridium*), which disrupt bacterial ATP level, thus inhibit the growth of bacteria (Majou & Christieans, 2018). Hence, with the presence of nitrite, nitrate and peroxide in HVACP treated QFC, the growth of LI and background microflora was inhibited throughout the 28-day storage. It is worth to note that after HVACP treatment, the level of nitrite, nitrate and peroxide in treated QFC is below the limit required by Code of Federal Regulations, in which nitrite, nitrate and peroxide should not exceed 200 ppm, 500 ppm and 500 ppm, respectively, in the finished products (CFR, 2018a, 2018b).

$$NO_2^- + H_2O_2 + H^+ \rightarrow ONOOH + H_2O \quad (15)$$

4. Conclusion

Direct HVACP in dry air at 100 kV demonstrated a continuous inhibition of *Listeria innocua* and background microflora with minor effects in pH, moisture and lipid oxidation of queso fresco after four and six minutes treatment followed by 28-day storage under refrigerated condition. At the end of 28 days storage, HVACP treated cheese exhibited a *Listeria innocua* population of 1.4 $\log_{10}$ CFU/g and 1.0 $\log_{10}$ CFU/g after four and six minutes treatment, respectively, compared to around 7.4 $\log_{10}$ CFU/g LI population in control samples. Similar

as inhibition of LI population, direct HVACP for four or six minutes in dry air inactivated and suppressed the growth of background microflora. After 28 days of storage, close to 1.0 log_{10} CFU/g survival mesophilic, psychrotrophic and enterobacteriaceae population was observed for treated cheese while greater than 6.0 log_{10} CFU/g for untreated samples. These results indicate a great potential for the future application of HVACP as a solution to not only ensure the safety of fresh cheeses like queso fresco, but also extend the shelf-life of the product. By increasing the shelf-life of perishable products like fresh cheese, HVACP can help reduce food waste due to spoilage and increase the value of the products.

Acknowledgement

Research funding support provided by the National Dairy Council and Innovation Center for US Dairy.

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Table 5.1. Lipid peroxidation (MDA), moisture content and pH of QFC treated with HVACP for four minutes in dry air for up to 28 days storage at 4 °C. Same small letter beside each measurement indicated no significant (p>0.05) different within the same day of storage.

Day	Treated			Control		
	MDA(mg/kg)	MC%	pH	MDA(mg/kg)	MC%	pH
0	1.37[a]	50.25[a]	5.69[a]	0.09[b]	50.26[a]	5.91[b]
1	1.29[a]	50.76[a]	5.82[a]	0.08[b]	50.65[a]	6.00[b]
5	0.86[a]	50.29[a]	5.77[a]	0.08[b]	50.64[a]	5.97[b]
10	0.98[a]	50.04[a]	5.67[a]	0.12[b]	50.87[a]	5.85[b]
15	1.10[a]	49.37[a]	5.59[a]	0.20[b]	50.33[a]	5.77[b]
20	1.21[a]	49.25[a]	5.53[a]	0.20[b]	49.32[a]	5.70[b]
23	0.93[a]	47.69[a]	5.54[a]	0.23[b]	49.17[a]	5.50[a]
25	0.94[a]	48.43[a]	5.57[a]	0.27[b]	48.94[a]	5.57[a]
28	0.77[a]	47.76[a]	5.57[a]	0.30[b]	49.86[a]	5.57[a]

Table 5.2. Lipid peroxidation (MDA), moisture content and pH of QFC treated with HVACP for six minutes in dry air for up to 28 days storage at 4 °C. Same small letter beside each measurement indicated no significant (p>0.05) different within the same day of storage.

Day	Treated			Control		
	MDA(mg/kg)	MC%	pH	MDA(mg/kg)	MC%	pH
0	1.56[a]	48.62[a]	5.52[a]	0.23[a]	49.48[a]	5.46[a]
1	2.17[a]	48.43[a]	5.40[a]	0.21[a]	49.51[a]	5.49[a]
5	1.72[a]	48.29[a]	5.26[a]	0.24[a]	49.17[a]	5.79[b]
10	1.11[a]	47.42[a]	5.56[a]	0.26[a]	49.16[a]	5.90[b]
15	2.86[a]	44.42[a]	5.72[a]	0.26[a]	45.73[a]	6.04[b]
20	1.36[a]	44.41[a]	5.64[a]	0.35[a]	42.76[a]	5.89[b]
23	1.11[a]	46.47[a]	5.60[a]	0.38[a]	44.31[a]	5.95[b]
25	1.31[a]	45.05[a]	5.57[a]	0.29[a]	44.17[a]	5.91[b]
28	1.79[a]	41.43[a]	5.53[a]	0.33[a]	44.87[a]	5.85[b]

Table 5.3. Peroxide (O_2^{2-}), Nitrite (NO_2^-) and Nitrate (NO_3^-) of QFC treated with HVACP for four minutes in dry air for up to 28 days storage at 4 °C. Same small letter beside each measurement indicated no significant ($p>0.05$) different within the same compound measured during 28 days storage

Day	peroxide (ppm)	nitrite (ppm)	nitrate (ppm)
0	100^{a}	12.5^{a}	250^{ab}
1	30^{b}	10^{a}	250^{ab}
5	30^{b}	10^{a}	262^{ab}
10	28^{b}	10^{a}	238^{b}
15	30^{b}	10^{a}	250^{ab}
20	30^{b}	10^{a}	250^{ab}
23	12^{c}	10^{a}	250^{ab}
25	15^{c}	10^{a}	275^{ab}
28	18^{c}	10^{a}	250^{ab}

