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Effectiveness of Atmospheric Cold Plasma in Inactivating Microorganisms

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EFFECTIVENESS OF ATMOSPHERIC COLD PLASMA IN INACTIVATING MICROORGANISMS

By

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THESIS ACCEPTANCE PAGE

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This thesis is approved as a creditable and independent investigation by a candidate for the master's degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ABBREVIATIONS

AT: After Treatment

BT: Before Treatment

CAP: Cold Atmospheric plasma

CFU: Colony forming Unit

CP: Cold Plasma

DBD: Dielectric Barrier Discharge

LA: Luria Agar

LB: Lysogeny Broth or Luria Bertani

PAW: Plasma Activated Water

PBS: Phosphate Buffered Saline

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

UV: Ultraviolet

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ABSTRACT

EFFECTIVENESS OF ATMOSPHERIC COLD PLASMA IN INACTIVATING MICROORGANISMS

NADEE SHANIKA KALUWAHANDI

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In the food processing industry, Cold Plasma (CP) is an emerging green process with a number of potential applications. Cold plasma has mostly been used to reduce microbial counts in foodstuffs and biological materials as well as in different levels of packaging, particularly in cases where there is thermal sensitivity. Recent studies have demonstrated that CP technology is being developed for use in the food and agriculture industries. The primary focus is on the interactions between reactive species and food-borne microbes to inactivate them. The literature review discusses both proven and potential applications for cold plasma in food processing, as well as the effects of antimicrobial efficiency and the different types of reactor designs. In the next chapter, the effect of ambient cold plasma treatment with varying treatment time and discharge frequency will be investigated, to determine its ability to inactivate *Escherichia coli K-12*, *Salmonella Cerro*, *Salmonella Dublin*, and *Enterotoxigenic Escherichia coli* cells on the beef surface. Furthermore, these studies show that cold plasma can be used for the surface decontamination of high moisture beef slices and for the effective use of cold plasma activated water using two types of reactors. A novel plasma leap water reactor was investigated in the next chapter for its antimicrobial efficacy against Escherichia coli, one of the most common bacteria. A range

of five different discharge frequencies between 500 Hz and 1500 Hz and six different treatment times between 2 seconds and 60 seconds was tested in distilled water in order to test the efficacy of the water reactor. The results suggest that all bacteria (6 log) are completely inactivated within 5 seconds of being treated with PAW generated with plasma leap water reactors. Cold plasma is an eco-friendly alternative to conventional methods of food preservation and is used in many applications.

Keywords: Cold Plasma, Food processing, Food pathogens, Plasma Activated Water

CHAPTER 01 LITERATURE REVIEW OF OPPORTUNITIES AND CHALLENGES OF COLD PLASMA IN FOOD PROCESSING

1.1 ABSTRACT

Traditionally, thermal processing methods have been utilized to ensure a safe food supply. In the past three decades, non-thermal processing methods, such as high-pressure processing (HPP), pulsed electric field (PEF), irradiation, ultrasound, ozone, ultraviolet (UV) radiation, and pulsed light, have been extensively researched. High-pressure processing, irradiation, ultrasound, and ozone technologies have been successfully commercialized in the food industry. Food can be subjected to high-pressure processing to destroy disease-causing microbes, which, when used properly, does not change the freshness of the food. Ozone gas can also be utilized by food processors to kill microbes while maintaining the freshness of fruits, vegetables, and meat. Additionally, UV light or pulse light can be used to make food safer without severely damaging its quality. Recently, cold plasma technology has attracted the attention of researchers in academia and the food industry. Cold plasma, also known as non-thermal plasma, is a well-ionized plasma generated under atmospheric conditions at near ambient temperatures or in vacuum at temperatures ranging from 30 ^{o}C to 60 ^{o}C, requiring less energy. It can be used for microbial inactivation or surface decontamination for food products such as meat, grains, dairy products, fresh vegetables and fruits, and the production of cold plasma activated water to address major food safety concerns worldwide. Cold plasma has also been successful in decontaminating herb and spice samples that contain fungi such as

Aspergillus flavus or *Aspergillus brasiliensis*. Additionally, the cold nature of plasma prevents volatiles from escaping, preserving its nutritional benefits, which is a problem encountered with several thermal processes already tested. The shelf-life of meat and fish products may also benefit from cold plasma treatment by reducing mold or yeast populations as well as killing *Listeria innocua* in ready-to-eat meats. Furthermore, it can modify the surface properties of food and packaging materials as well as enhance mass transfer on the surface. Based on a review of previous studies on cold plasma in various food products, the current status of the cold plasma process, new reactor designs, the chemistry of cold plasma interactions with various food constituents, antimicrobial efficiency, and the effectiveness of plasma activated water are discussed. While cold plasma offers numerous opportunities for the agri-food industry, further research is still needed. This includes the examination of the degradation products of targeted compounds after plasma treatment, understanding the chemical mechanisms by which the process takes place, and determining whether the toxicity of degraded compounds is greater than that of the original compounds, which could compromise the safety of the food supply.

Keywords: cold plasma, decontamination, food processing, food quality, food safety

1.2 INTRODUCTION

In 1928, Irving Langmuir introduced the term "plasma" by describing the oscillation in ionized gases with the same number of ions and electrons by using the early experiment (Bermudez-Aguirre, 2019), which were conducted with air and argon done by Penning in 1926. During the three decades beginning in 1960, cold plasma was primarily used for bacterial inactivation through a dielectric discharge device. Moreover, the first cold plasma application for inactivating bacteria was done by a dielectric barrier discharge device in 1990. Since 1990, there has been extensive research being conducted to explore the applications of cold plasma in the food processing sector as cold plasma is a unique, economical, and environment friendly green technology.

In the food manufacturing sector, cold atmospheric plasma has the potential to inactivate microorganisms, thus improving food safety. The food industry must supply safe foods with minimal processing to meet the growing demand for fresh produce. As many products are eaten raw, it is crucial that they do not contain any microbial contamination. There is a growing interest in developing new methods for preserving foods and destroying microorganisms without affecting its quality. The cold atmospheric plasma (CAP) treatment is an emerging technology that has proven to be a successful disinfectant method in the food industry. An overview of cold plasma technology is presented along with its potential applications in the food processing sector.

The science of plasma is known as the fourth state of matter and it is energized electrically (Mandal et al., 2018). Plasma can be classified into two major types: thermal plasma and non-thermal or cold plasma (Thirumdas et al., 2015). Cold plasma is generated at

temperature ranging between 30 °C and 60 °C under atmospheric pressure and consists of many excited atomic, molecular, ionic and radical species, which co-exist with numerous reactive oxygen species (ROS) such as O, O2, O3, and OH and reactive nitrogen species (RNS) such as NO, NO2, NOx and ultraviolet radiation (UV) (Paula Bourke et al., 2018; Tolouie et al., 2018). These include electrons, positive and negative ions, free radicals, gas atoms, molecules in the ground or excited state and electromagnetic radiation. A plasma is typically created by applying electrical energy to a gas that exists between two electrodes with a large electrical potential difference, causing gas to ionize (Mandal et al., 2018). Among the emerging technologies, cold plasma is leading as a novel technology for food processing and preservation.

This study mainly presents a brief introduction regarding the cold plasma technology and how it is related to food processing and preservation, such as microbial inactivation, disinfection of different types of food, mechanisms of cold plasma treatment, principles of different cold plasma generating devices, different types of cold plasma treatment technologies, benefits of cold plasma and the present status of this novel technology.

1.3 PLASMA SCIENCE AND TECHNOLOGY

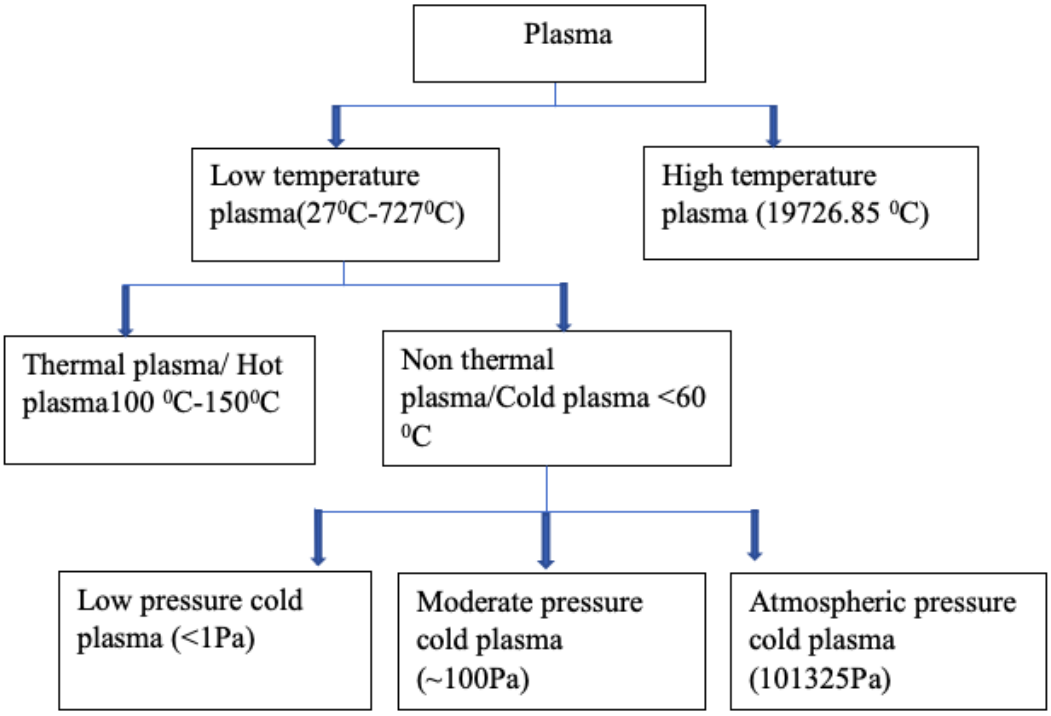


Figure 1.1: Classification of plasma

As shown in Figure 1.1 plasma can be divided into two main categories: low temperature plasma, also known as thermally non-equilibrium plasma, and high temperature plasma, also known as thermally equilibrium plasma (Sonawane, 2020). High temperature plasma is generated when a gas is heated to almost 19,726.85 °C, or 20000 K. Low temperature plasma is further divided into two subcategories: thermal plasma, also known as Quasi equilibrium plasma, and non-thermal plasma, also known as non-equilibrium plasma or cold plasma. Thermal plasma operates at high gas temperatures and particle species exist