Table 5.4. Peroxide (O_2^{2-}), Nitrite (NO_2^-) and Nitrate (NO_3^-) of QFC treated with HVACP for six minutes in dry air for up to 28 days storage at 4 °C. Same small letter beside each measurement indicated no significant ($p>0.05$) different within the same compound measured during 28 days storage

Day	peroxide (ppm)	nitrite (ppm)	nitrate (ppm)
0	100^{a}	20^{a}	300^{c}
1	80^{b}	20^{a}	275^{c}
5	58^{c}	18^{a}	263^{c}
10	30^{e}	20^{a}	275^{c}
15	30^{e}	15^{ab}	500^{a}
20	50^{cd}	10^{b}	500^{a}
23	33^{e}	10^{b}	425^{b}
25	40de	10^{b}	463^{ab}
28	40de	10^{b}	475^{ab}

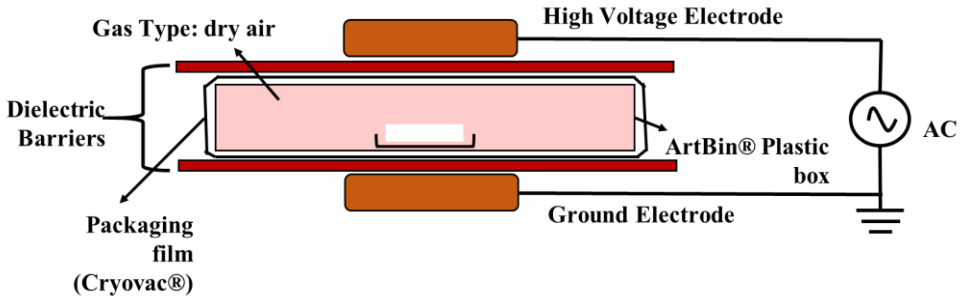

Figure 5.1. Schematic of plasma set-up

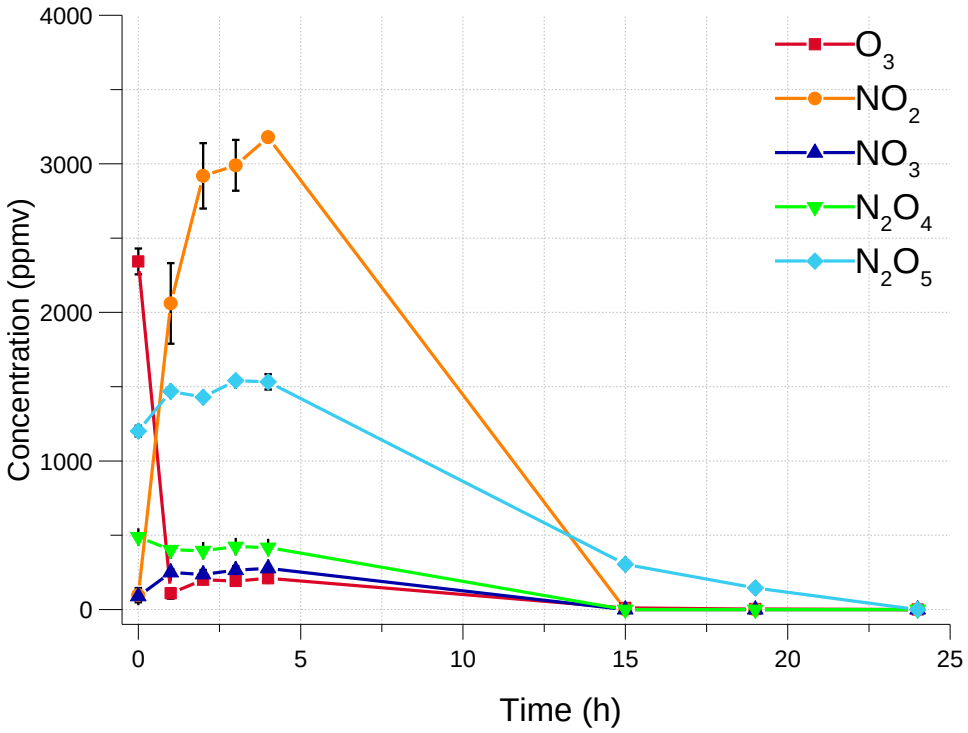

Figure 5.2. Post-discharge composition of the gas for direct four minutes HVACP treatment of QFC in dry air at 100 kV with 24-hr storage

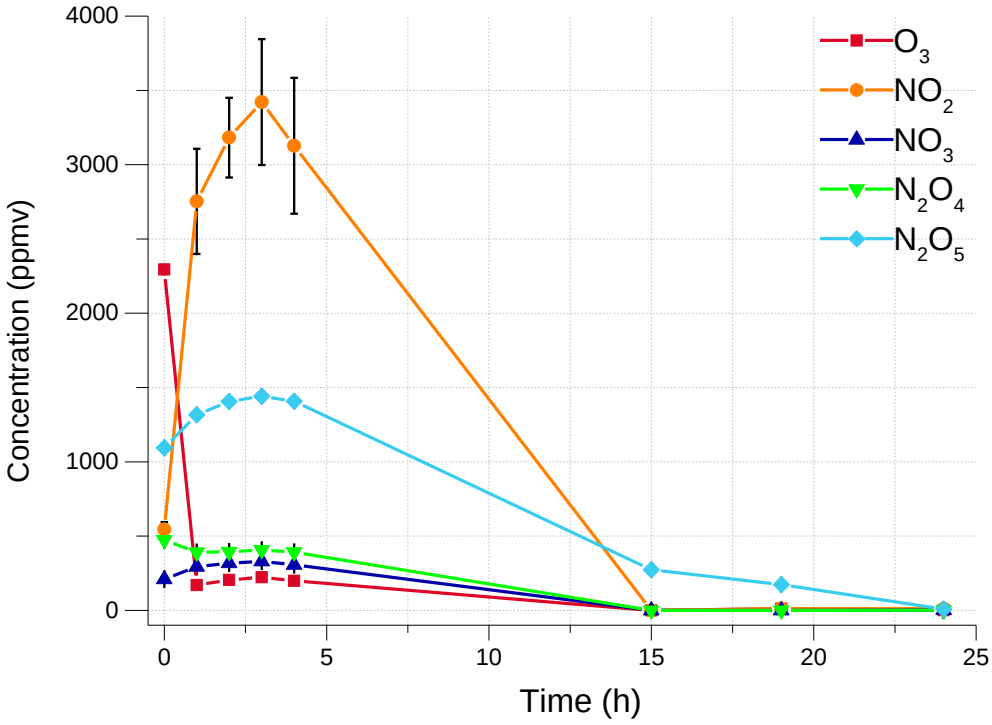

Figure 5.3. Post-discharge composition of the gas for direct six minutes HVACP treatment of QFC in dry air at 100 kV with 24-hr storage

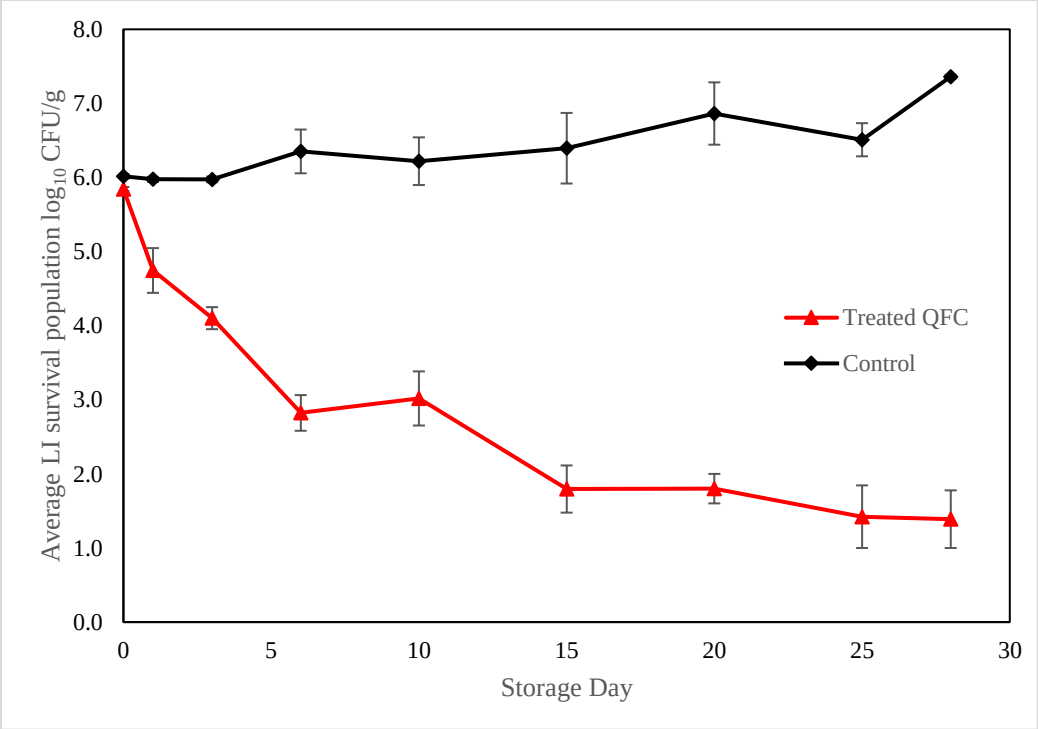

Figure 5.4. *Listeria innocua* population (log_{10} CFU/g) for 10.0 g crumbled queso fresco cheese (QFC) treated with HVACP (Direct) in dry air at 100 kV for four minutes within 28 days storage at 4 °C recovered on *Listeria* selective agar (LSA). The detection limit of the applied enumeration method was 1.0 log_{10} CFU/g.