in a local thermal equilibrium state. In contrast, cold plasma can be generated at low gas temperatures and is characterized by non-equilibrium particle distributions. Cold plasma can also be generated at different pressures, including low-pressure cold plasma (<1Pa), moderate cold plasma (~100Pa), and Atmospheric cold plasma (101,325Pa). Among these, atmospheric cold plasma has played a significant role in the food industry.

1.4 PRINCIPLES OF COLD PLASMA

According to Laroque et al. (2022), the formation of free radicals, including reactive oxygen species and reactive nitrogen species, as well as UV light, positive and negative ions, and electrons, can occur in air. These contribute to the strong oxidation of lipids and proteins. Understanding the plasma chemistry in liquid and gaseous phases is crucial, as it involves thousands of reactions and dozens of species. Commonly associated with antimicrobial activity and inactivation cascades are reactive oxygen species such as hydrogen peroxide (H2O2), ozone (O3), superoxide anion (O2•−), hydroperoxyl (HO2•), alkoxyl (RO•), peroxyl (ROO•), singlet oxygen (1O2), hydroxyl radical (•OH), and carbonate anion radical (CO3•−). Examples of reactive nitrogen species include nitric oxide (NO•), nitrogen dioxide radical (•NO2), peroxynitrite (ONOO−), peroxynitrous acid (OONOH), and alkylperoxynitrite (ROONO) (S. K. Pankaj et al., 2014). Additionally, the antimicrobial process involves a variety of molecules such as atoms, metastable, radicals, and molecules that are electronically and vibrationally excitable (Bourke, 2017). This paragraph aims to summarize the mechanisms behind the inactivation of microorganisms under plasma treatment.

1.5 APPLICATIONS OF COLD PLASMA

Cold plasma technology has been found to have a range of potential applications in non-food industries, such as enhancing the adhesion and printing properties of polymers, increasing the surface energy of materials, and improving glass and paper properties (Varilla et al., 2020). Recently, there has been increasing interest in the use of cold plasma technology in agri-food industries. Cold plasma technology is considered to be a green process and has been noted for its potential role in microbial deactivation, which could improve the shelf-life, food quality, and safety of agricultural products (Gavahian & Khaneghah, 2020). As research in the field of plasma technology continues to develop, there is a growing emphasis on gaining a deeper understanding of the mechanisms behind the inactivation of different pathogens in various food products. Researchers have conducted studies to test the effectiveness of cold plasma in decontaminating a wide range of food groups, including fresh fruits and vegetables, meats and meat products, milk and dairy products, eggs and egg products, seafood, fruit juices, powdered products, nuts, cereals, grains, etc. Additionally, cold plasma processing can be used to alter or modify packaging materials, making it a chemical-free, fast, and safe method of sterilization. This method yields no residue and is quick and easy to perform (Pankaj, 2014). Cold plasma technology is also used for surface treatment such as cleaning, coating, printing, painting, and adhesive bonding.

1.6 COLD PLASMA TREATMENT

Several systems, processes, and target parameters have been identified as determining the interaction of cold atmospheric plasma (CAP) treatment with effector molecules and microorganisms. These include the type of device, voltage level, frequency, working gas, gas flow rate, humidity level, distance between the target and the plasma emitter, as well as the type, concentration, and physiological state of the microorganism. The complexity of these factors makes comparisons of reported efficacies difficult. Plasma is formed in the air above the food and in the area beneath the plasma by passing electricity through atmospheric air or another type of gas, such as oxygen, nitrogen, or helium. Gas particles are energized by the electricity, creating reactive species, charged particles that collide with bacteria in food and destroy the chemical bonds that hold the protective cell wall in place (Bourke et al., 2017). As a result of damage to the cell wall, particles penetrate bacteria and destroy their interior organs, including DNA and proteins, lipids, membranes, and other components in microorganisms, thereby killing the bacteria. Generally, three different action mechanisms have been proposed for plasma-mediated bacterial cell inactivation: firstly, direct permeabilization of the cell membrane or cell wall; secondly, critical damage of intracellular proteins from oxidative or nitrosative species; and finally, direct chemical damage of DNA (Rød et al., 2012). As shown in Figure 1.2, a picture of a plasma beam coming from the plasma leap system illustrates the two distinct regions: the active region and the remote region. Research in the fields of food and bio-decontamination uses direct (active) and indirect (remote) plasma treatments for practical reasons.

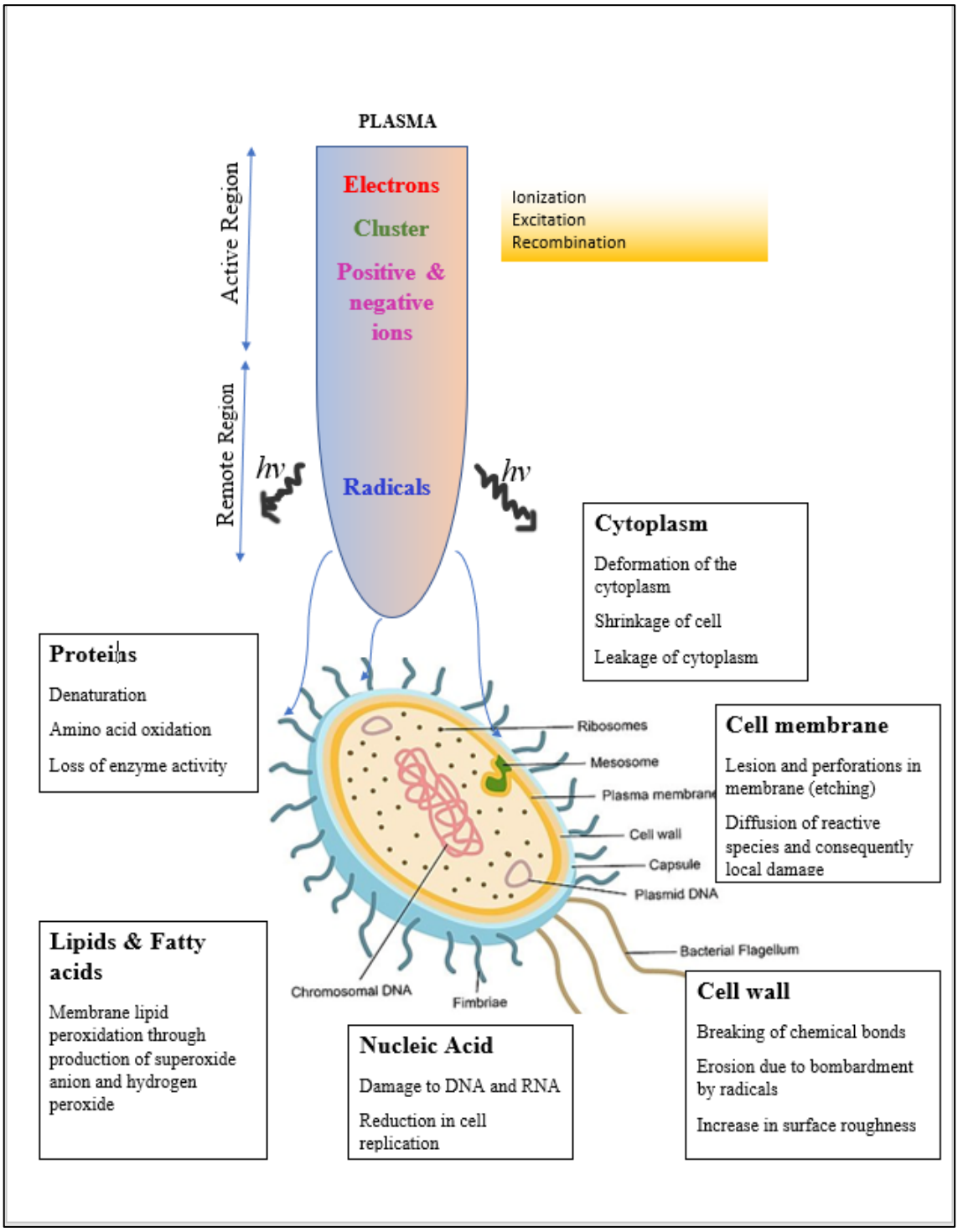


Figure 1.2: Effect of cold plasma on bacterial cell

1.7 EFFECTS OF PLASMA TREATMENT OF FRESH FOOD PRODUCTS

1.7.1 Color Parameters

Among the various parameters considered by consumers, the color of fresh food is considered to be an indicator of its freshness and quality. Appearance plays a direct role in the acceptance of fruits, vegetables, and meat products, and it influences consumers' preferences for purchasing these products. Previous literature has shown that after plasma exposure, the L* (brightness) and b* (yellow- blue) values of a meat sample do not show significant changes compared to the untreated control sample (Bermudez-Aguirre, 2019). However, the a* (red-green) values of meat samples are significantly lower than those of untreated samples.