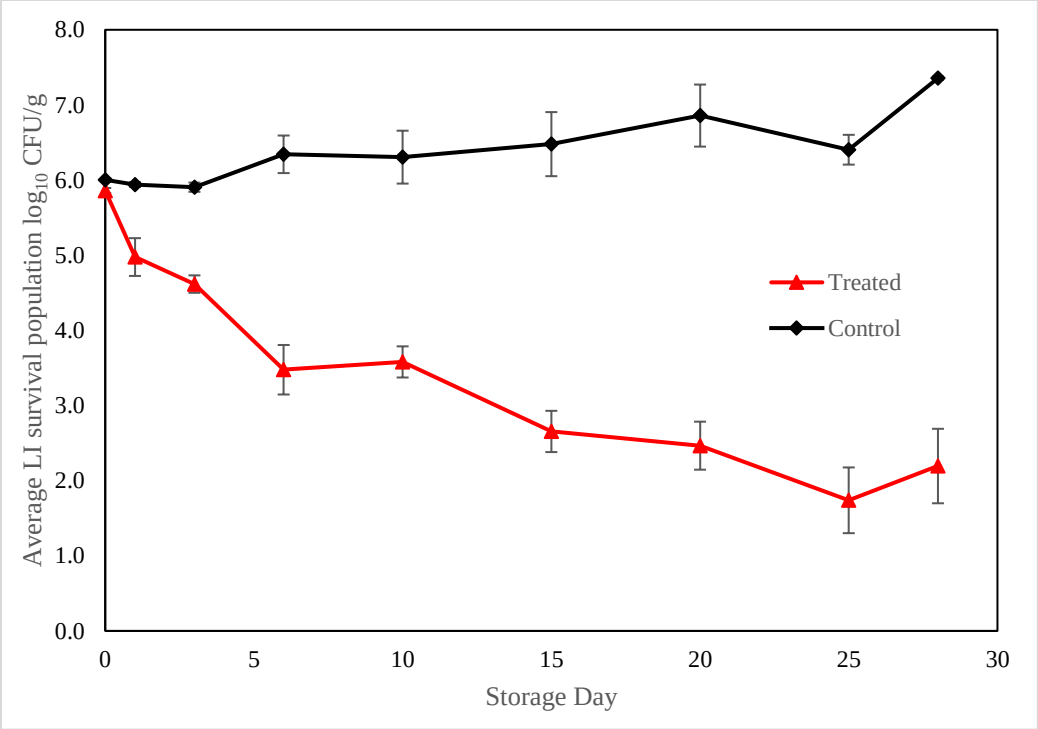

Figure 5.5. *Listeria innocua* population (log_{10} CFU/g) for 10.0 g crumbled queso fresco cheese (QFC) treated with HVACP (Direct) in dry air at 100 kV for four minutes within 28 days storage at 4 °C recovered using Thin agar layer method (TAL). The detection limit of the applied enumeration method was 1.0 log_{10} CFU/g.

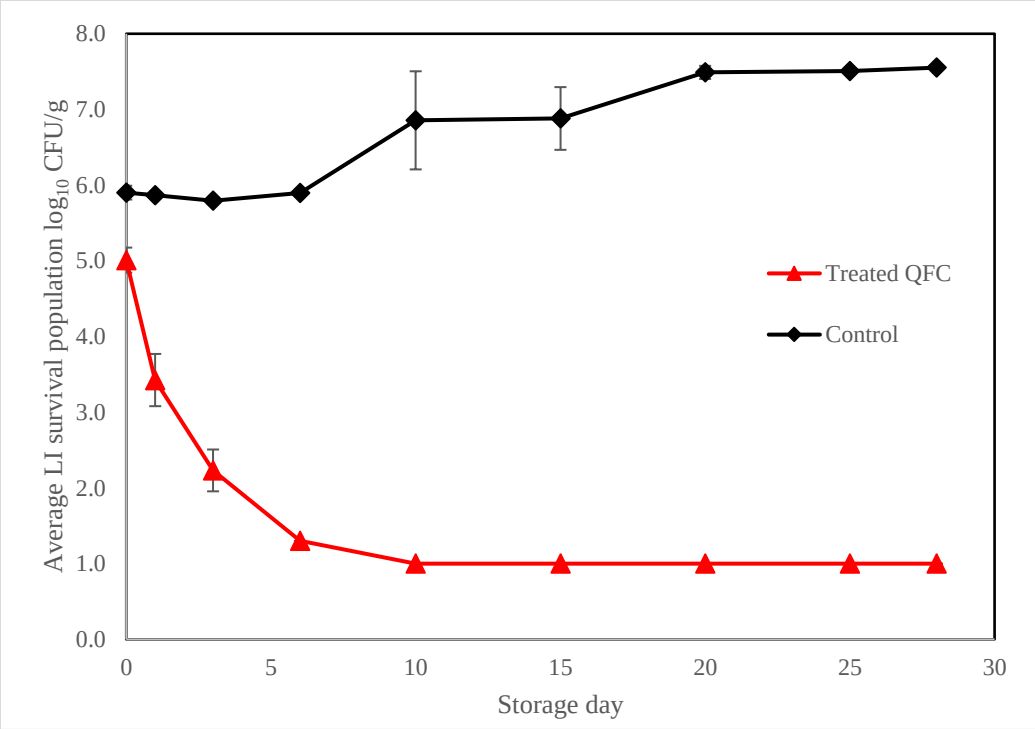

Figure 5.6. *Listeria* innocua population (log_{10}CFU/g) for 10.0 g crumbled queso fresco cheese (QFC) treated with HVACP (Direct) in dry air at 100 kV for six minutes within 28 days storage at 4 °C recovered on *Listeria* selective agar (LSA). The detection limit of the applied enumeration method was 1.0 log_{10} CFU/g.