1.7.2 Water Content and Water Activity

A significant decrease in the water content of fruits, vegetables, meat and food products during cold plasma treatment was observed in the previous studies. In the similar manner, the water activity values were also decreased during the cold plasma treatment (Yadav et al., 2019).

1.8 OPERATING PRINCIPLES OF DIFFERENT COLD PLASMA GENERATING DEVICES

The types of discharge depend on some of the properties of the power sources, such as direct current and alternating current, low-pressure plasma, atmospheric pressure plasma, and the different types of electrodes.

1.8.1 Corona Discharge

Corona discharge is a stream of charged particles such as electrons and ions that are accelerated by an electric field (Bermudez-Aguirre, 2019). This is created at atmospheric pressure with low frequency or pulsed high voltage or high voltage over two electrodes which have a big difference in size. This type of plasma contains a series of small lighting type discharges. It is a promising technology for controlling postharvest mold rot during cold storage or preventing contamination in a controlled growth environment (Munekata et al., 2020).

1.8.2 Dielectric Barrier Discharge

Dielectric Barrier Discharge (DBD) is a method that has been utilized by scientists, including Siemens, to generate cold plasma at atmospheric pressure (Varilla, 2020). This method has various applications, such as sterilization, inactivation of microorganisms/bacteria, and surface treatments. Typically, DBD consists of two metal electrodes, one of which is high voltage and the other grounded, which are covered with dielectric materials. A carrier gas is utilized to move between the electrodes to create plasma. The high voltage electrode plays a crucial role in producing the discharge and generating the plasma (Bermudez-Aguirre, 2019). Although there are various types of electrodes used in DBD, the fundamental concept behind them remains the same. It was discovered in 1990 that cold plasma had the potential to inactivate bacteria using DBD,

thereby reducing the concentration of an initial load of bacteria such as E. coli or Pseudomonas aeruginosa (Braný et al., 2020).

1.8.3 Plasma Leap System

According to the Plasma leap Technologies Leap 100 user manual, the Plasma leap system is non-thermal and protects bio molecules against thermal damage in the plasma source system. Unlike other systems, this system does not require an expensive noble gas, but instead uses atmospheric air. The system boasts a power supply unit with a discharge voltage of up to 80 kV, a resonance frequency of 30-125 kHz, a discharge frequency of 50 Hz-3000 Hz, and power ranging from 50 W to 700 W. Additionally, the Plasma leap system features two types of reactors, including a pin reactor for discharge in open air and a water reactor for discharge under water. One of the major advantages of this system is its ability to treat 96 well plates for data-rich experiments. Furthermore, it is considered one of the most technologically advanced systems compared to other plasma systems.

1.8.4 Plasma leap pin Reactor

A novel large gap pin-to-plate plasma reactor (Leap100, Plasma Leap Technologies, Sydney, Australia) was utilized in the experiment. Two steel plates were connected to a high voltage electrode using a pin array (11 * 8) and a flat ground electrode. The electrode pins were placed in a convex pattern, resulting in a homogeneous plasma discharge across the array, with the core pins closer to the ground electrode. The power supply unit of the

Plasma Leap pin reactor included a discharge voltage of up to 80 kV at pulse frequencies in the range of 100Hz to 3000Hz, a resonance frequency of 30-125 kHz, a voltage of 50-325 V, a time range of 0-900 seconds, and a power range of 50 W to 700 W.

1.8.5 Plasma Leap Water Reactor

The Plasma Leap water reactor, also known as the bubble water reactor, is manufactured by Plasma Leap Technologies in Sydney, Australia. According to the user manual of the Plasma Leap 100, the system is composed of several major components: a high voltage power unit, a controller, a bubble water reactor, a ground electrode, and a rotameter. The high voltage power supply unit provides high voltage pulses of up to 80kV at pulse frequencies in the range of 100 Hz to 3000 Hz, with an output power range of 50 W to 700 W. The controller features digital and touch screen controls and allows for the alteration of parameters during operation. The water reactor, which is constructed of stainless steel, is covered with acrylic spacers and features several holes to allow plasma to escape into the water. The reactor is equipped with Polytetrafluoroethylene (PTFE) T-fittings. The rotameter is used to control the airflow rate and is attached to a machined Teflon fitting on the outside tubes of the gas source. The high voltage supply is connected to the stainless-steel bubble reactor, while the ground electrode of the high voltage supply is attached to the stainless-steel ground probe.

1.8.6 Microwave Discharge

Microwave discharge is a method of generating plasma that uses frequencies between 300 MHz and 300 GHz. Unlike other methods, it does not require electrodes as plasma is generated within the process chamber through the use of waveguides, coaxial cables, or antennas to excite electrons in the process gas. This method is considered to be easy to handle as a result of the electron absorption of microwaves, which increases the kinetic energy of the electrons and leads to ionization reactions through inelastic collisions. (Coutinho et al., 2018).

1.8.7 Radio Frequency Discharge

Radio frequency discharge is commonly used in the food processing industry for various applications such as drying, baking, thawing of frozen food, pasteurization, sterilization, and disinfection. The radio frequency wavelengths used in this process cover the range of the electromagnetic spectrum from 3 kHz to 300 MHz, as reported by McHugh (2016). In this method, radio frequency energy is generated by a triode valve and applied to the food via a pair of electrodes, resulting in the generation of plasma around the electrodes. The plasma generated in this process can be either capacitively coupled plasma, generated around the electrodes when applying the RF power, or inductively coupled plasma, generated inside the coil of the antenna of coiled shape electrode when supplying with radio frequency power. As noted by Bermudez-Aguirre (2019), the system produces high-frequency plasma inside the coil. The main advantage of this reactor is that materials are disinfected with minimal damage, and a highly dense plasma is generated using minimal heating under low atmospheric pressure plasma.

1.8.8 Plasma Jet

The Plasma jet is commonly used in medical technology with the aim of targeting biological tissue in normal room conditions. The Plasma jet travels a small amount of plasma at supersonic velocity, similar to plasma bullets. Atmospheric pressure plasma jets are operated in open air at moderate gas temperatures, but with high plasma-chemical activity, triggering a variety of interesting applications. These sources produce charged particles, neutral metastable species, radicals, and UV radiation at biologically tolerable gas temperatures, making them important for biomedical applications and the food processing industry. Studies on the treatment of food-related materials can be conducted using a plasma jet. The plasma source, for example, consists of a ceramic nozzle with a needle electrode at its center and an outer electrode grounded to Earth. Ionized gas is ejected from the source after flowing between the electrodes (Knorr et al., 2011). Additionally, plasma jets have enormous potential in the fields of surface activation and modification, as well as the deposition of thin films.

1.9 EFFECT OF COLD PLASMA ON REDUCING MICROORGANISMS

1.9.1 Bacterial Inactivation Under Cold Plasma

In the United States, there are numerous instances of foodborne outbreaks associated with fresh produce. While several disinfection methods exist, emerging pathogens are becoming increasingly resistant to them. Additionally, consumers are increasingly seeking food that is free of chemicals. Cold atmospheric plasma technology has emerged as a solution in the

food industry for deactivating bacteria on food surfaces without causing harm to the food materials. Cold plasma generates a variety of physical and chemical effects on cells that result in microbial death. During a cold plasma treatment, multiple events occur simultaneously, including the emission of UV radiation, the generation of ozone, other free radicals, ions, and electrons. Cold plasma has been shown to affect DNA, proteins, and cell membranes (Thirumdas et al., 2015). The effects of OH radicals on microbial cells can initiate the oxidation process, leading to damage to the cell membrane. Free radicals can oxidize unsaturated fatty acids, damage the unsaturated lipid bilayer of the cell wall (Bermudez-Aguirre, 2019), and break peptide bonds and oxidize amino acid chains through peroxide radicals.

1.9.2 Mold and Yeast Inactivation Under Cold Plasma

In the food and food supplement industry, mold and yeast play a significant role in various types of products. Molds are also responsible for a significant amount of food spoilage worldwide. To prevent mold contamination, various methods are employed, including the use of fungicides, disinfectants, and liquid disinfectants. However, the Cold Atmospheric Plasma (CAP) technology has emerged as a promising and effective method for inactivating mold without the use of chemicals. Studies have already proven its effectiveness in inactivating microbial pathogens and removing them from food surfaces (Park et al., 2020). The principle of this method involves the generation of nonequilibrium atmospheric pressure plasma, which results in the production of a wide spectrum of reactive oxygen and nitrogen species at low temperatures. These species, due to their high oxidative potential, play a significant role in the biological activity of CAP (Hojnik,2019).

1.9.3 Spores' Inactivation Under Cold Plasma

The factors that affect the resistance of spores to cold plasma, such as processing parameters, environmental elements, and spore properties, have been well-studied. Previous research has shown that the inactivation targets in spore cells, such as outer structure, DNA, and metabolic proteins, can be inactivated by cold plasma (Bermudez-Aguirre, 2019). Different models, such as nonlinear biphasic inactivation models and linear first-order kinetic models, have been used to investigate the inactivation kinetics of spores treated with cold plasma. The establishment of predictive mathematical models, which take into account key affecting factors such as gas types, humidity, and spore resistance, is important for accurate description of the sporicidal activity of cold plasma and its industrial applications. Cold plasma is likely to directly destroy spore cells, inhibiting germination instead of promoting it (Liao et al., 2019). However, a lack of understanding persists concerning the biochemical mechanisms behind cold plasma's inactivation of spores.

1.9.4 Viruses' Inactivation Under Cold Plasma

The current methods of disinfection for viruses have several disadvantages, such as contamination with products and difficulties associated with their use. These methods include UV radiation and chemical disinfectants, both of which have limitations. UV radiation treatment can take a long time and chemical disinfectants can leave behind byproducts of contamination (Guo et al., 2018). Cold plasma, however, when used with argon mixed with air and plasma activated water, has been found to have strong antiviral activity after long-term storage (Bermudez-Aguirre, 2019).

Viruses are individual structures that consist of a virus genome and capsid. The mechanism of inactivation of viruses on food surfaces is related to the capsid and genome. Reactive

oxygen species generated during cold plasma treatment can potentially damage the polypeptide chains of the virus, resulting in perforation of the viral capsid (Liao et al., 2019).

To inactivate viruses, various physical and chemical treatments have been used. UV irradiation, filtration, pressure, and temperature are physical methods of disinfection. The choice of method depends on the matrix to be disinfected and the virus to be inactivated. Waterborne viruses and plant viruses are among the most stable, and the disinfection method must be strong enough to inactivate such stable viruses in a delicate matrix while also being non-toxic to maintain the quality and properties of the matrix. Traditional methods of disinfecting water, such as chlorination, do not effectively kill some viruses, and in the long run may pose a health risk due to the release of toxic byproducts. Recently, new technologies for inactivating waterborne viruses have been developed, such as membrane filtration, reverse osmosis, UV and ozone treatments, and hydrodynamic cavitation. These technologies have a number of disadvantages, including inefficiency, scalability problems, and unsustainable energy consumption (Filipić et al., 2020). Laboratory-scale studies suggest that cold plasma has the potential to overcome these problems, but confirmation will require studies focused on pilot or industrial-scale deployment of plasma-based disinfection devices and new technological methods.

1.9.5 Parasites Inactivation Under Cold Plasma

There is limited research on the inactivation of parasites using various new and old technologies. For example, ultraviolet light can cause minor damage to the organism, but it greatly reduces the rate of reproduction (Bermudez-Aguirre, 2019). Ozone has been found to inactivate some types of parasites due to its effect on protein degradation and

modification of the cyst wall structure. While UV and Ozone have been shown to be effective in inactivating certain parasites, it is highly probable that cold plasma could also be effective in inactivating emerging parasites on food surfaces. This is an important area of research that should be addressed in the near future.

1.10 EFFECT OF COLD PLASMA IN DISINFECTION OF MICROBES IN HIGH MOISTURE FOODS

Cold plasma food processing has been found to be effective in inactivating microorganisms in a variety of food products, as demonstrated in Table 1.1.

1.10.1 Fresh Vegetables

Cold plasma, when used in combination with nitrogen gas, has been demonstrated to effectively inactivate E. coli cells in fresh vegetables, as reported in a study by Chen et al. (2020). Following the treatment, a sensory evaluation was conducted on the treated vegetables, and no significant differences were observed between the control and treated samples in terms of color, appearance, and taste.

1.10.2 Fresh Fruits

Blueberries were treated with a plasma jet for varying treatment times, with a maximum of 120 seconds, and under atmospheric pressure. The berries were stored at 4 ^{0}C for 7 days.

According to previous literature, it was observed that treatment times longer than 60 seconds had an impact on the quality of the berries, particularly in terms of firmness (Lacombe et al., 2015).

1.10.3 Meat

Cold plasma treatment has been shown to be an effective method for inactivating microorganisms in meat. Research has demonstrated that replacing the working gas of oxygen with other gases can lead to more effective inactivation without causing significant quality issues, such as color degradation or lipid oxidation (Rød et al., 2012). As such, further research investigating the use of higher voltage cold plasma treatments as an alternative method for eliminating microbial problems in meat while maintaining its quality is warranted.

1.10.4 Fish

Research has shown that cold plasma has strong oxidative potential and that fish, due to its high amount of fatty acids, can be easily oxidized using cold plasma processing (Bermudez-Aguirre, 2019). Studies involving the Cold Plasma treatment of Herring fillets with dielectric barrier discharge have demonstrated that high voltages up to 80kV are most effective for microbial inactivation and extending shelf life. However, it has also been noted that this method can lead to some quality issues such as oxidation and color changes. Furthermore, it has been observed that when cold plasma treatment is applied at lower voltages, while microbial inactivation may be less effective, the resulting quality of the fillets is much better than that of the fillets treated with high voltage cold plasma.

Table 1.1: Microbial Inactivation in Different Food Products and Processing Conditions Using Cold Plasma Food Processing.

Product	**Microorganism**	**Processing condition**	**Reference**
Blueberries	*Escherichia coli*	Cold plasma jet 120s Dielectric barrier discharge 5, 10 & 20 mins	(Bermudez-Aguirre, 2019)
Strawberries	Aerobic bacteria and yeast and mold	DBD 60kV,50Hz, 42%relative humidity, 5min	(Misra et al., 2014)
Apple juice	*Citrobacter freundii*	Cold plasma jet 480s Dielectric barrier discharge 15, 60 & 300 s.	(Surowsky et al., 2013)
Pork	*Escherichia coli*	Indirect microwave powered cold plasma 2 x 2.5 and 5 x 2mins.	(Bermudez-Aguirre, 2019)

Chicken	*Escherichia coli*	Dielectric barrier discharge 3mins 30kV,0.5kHz0.15W/cm^ 2	(Bermudez-Aguirre, 2019)
Brown rice	*Escherichia coli*	Dielectric barrier discharge 20min, 250W, 15kHz	(Liu et al., 2021)
Cantaloupe	*Sallmonella spp*	OAUGDP,9kV, 6kHz, 25 ^{0}C, 1min	(Song et al., 2020)
Cabbage	*Listeria monocytogens*	Microwave powered cold plasma 400-900w, 667kPa, 1-10min,	(Lee et al., 2015)
Mandarins	*P. italicum*	Microwave powered cold plasma, 0.7kPa, 900W, N2, 10min.	(Won et al., 2017)
Iceberg lettuce	*Listeria monocytogens*	OAUGDP,9kV, 6kHz, 25 ^{0}C, 3-5mins	(Ziuzina et al., 2015)
Mangoes and melons	*Escherichia coli*, *Saccharomyces cerevisiae*	Cold Plasma pen 12-16kV, 30kHz	(Perni et al., 2008)
Tomato	*Escherichia coli*	DBD 15and 60kv from 5 to 30min.	(Prasad et al., 2017)
Radish	*S. typhimurium*	Microwave powered plasma 900W,667Pa, from 2 to 20 min	(Bermudez-Aguirre, 2019)
Chicken breast fillet	*Campylobacter jejuni*	Cold plasma jet, 30-180s 1MHz, 2-3kV, argon, air	(Wang et al., 2016)
Egg Shells	*Salmonella enteritidis* and *Salmonella typhimurium*	Cold nitrogen plasma (400W,20min	(Wan et al., 2017)

Pork Slices	Aerobic total plate count	Microwave-powered plasma 2.45 GHz, 1.2 kW, air, indirect treatment 2 x 2.5 and 5 x 2mins.	(Fröhling et al., 2012)
Fresh and frozen pork	*Escherichia coli*	Corona discharge plasma jet, 20kV, 58kHz	(Choi et al., 2016)
Bacon	*Listeria monocytogens*	DBD, 13.56MHz, 125W, He + O2, 90s	(B. Kim et al., 2011)
Fresh beef lion	*Listeria monocytogens*	Flexible thin layer DBD plasma 15kHz, 100W, 10 min N2+02.	(Jayasena et al., 2015)
Milk	Aerobic bacteria *Escherichia coli*	Encapsulated air DBD, 250W, 15kHz, 5 and 10 min	(H. J. Kim et al., 2015)

1.11 CHALLENGES FOR COLD PLASMA IN FOOD PROCESSING

In the short period of time that cold plasma (CP) has been used, it is encouraging to see a gradual appreciation of its potential, particularly in a country that relies heavily on agriculture and food production. However, the technique is still in its infancy and further research is required to fully understand the potential of cold plasma in the agri-food industry. This includes understanding the chemical mechanisms that occur throughout the process and whether the toxicity of the degraded compounds is more harmful than the toxicity of the original compounds, potentially compromising food safety. Additionally, despite being energy efficient with a low energy output, cold plasma processing costs are

largely determined by the expensive noble gases, helium and argon. If nitrogen can be identified as a profitable alternative, such costs will be significantly reduced. Furthermore, appropriate gas destruction and exhaustion are required, as are the necessary safety precautions when using extremely high voltages. Cold plasma has the potential to alleviate allergen, pesticide, microbial, and other public health concerns, as well as to treat infected animals, improve seed germination, and clean bacteria-infested surfaces. At a time when food safety and waste reduction are under intense public scrutiny, cold plasma technology could be one of the most important emerging techniques to help restore consumer trust.