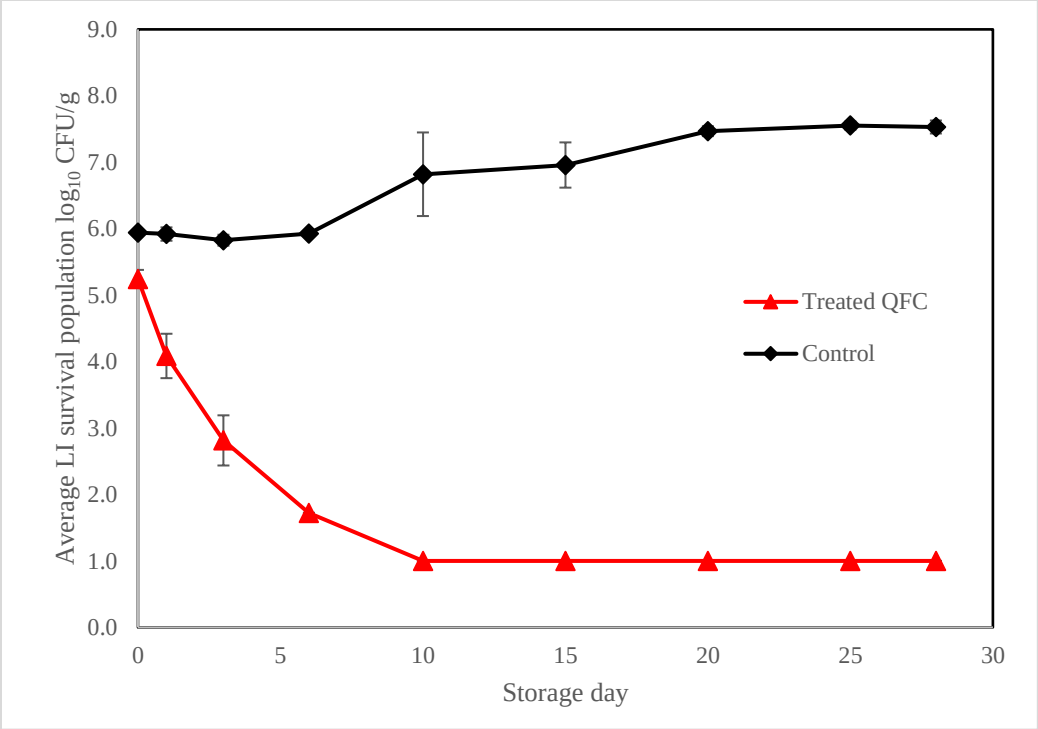

Figure 5.7. *Listeria* innocua population (log_{10} CFU/g) for 10.0 g crumbled queso fresco cheese (QFC) treated with HVACP (Direct) in dry air at 100 kV for six minutes within 28 days storage at 4 °C recovered using Thin agar layer method (TAL). The detection limit of the applied enumeration method was 1.0 log_{10} CFU/g.

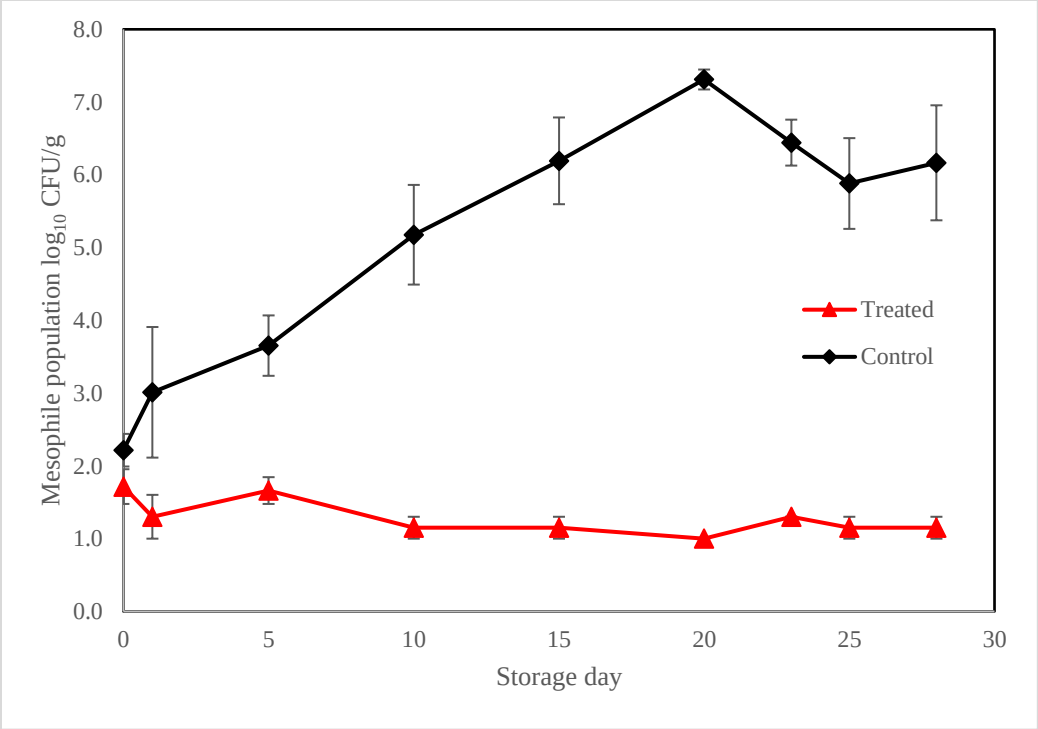

Figure 5.8. Mesophilic microorganism population (log_{10} CFU/g) for 10.0 g crumbled queso fresco cheese (QFC) treated with HVACP (Direct) in dry air at 100 kV for four minutes within 28 days storage at 4 °C. The detection limit of the applied enumeration method was 1.0 log_{10} CFU/g.