1.12 EFFECT OF COLD PLASMA ON PACKED FOOD AND PACKAGING MATERIALS

Cold plasma technology has been applied in the food industry to decontaminate packaged foods and sterilize packaging materials without altering the properties of the food or package. Cold plasma technology can decontaminate in packaged foods and to sterilize packaging materials without changing any significant property either the food or the package (Pankaj, 2014). The use of dielectric barrier discharge has been particularly effective in inactivating bacteria and reducing pesticides in fresh foods. Previous studies have focused on the in-package treatment of fresh produce such as fruits and vegetables, while recent studies have explored the coating of antimicrobial compounds onto food packaging materials to enhance shelf life. A recently developed plasma sanitation system has been used to effectively decontaminate food packaging from SARS-CoV-2 RNA (Capelli et al., 2021). This treatment did not significantly affect packaging material

performance or shelf life of packaged products, indicating that it could be used for the decontamination of packaged products without compromising their safety or quality.

1.13 EFFECT OF COLD PLASMA ON FOODBORNE DISEASES

According to data from the Centers for Disease Control and Prevention (CDC), foodborne illnesses caused by known pathogens are estimated to result in approximately 14 million illnesses, 60,000 hospitalizations, and 1,800 deaths in the United States each year (Borchers et al., 2010). Common bacterial pathogens associated with foodborne diseases include over 2,300 types of *Salmonella*, over 30 types of *Shigella*, *Campylobacter jejuni*, strain 0157: H7, and several strains of *E. coli*. Additionally, *Listeria monocytogenes*, *Clostridium botulinum*, *Vibrio*, and *Yersinia*, as well as parasites such as *Cryptosporidium*, *Cyclospora*, and *Giardia* can also cause foodborne illnesses (Borchers et al., 2010). In recent years, non-thermal methods such as ozone and cold plasma have gained increasing importance in the decontamination of food-borne pathogens in order to develop food products with longer shelf lives and preserve their nutritional and organoleptic characteristics (Niveditha et al., 2021).

1.14 COLD PLASMA ACTIVATED WATER

The discharge of cold plasma to a water surface or through plasma gas bubbles can produce plasma activated water (PAW). The interaction of the plasma with the water at the liquid interface generates reactive species. The concentration and type of reactive species

generated is determined by the reactor design and the parameters used for PAW generation. Reactive oxygen and nitrogen species are the main bactericidal agents in PAW (Rothwell et al., 2021). Depending on the storage conditions, nitrite, nitrate, and hydrogen peroxide can last for several months and can also be preserved by freezing. The presence of hydrogen peroxide, nitric and peroxy nitrous acids, makes PAW acidic and bactericidal. As a result of the individual and synergistic effects of each reactive species, PAW is antimicrobial. PAW has a wide range of potential applications and has a significant impact on health and the food environment. In terms of overall effect, PAW shows similar characteristics to cold plasma and is useful in disinfection, decontamination, and seed germination (Thirumdas et al., 2018) and growth enhancement. Therefore, PAW can be considered an eco-friendly disinfectant.

1.15 ECONOMICS OF COLD PLASMA

According to a Region-Global forecast report, the global cold plasma market is projected to reach USD 3.1 billion by 2024, growing at a CAGR of 15.6% from 2019 to 2024. Cold plasma technology is utilized to extend the shelf life of food products and to enhance their appearance. As a non-thermal processing technology, cold plasma serves the purpose of food safety and preservation by inactivating microbial cells without degrading the nutritional quality of food (Bermudez-Aguirre,2019).

1.16 FUTURE PROSPECTS

Further studies are needed to better understand the application of cold plasma technology in the processing of high moisture foods, such as beef, which is a highly economically important food in the United States. Literature on the UV and ozone treatment for the inactivation of microorganisms suggests that it is highly probable that cold plasma could be effective in the inactivation of emerging parasites on food surfaces. Therefore, further research should be conducted to explore and establish industrial procedures for cold plasma treatment.

1.17 CONCLUSION

In conclusion, the literature suggests that cold plasma technology holds the potential to improve food safety, enhance food quality, and improve packaging properties. The technique can be used to deactivate intercellular food microorganisms where conventional methods cannot be applied. The CP technology operates at atmospheric pressure and ambient temperature, minimizing or eliminating any loss of nutritional or sensory qualities in food products. Cold plasma has been found to have positive effects not only on sensory and nutritional quality, but also on microbial inactivation. It can therefore be considered a promising, economical, and environmentally friendly technology with great industrial potential. However, there is still a need for further research to better understand the potential consequences of plasma treatment, including the chemical mechanisms involved,

as well as why degraded compounds may be more toxic than the original compounds and compromise food safety.

CHAPTER 02: INACTIVATION OF ESCHERICHIA COLI K-12, SALMONELLA CERRO, SALMONELLA DUBLIN, ENTEROTOXIGENIC ESCHERICHIA COLI AND QUALITY CHANGES IN COLD PLASMA TREATED BEEF

2.1 ABSTRACT

Over the past three decades, non-thermal food processing methods such as high-pressure processing, pulsed electric field, irradiation, ultrasound, ozone, UV-Light, pulsed light, and plasma treatment have been studied to improve the shelf life of foods while maintaining good sensory and textural characteristics. Recently, food scientists have become increasingly interested in cold plasma technology for reducing the microbial load in or on food due to its non-thermal, economical, versatile, and environmentally friendly nature. In a recent study, the effect of ambient cold plasma treatment with varying treatment times and discharge frequencies was investigated on inactivating *Escherichia coli K-12*, *Salmonella Cerro*, *Salmonella Dublin*, and *Enterotoxigenic Escherichia coli* cells on beef surfaces. The study also evaluated the effect on quality attributes such as surface color, temperature, and moisture content. Results showed that as the treatment time and discharge frequency increased from 60s to 300s and 2000Hz to 2500Hz, respectively, the log reduction of microbes increased from 0.262 log CFU/ml to 0.567 log CFU/ml in 2000Hz, 0.359 log CFU/ml to 1.3702 log CFU/ml in 2250Hz, 0.3806 log CFU/ml-1.4687 log CFU/ml for *Escherichia coli K-12* and from 0.518 log CFU/ml to 1.13 log CFU/ml for *Salmonella Cerro* in 2000Hz, from 0.550 log CFU/ml to 1.080 log CFU/ml in 2250Hz, and

from 0.66 log CFU/ml to 1.711 log CFU/ml in 2500Hz. Similarly, for *Salmonella Dublin*, log reductions ranged from 0.536 to 0.796 log CFU/ml in 2000Hz, from 0.221 to 0.920 log CFU/ml in 2250Hz, and from 0.294 log to 1.651 log CFU/ml in 2500Hz. For *Enterotoxigenic Escherichia coli*, log reductions ranged from 0.160 to 0.219 log CFU/ml in 2000Hz, from 0.249 to 0.290 log CFU/ml in 2250Hz, and from 0.292 log to 0.367 log CFU/ml in 2500Hz on beef surfaces. No significant differences in the physical properties such as surface color, temperature, and moisture content of beef were observed after cold plasma treatment. However, cold plasma technology in food processing is still in its early stages and requires further research before it can be applied industrially.

Keywords. cold plasma, decontamination, food pathogens, food processing, food quality, food safety

2.2 INTRODUCTION

Innovative technologies in food processing are being developed to address the unique requirements of consumers with a view to ensuring the safety, healthiness, and minimal processing of their food. These innovations have also led to the creation of low-energy consuming food production techniques, thereby overcoming some of the limitations posed by conventional food processing methods. The increasing global population, which is driving the demand for food, water, and energy resources, has led to a corresponding increase in the demand for innovative and sustainable technologies in agriculture and food.

In the meat industry, food processing typically involves freezing, cooling, dehydration, and thermal processes, along with hurdle technologies such as pasteurization that are mainly used for food safety purposes (Misra & Jo, 2017). Thermal processing of meat often results in changes to the meat structure and texture. Over the past two decades, food engineers and scientists have made use of both thermal and non-thermal processing techniques to decontaminate meat. Of all the non-thermal methods, cold plasma is the newest and most effective in enhancing meat quality and safety.