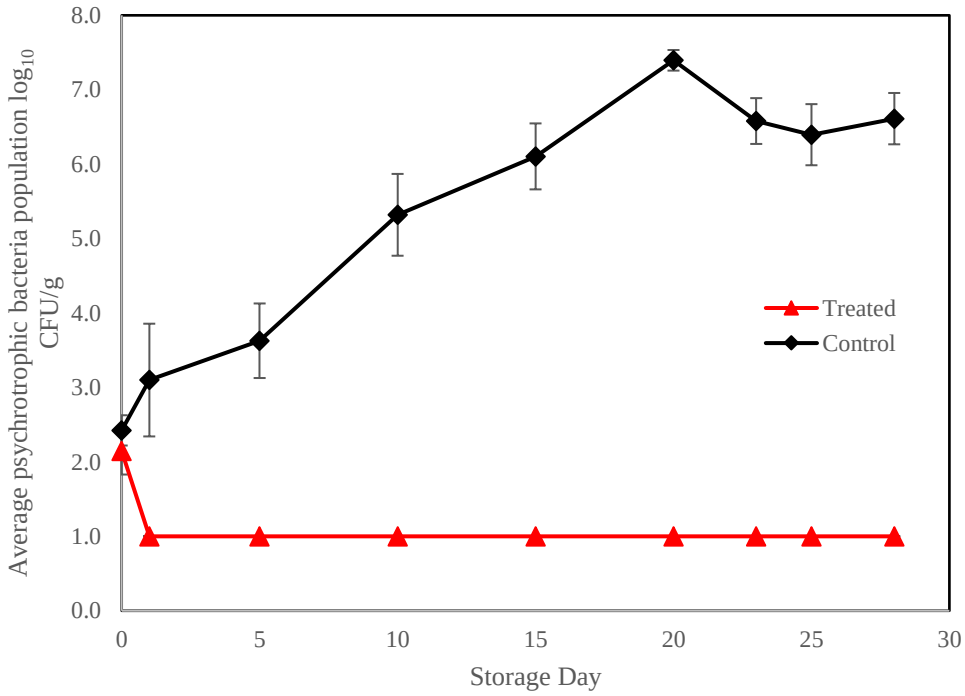

Figure 5.9. Psychrotrophic microorganism population (log_{10} CFU/g) for 10.0 g crumbled queso fresco cheese (QFC) treated with HVACP (Direct) in dry air at 100 kV for four minutes within 28 days storage at 4 °C. The detection limit of the applied enumeration method was 1.0 log_{10} CFU/g.

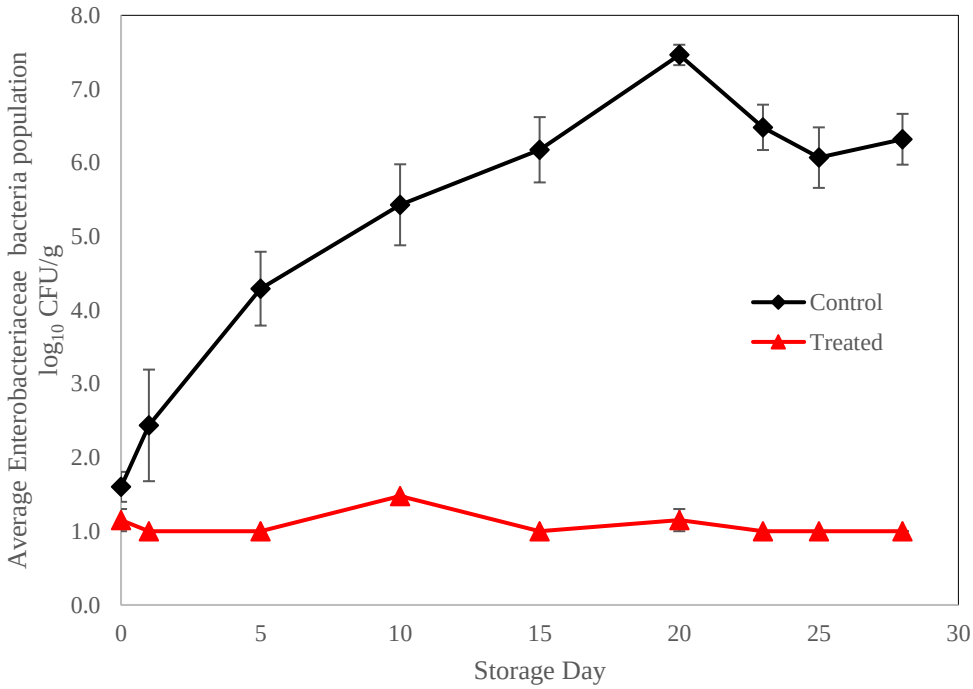

Figure 5.10. Enterobacteriaceae population (log_{10} CFU/g) for 10.0 g crumbled queso fresco cheese (QFC) treated with HVACP (Direct) in dry air at 100 kV for four minutes within 28 days storage at 4 °C. The detection limit of the applied enumeration method was 1.0 log10 CFU/g.