Plasma is an ionized quasi-neutral gas primarily composed of free electrons, protons, and photons, as well as atoms in their fundamental or excited states with a net neutral charge (Shashi K. Pankaj & Keener, 2017). Cold plasma is made up of molecules at temperatures between 30°C and 60°C and at atmospheric pressure (P. Bourke et al., 2017). This study utilized the plasma leap pin reactor, with atmospheric air instead of the more expensive noble gas. The plasma leap system comprises a power supply unit with a discharge voltage of 80Kv, a resonance frequency of 30-125kHz, a discharge frequency of 50Hz-3000Hz, and a power range of 50W-700W. There are two types of reactors within the plasma leap system: a pin reactor, used for open-air discharge, and a water reactor, used for underwater discharge (Kaluwahandi et al., 2020). This study utilized several different types of food-borne pathogenic and non-pathogenic bacteria, including *Escherichia coli K-12*, *Salmonella cerro*, *Salmonella dublin*, and *Enterotoxigenic Escherichia coli*. For example, salmonella infection in dairy cows can be transmitted to humans through contaminated food such as raw milk and beef, and in the US, salmonella is responsible for more foodborne deaths than any other pathogen (Lee, 2015). The prevalence of *Salmonella Dublin* in boneless beef was found to be 52.8%, while its prevalence in ground beef was 20.5%. Salmonella Dublin had lower indicator levels compared to other salmonella strains. It is estimated that salmonella causes 1,000,000 cases, 19,000 hospitalizations, and 350 deaths annually in the United States (Vial et al., 2020). Over the past decade, foodborne outbreaks have been largely caused by pathogenic bacteria, with *Enterotoxigenic Escherichia coli* (ETEC) being one of the major pathogens. ETEC contamination can occur at any point along the farm-to-table process and can affect fresh vegetables, fruits, meat, and meat products such as ground beef and raw beef (Yang et al., 2017). Pathogens can be

spread through contaminated environments, animals, and humans. In most foodborne *Escherichia coli* outbreaks, undercooked and contaminated foods such as ground beef, hamburgers, and salads were consumed.

The study used cold plasma technology, which is a novel and environmentally friendly approach to food processing and safety. This technology uses energetic reactive gases to inactivate contaminants on high moisture foods such as meats, poultry, fruits, and vegetables. The use of atmospheric air in the plasma leap pin reactor was found to be a cost-effective and user-friendly means of generating reactive species, making it a promising solution for food safety and quality improvement.

The purpose of this study is to eliminate non-pathogenic and pathogenic bacteria on beef surfaces with the intention of enhancing the quality of the meat, increasing its shelf life and reducing food waste. Cold plasma, a more environmentally conscious and sustainable food safety technology, is used for this purpose. The study evaluates the physical properties of beef slices by using this technology. The results of this study may serve as a basis for future research and advancements in food safety and quality improvement.

2.3 OBJECTIVES

1. To determine the efficacy of cold plasma in inactivating *Salmonella cerro*, *Salmonella dublin*, *Enterotoxigenic Escherichia coli* and *Escherichia coli* K-12 at different treatment times and discharge frequencies.

2. To investigate the effectiveness of cold plasma on quality changes in the beef surface such as temperature, moisture content, and surface color.

2.4 MATERIALS AND METHODS

2.4.1 Source and design of plasma leap pin reactor

The Plasma Leap Pin Reactor, referred to as the cold plasma generator, is depicted in Figure 3.1. It was designed at Plasma Leap Technologies located in Sydney, Australia, and features a high-voltage electrode consisting of two steel plates connected through a pin array of 11 * 8 and a ground electrode. The user manual for the Plasma Leap 100 explains that the system is composed of several key components, including a High Voltage Power Supply Unit capable of producing high voltage pulses up to 80 kV with repetitive pulse frequencies ranging from 100Hz to 3000Hz. The treatment time can be extended 0-900 seconds, with an output power capacity of between 50W to 400W. The system also includes a controller with digital and touch screen control capabilities, which allows for adjustments to parameters during operation.

2.4.2 Bacterial Strain and Inoculation Preparation

The study utilized a few pathogenic bacteria species, including *Salmonella Cerro*, *Salmonella Dublin*, and *Enterotoxigenic Escherichia coli*, which were obtained from glycerol stocks stored in a -80°C freezer at a Biosafety Level III laboratory in the Veterinary and Biomedical Science Department of South Dakota State University. The non-pathogenic bacterial culture, *Escherichia coli K-12*, was procured from the Biology and Microbiology Department at South Dakota State University. These cultures were grown on appropriate media, including Luria agar for *Escherichia coli K-12*, HiChrome agar for *Enterotoxigenic Escherichia coli*, and XLT4 agar for *Salmonella Cerro* and *Salmonella Dublin*. All experiments were performed in a Biosafety Level III laboratory, with the bacterial cultures maintained in glycerol at -80°C as stock cultures. Inoculum preparation was performed in the Biosafety Level III laboratory, and all procedures were conducted under Biosafety Level III conditions using a laminar airflow hood. The expected population of the bacterial cultures was estimated through optical density measurements at 603nm using a UV Spectrophotometer. The optical densities of the cultures were as follows: *Escherichia coli K-12* (1.0), *Salmonella Cerro* (1.47), *Salmonella Dublin* (1.13), and *Enterotoxigenic E. coli* (1.3), as determined using the Unico SpectroQuest SQ2800 UV-Visible Spectrophotometer.

2.4.3 Purity Test of Bacteria Culture

The washed strain of Escherichia coli was grown overnight in LB broth at 37 °C with continuous agitation. The growing culture of *Escherichia coli* was then serially diluted by combining 0.1 ml of the culture with 0.9 ml of PBS (Han et al., 2016). After each dilution, the diluted culture was plated on Luria agar and incubated at 37 °C overnight to obtain colonies. The plate count method was used to determine the microbial count of E. coli using Luria Agar, which is the selected media for this bacteria (Liao, Li, et al., 2018). The E. coli spread plates were incubated in a 37 °C incubator for 24 hours to allow colonies to grow and be counted (Rød et al., 2012). *E. coli* colonies are characterized by their beige color and shiny surface. *Salmonella Cerro* and *Salmonella Dublin* bacteria were transferred into separate 20 ml of LB broth for 16-24 hours at 37 °C with continuous shaking at 150 RPM. 0.1 ml of each culture was then transferred into separate 0.9 ml of PBS (Phosphate Buffered Saline) and diluted via serial dilution. Plate counts for *Salmonella Cerro* and *Salmonella Dublin* were performed using XLT4 Agar, a highly selective medium for detecting and isolating non-typhi salmonella. The Salmonella were spread on agar plates and incubated for 24 hours at 37°C to grow colonies. The colonies were counted after 24 hours. *Enterotoxigenic Escherichia coli* bacteria were placed in LB broth and shaken continuously at 150 RPM for 16-24 hours. 0.1 ml of the bacteria was transferred into 0.9 ml of PBS and serially diluted. The HIChrome *Escherichia coli* Agar plate counting method was used to determine the bacterial count of *Enterotoxigenic Escherichia coli*, which produces tiny dark red colonies on Hi-Chrome Agar. The colony forming units per 1ml were calculated for all four types of bacteria using Equation 2.1.

$$\frac{\text{CFU}}{\text{ml}} = \frac{(\text{No of Colonies X Dilution factor})}{\text{Volume of culture plate}} \quad \text{----------2.1}$$

2.4.4 Beef Sample Preparation

On the day of the experiment, 25 cm^2 of surface area and 10 g of weight of beef steaks were purchased from a local Walmart store located in Brookings, South Dakota. The surface of the package was sanitized using 70% alcohol in a laminar airflow hood. Using a sterilized measuring ruler, disposable knife, and scale, 25 cm^2 of the beef sample was measured and 10 g of its weight was recorded. Each piece of meat was placed in a sterilized, large-sized (100 mm x 15 mm) petri dish and labeled according to the different treatment categories. A disposable plastic pipette and sterilized pipette tip were used aseptically to apply 0.1 ml of bacteria culture to the beef slices, which were then spread using a sterile plastic spreader. The inoculated beef samples were stored in a refrigerator at 4°C for 20 minutes to allow the bacteria to absorb to the surface of the beef.

2.4.5 Treatment of the Sample

The aseptically prepared and labeled Petri dishes containing beef samples were individually moved to the treatment chamber of the plasma leap system for direct cold plasma treatment. The treatment was performed using cold atmospheric pressure plasma at different time intervals (1 min, 3 min, and 5 min) and discharge frequencies (2000Hz, 2250Hz, and 2500Hz). Control samples, consisting of beef slices with bacteria that were

not exposed to cold plasma, were also included in each test run, which was replicated three times. The plasma leap system was operated within a sterilized laminar flow hood located in a biosafety level III lab in the Biomedical Science Department at South Dakota State University. The plasma leap system was controlled from the power supply unit's main screen, where the parameters for different treatments were adjusted. Upon completion of the exposure time, the plasma leap chamber automatically stopped, and the samples were removed aseptically for microbial analysis.