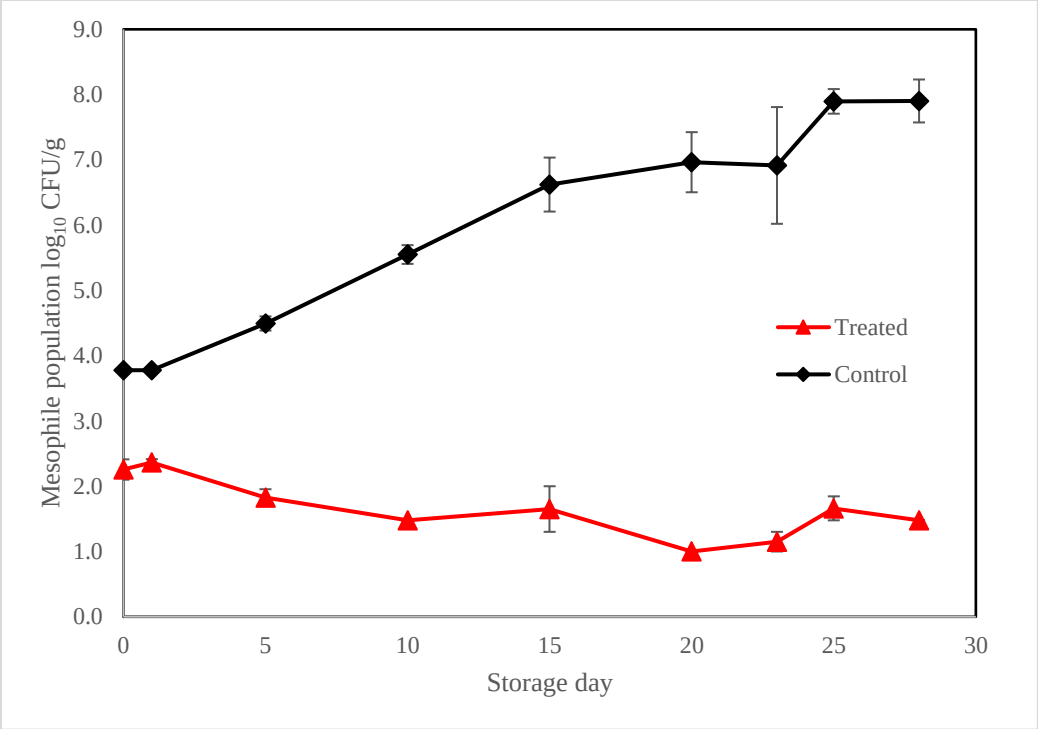

Figure 5.11. Mesophilic microorganism population (log_{10} CFU/g) for 10.0 g crumbled queso fresco cheese (QFC) treated with HVACP (Direct) in dry air at 100 kV for six minutes within 28 days storage at 4 °C. The detection limit of the applied enumeration method was 1.0 log_{10} CFU/g.

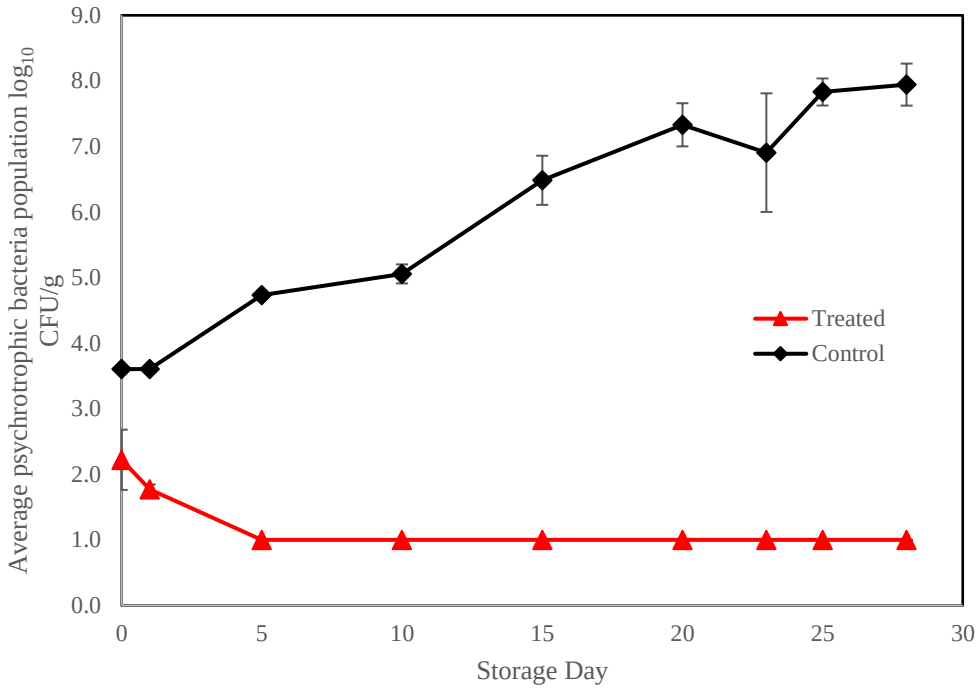

Figure 5.12. Psychrotrophic microorganism population (log_{10} CFU/g) for 10.0 g crumbled queso fresco cheese (QFC) treated with HVACP (Direct) in dry air at 100 kV for six minutes within 28 days storage at 4 °C. The detection limit of the applied enumeration method was 1.0 log_{10} CFU/g.