2.4.6 Measurement of Physical Properties of Beef Sample

A study was conducted to evaluate the effects of cold plasma exposure time and discharge frequency on the physical properties of beef slices, specifically, surface color, temperature, and moisture content. The beef steak samples, with 25 cm2 of surface area and 10 g of weight, were treated with cold plasma using the plasma leap system at room temperature and atmospheric pressure for 1 minute, 3 minutes, and 5 minutes, and with discharge frequencies of 2000Hz, 2250Hz, and 2500Hz. A three-replicate experiment was conducted to measure the surface color of the beef samples using a Minolta colorimeter. The results were expressed as L*, a*, b* values for lightness, redness, and greenness, respectively (Bauer et al., 2017), with a* being the most critical color value. The colorimeter was calibrated with standard black and white tiles before taking measurements (Speckhahn et al., 2010).

The temperature of the same treated beef samples was measured before and after the cold plasma treatment using a Fluke 51-2 60Hz Handled Digital Probe Thermometer, and the measurements were performed in triplicate.

The moisture content of the beef samples treated with cold plasma was determined through the oven-drying method, which follows the ASABE standard (Xiang, Liu, et al., 2018). This method measures the total amount of water present in the sample (Rød et al., 2012). The moisture content can be expressed as wet basis, which describes the percentage of the ratio of the weight of water to the total weight of the material, or as dry basis, which describes the percentage of the ratio of the weight of water to the weight of the dry matter. In the oven-drying method, a 10g sample of treated beef was weighed, placed into a dryer at 105°C for 24 hours, and then weighed again to calculate the weight loss and determine the moisture content. The final solid percentage was also calculated using the appropriate equation (Feizollahi et al., 2020).

$$\text{Moisture \%} = \left(\frac{\text{Weight loss}}{\text{Weight of the sample}}\right) * 100 \text{----------2.2}$$

$$\text{Total solid \%} = 100 - \text{Moisture \%} \text{----------2.3}$$

2.4.7 Microbial Analysis

The analysis of microorganisms in the beef samples was carried out using a previously described method (Lis et al., 2018). The plasma-treated beef slices were placed in a sterilized stomacher bag, filled with 40 ml of sterile phosphate buffer, and blended for two minutes in a stomacher blender to achieve homogeneity. The homogenized samples were then transferred to 50 mL conical tubes. A serial dilution of the homogenized beef sample was performed, and 0.1 ml of each dilution was spread on LA agar medium to obtain

colonies. Afterward, the spread plates were incubated for 24 hours at 37°C. The microbial counts were expressed as colony-forming units per milliliter (log CFU/ml) and the inactivation rate was represented as the log difference between the initial microbial count and the microbial count after cold plasma treatment (Lis et al., 2018).

Log reduction was calculated as follows:

$$\text{Log Reduction} = \text{Log } 10 \left[\frac{\text{Number of viable microorganisms before the treatment}}{\text{Number of viable microorganisms after the treatment}}\right] \text{----------2.4}$$

2.4.8 Statistical Analysis

In this study, all measurements were repeated three times to ensure accuracy and precision. The results were presented as mean values along with their standard deviations (SDs). To determine the significance of the differences between the mean values, an Analysis of Variance (ANOVA) and t-test were performed. The results were considered statistically significant if $p < 0.05$.

2.5 RESULTS AND DISCUSSION

2.5.1 Effect of cold plasma-treatment time and discharge frequency on microbiological qualities of meat slices

A lower efficacy of bacterial inactivation was observed for a 1-minute exposure to cold atmospheric plasma, while a higher efficacy was observed for a 5-minute exposure. The Escherichia coli K-12 microbial strain was treated with cold plasma at different parameters, and as the exposure time increased, the log reduction or bacterial inactivation rate also

increased for all three discharge frequencies. For the 2000Hz discharge frequency, the log reduction was 0.2626 log CFU/ml in 1 minute, 0.4274 log CFU/ml in 3 minutes, and 0.5672 log CFU/ml in 5 minutes. For the 2250Hz discharge frequency, the log reduction was 0.359 log CFU/ml in 1 minute, 0.59 log CFU/ml in 3 minutes, and 1.3702 log CFU/ml in 5 minutes. The highest log reduction of 1.4686 log CFU/ml was achieved in 5 minutes for the Escherichia coli K-12 strain, compared to 0.3806 log CFU/ml in 1 minute and 1.1193 log CFU/ml in 3 minutes. These results are illustrated in Figure 2.2.A.

The second bacterial strain of Salmonella cerro on beef slices was inactivated by cold plasma, with the log reduction increasing with both time and discharge frequency. At 2000 Hz discharge frequency, the log reduction was 0.5180 log CFU/ml in 1 minute, 0.5493 log CFU/ml in 3 minutes, and 1.1346 log CFU/ml in 5 minutes. The log reduction increased as the discharge frequency was increased to 2250 Hz, with values of 0.5509 log CFU/ml in 1 minute, 0.8027 log CFU/ml in 3 minutes, and 1.0805 log CFU/ml in 5 minutes. The highest log reduction, 1.7115 log CFU/ml, was achieved when the discharge frequency was increased to 2500Hz. Over the course of 5 minutes, the log reduction increased from 0.6616 log CFU/ml in 1 minute to 1.1139 log CFU/ml in 3 minutes and finally 1.7115 log CFU/ml in 5 minutes (Figure 2.2.B).

Accordingly, *Salmonella dublin* was the third type of bacteria strain that was inactivated by cold plasma treatment, resulting in a log reduction of 0.5363 log CFU/ml, 0.6466 log CFU/ml, and 0.7958 log CFU/ml after 1 minute, 3 minutes and 5 minutes of treatment time at 2000Hz, respectively (Figure 2.2.C). For 2250Hz, a log reduction of 0.2215 log CFU/ml was observed after 1 minute, 0.7959 log CFU/ml after 3 minutes, and 0.9208 log CFU/ml

after 5 minutes. For 2500Hz, a log reduction of 0.2940 log CFU/ml was observed after 1 minute, 1.5503 log CFU/ml after 3 minutes, and 1.6511 log CFU/ml after 5 minutes.

In the study, *Enterotoxigenic Escherichia coli* was inactivated by cold plasma. The inactivation of the bacteria strain was observed after 1, 3, and 5 minutes, with the use of 2000Hz discharge frequency. At the end of 1 minute, a log reduction of 0.1600 log CFU/ml was recorded. After 3 minutes, the log reduction increased to 0.1874 log CFU/ml. At the end of 5 minutes, the log reduction was 0.2197 log CFU/ml. When the discharge frequency was increased to 2250Hz, the log reduction after 1 minute was 0.2494 log CFU/ml, and 0.2702 log CFU/ml after 3 minutes. After 5 minutes, the log reduction was 0.29014 log CFU/ml. Furthermore, the log reduction was found to increase with the increase in discharge frequency to 2500Hz. After 1 minute, the log reduction was 0.2753 log CFU/ml, and after 3 minutes, it was 0.2929 log CFU/ml. At the end of 5 minutes, the log reduction was 0.3675 log CFU/ml (Figure 2.2.D).

2.5.2 Effect of Cold Plasma Treatment on Surface Color of Meat Slices

This study investigated the effect of different cold plasma discharge frequencies on the beef surface color (L-value), which was observed differently before and after the treatment. Before the “1-minute” treatment and at a frequency of 2000 Hz, the L-value was 31.96 and after the treatment, it decreased to 30.6. At a frequency of 2250 Hz, the L-value increased from 24.86 to 29.33, and at a frequency of 2500 Hz, it increased from 27.84 to 29.33. After the 3-minute treatment, at a frequency of 2000Hz, the L-value changed from 29.34 to 34.14, at 2250Hz from 32.93 to 41.78, and at 2500Hz from 28.3 to 34.12. The treatment time increased the L-value from 2000Hz, from 28.44 to 28.1, from 2250Hz from 33.63 to

40.10, and from 2500Hz from 28.65 to 32.47 (as shown in Figure 2.3). The effect of different cold plasma discharge frequencies on the beef surface color (a*-value) was also observed differently before and after the treatment (as shown in Figure 2.4). Before the 1-minute treatment and at a frequency of 2000 Hz, the a*-value was 15.28 and after treatment, it decreased to 12.69. At a frequency of 2250 Hz, the a*-value decreased from 13.56 to 11.97, and at a frequency of 2500 Hz, it increased from 14.21 to 14.89. After the 3-minute treatment, at a frequency of 2000Hz, the a*-value changed from 15.33 to 13.64, at 2250Hz from 13.35 to 13.23, and at 2500Hz from 15.13 to 13.36. The treatment time increased the a*-value from 2000Hz from 15.35 to 10.43, from 2250Hz from 12.31 to 12.10, and from 2500Hz from 11.90 to 11.54. Similarly, the effect of different cold plasma discharge frequencies on the beef surface color (b*-value) was observed differently before and after the treatment (as shown in Figure 2.5). Before the 1-minute treatment and at a frequency of 2000 Hz, the b*-value was 6.10 and after the treatment, it increased to 07.09. At a frequency of 2250 Hz, the b*-value decreased from 12.41 to 6.63, and at a frequency of 2500 Hz, it decreased from 9.53 to 5.056. After the 3-minute treatment, at a frequency of 2000Hz, the b*-value changed from 6.16 to 3.61, at 2250Hz from 4.25 to 3.09, and at 2500Hz from 8.33 to 3.03. The treatment time increased the b*-value from 5 minutes at 2000Hz from 9.43 to 8.023, from 2250Hz from 4.37 to 5.24, and from 2500Hz from 7.02 to 5.12.