CHAPTER 6. GENERAL CONCLUSION

The overall objective of this research was to evaluate the applicability of high voltage atmospheric cold plasma (HVACP) for destroying post-processing pathogenic contaminants from soft cheeses (e.g. queso fresco). The first phase of this study (Chapter 2) focused on examining the effect of product composition, surface topography and mode of exposure on inactivation of *Listeria innocua* by HVACP treatment. Queso fresco, cheese model and tryptic soy agar was examined for comparison. Direct mode of exposure was found to be more effective in *Listeria innocua* inactivation on queso fresco and cheese model. Moreover, it was found that products with a more complex surface structure and higher nutrients were more resistant for *Listeria* inactivation by HVACP treatment. A 1.6 $\log_{10}$ CFU/g, 3.5 $\log_{10}$ CFU/g, and 5.0 $\log_{10}$ CFU/g *Listeria innocua* reduction was achieved after five minutes direct HVACP treatment in dry air followed by 24-h refrigerated storage, for queso fresco, cheese model and tryptic soy agar, respectively.

The second phase of study (Chapter 3) investigated the effect of applied voltage, gas composition and bacteria species on the efficacy of direct HVACP in microbial inactivation on sliced queso fresco. From this study, it was observed that increasing applied voltage from 60 kV to 100 kV resulted in a higher microbial inactivation. HVACP treatment was found to be more effective in inactivation of the gram-negative *E.coli* K-12 than gram-positive *Listeria innocua*. After five minutes direct HVAPC treatment in dry air followed by 24-h storage at 4 °C, a 1.4 $\log_{10}$ CFU/g and 3.5 $\log_{10}$ CFU/g reduction was achieved for *Listeria innocua* and *E.coli* K-12, respectively. Slight decrease of pH and minor increase in lipid oxidation was observed with no significant changes in texture and minimal changes in moisture of queso fresco after HVACP treatment.

The third phase of the study (Chapter 4) examined the effect of direct HVACP treatment in inactivation of *Listeria innocua* on queso fresco crumbles using two gas environments (air and MA50: 50% N_2, 50% CO_2). Air HVACP treatment was found to be more effective in *Listeria innocua* inactivation over MA50 plasma treatment, which is likely due to the higher amount of reactive oxygen species (ROS) generated in air plasma. As discussed in the early sections, ROS play an essential role in microbial inactivation, which result severe oxidative stress to cell membrane and intracellular components leading to cell death. In this study, the optimal HVACP treatment conditions were identified as dry air for four minutes and six minutes at 100 kV. Under these two conditions, a 0.8 $\log_{10}$ CFU/g and 2.6 $\log_{10}$ CFU/g LI reduction was achieved after four and six minutes treatment, respectively, while the quality of the cheese still retained. Thus, these two conditions were utilized for the 28 days storage study.

The fourth phase of the study (Chapter 5) assessed the effect of cold plasma treatment in reduction of *Listeria innocua* as well as background microflora on queso fresco crumbles with 28 days storage at 4 °C. Four and six minutes direct HVACP treatments in dry air demonstrated its capability in inhibiting the growth of both *Listeria innocua* and background microflora on queso fresco until the end of the storage period. At the end of the storage, ~1.0 $\log_{10}$ CFU/g *Listeria innocua* and background microflora was observed in treated cheese, while greater than 6.0 $\log_{10}$ CFU/g population was found in untreated cheese.

Overall, we demonstrated the efficacy of HVACP treatment in microbial inactivation, for both *Listeria innocua* and background microflora, on queso fresco while still maintaining the quality of the cheese. These findings demonstrate a potential of HVACP treatment to remove post-processing pathogenic contaminants from soft cheeses delivering a pathogen-free product with an extended shelf-life.

Future work

HVACP treatment can be further optimized by examining a wider range of gas blends for microbial inactivation along with quality assessment. In this study, reactive oxygen species (ROS) were found to play a significant role in microbial inactivation during HVACP treatment. Dry air (~21% O_2) was observed being the most effective gas blend among the three gas blends utilized in this study. It would be beneficial to investigate gas blends with various O_2 concentration (5% - 20%) identifying the optimal concentration of O_2, which would result an adequate microbial inactivation in a reduced treatment time with minimal effects on cheese quality (such as lipid oxidation resulted from reaction with ROS).

After successfully establishing the optimal HVACP treatment condition, toxicology screening should be also conducted to identify any potential hazardous chemicals in cheese after HVACP treatment. Mutagenicity testing can be carried out *in vitro* in different bacteria (e.g. Ames test) and mammalian cells examining any mutations in DNA (Parasuraman S., 2011). Amounts of nitrate, nitrite, and peroxide need to be evaluated in treated cheeses ensuring that they are within the limits required by Code of Federal Regulations. After assuring that there are no potential hazardous chemicals in HVACP treated cheese, descriptive sensory evaluation, including appearance, color, flavor and overall acceptance, can be performed to understand the acceptability of HVACP treated cheese. Paired comparison tests can also be carried out to evaluate any differences between HVACP treated cheeses and commercial cheeses.

Moreover, inactivation of yeasts and molds on cheese by HVACP treatment should be investigated, as they are the major cause for spoilage of dairy products (Garnier et al., 2017). Cheese could be inoculated with *Penicillium* and *Aspergillus*, two of the major spoilage fungi in cheeses (Hymery, N. et al., 2014) and treated by HVACP treatment for evaluating the efficacy of

HVACP in inactivation of fungi in cheeses. Successful inactivation of spoilage fungi in dairy products would really help reduce the economic losses. Lastly, in-depth studies on plasma-chemical kinetics should be performed to further understand the inactivation mechanisms and the interaction between reactive gas species and the food products allowing to fine-tune this technology for industrial application.

References

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