The beef slice was observed to have no significant difference in color on its surface after and before the cold plasma treatment. In comparison to untreated meat, the a* and b* values of beef were slightly changed, but these changes were not significant. This study

investigated the effect of different cold plasma discharge frequencies on the beef surface color (L-value), which was observed to have slight variations before and after the treatment, but these differences were not significant. The a* and b* values also showed slight changes with different cold plasma discharge frequencies, but these changes were not significant. The results indicated that the cold plasma treatment did not result in significant changes to the beef surface color in terms of L, a*, and b* values.

2.5.3 Effect of Cold Plasma Treatment on Surface Temperature of Meat Slices

The temperature of beef samples was measured using a thermocouple before and immediately after cold plasma treatment. Results showed that all nine parameters were not significantly different before and after treatment. As shown in Figure 2.6 A, an increase in discharge frequency from 2000Hz to 2250Hz and 2500Hz resulted in a decrease in temperature for some observations, with the temperature decreasing from 21.23°C to 20.06°C at 2000Hz, 19.93°C to 19.23°C at 2250Hz, and increasing from 19.3°C to 19.5°C at 2500Hz at 1min. Figure 2.6 B, when the treatment time was increased to 3 minutes and the discharge frequency was set at 2000Hz, the surface temperature of the beef slices decreased from 20.3°C to 19.33°C, from 21.5°C to 19.96°C, and from 22.73°C to 19.65°C. As shown in Figure 2.6 C when the treatment time was increased to 5 minutes and the discharge frequency was set at 2000Hz, the temperature increased from 20.13°C to 21.26°C, from 20.9°C to 21.1°C at 2250Hz and decreased from 20.9°C to 20.6°C at 2500Hz.

2.5.4 Effect of Cold Plasma Treatment on Moisture Content of Beef Slices

As depicted in Figure 2.7 A, the findings indicate that there were no substantial alterations in the wet-based moisture content of the beef prior to and after 1-minute cold plasma treatment at the three different discharge frequencies. Likewise, the results from Figure 2.7.C reveal that no significant modifications occurred after 5 minutes of treatment. However, for the 3-minute treatment time Figure 2.7.B, there was no significant difference observed in moisture content for all discharge frequencies. It should be noted that the decrease in moisture content from 80 to 70, when the treatment was performed for 3 minutes at a frequency of 2000Hz (Figure 2.7.B), does not necessarily indicate a significant difference.

2.6. CONCLUSIONS

In this study, the aim was to investigate the effects of cold plasma treatment on microbial inactivation and surface decontamination of beef slices. The results showed that the treatment effectively achieved microbial inactivation and surface decontamination, as well as altered the surface properties of the beef slices. The findings also indicated that as the exposure time increased, the log reduction and bacterial inactivation rate increased for all three discharge frequencies. Furthermore, there were no significant differences observed in the physical properties of the meat slices, such as moisture content, temperature, and surface color, before and after the treatment.

In conclusion, cold plasma treatment showed promising results for the inactivation of microorganisms and decontamination of the beef slices in this study. However, further

research is needed to scale up the technology for industrial and commercial applications and to fully understand its effects on the quality of food.

2.7. FIGURES

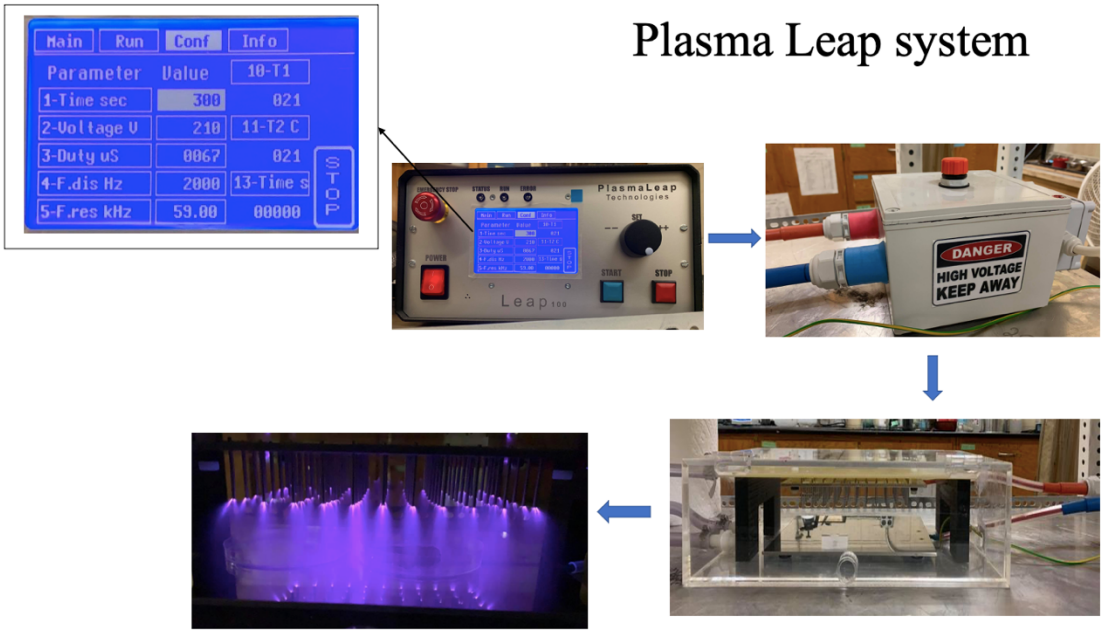
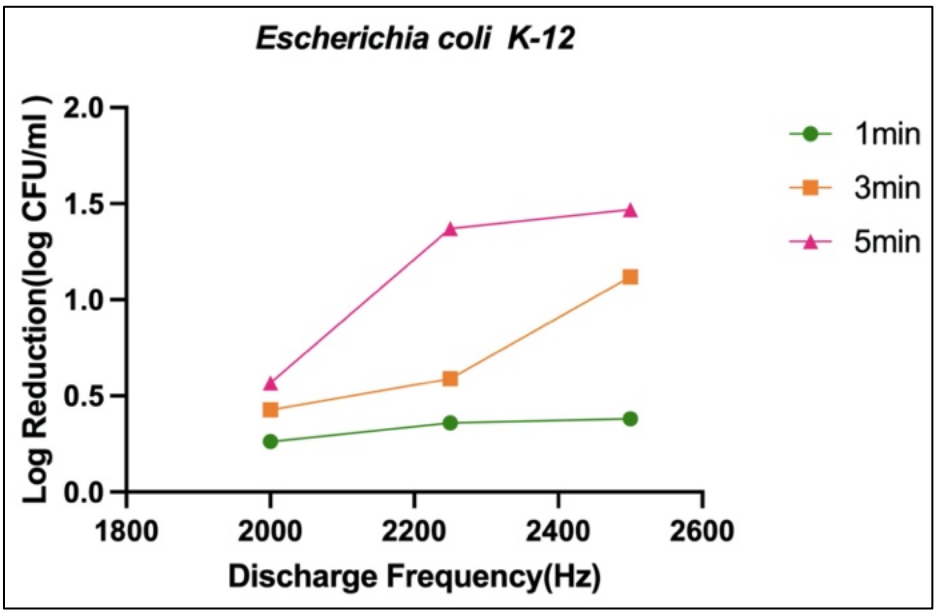
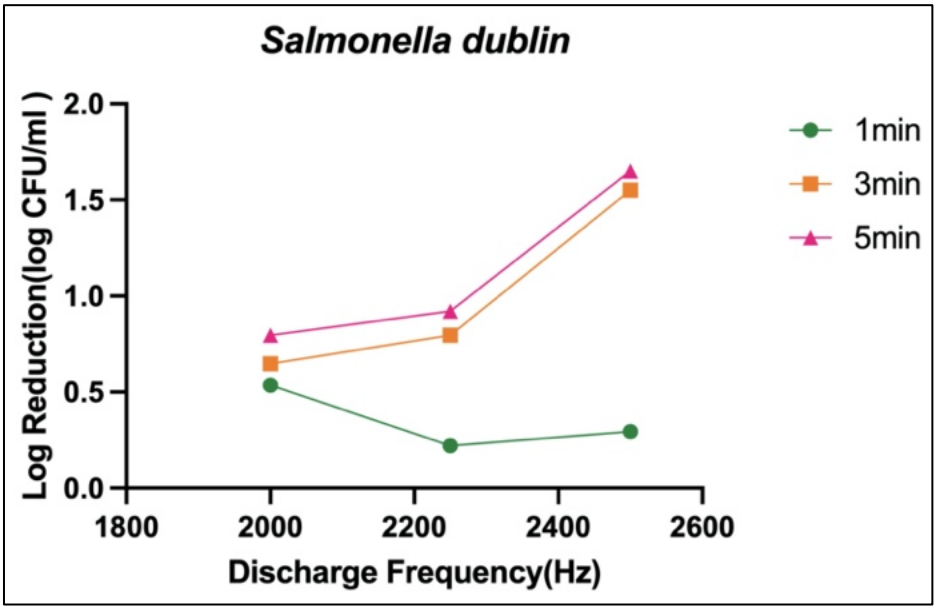


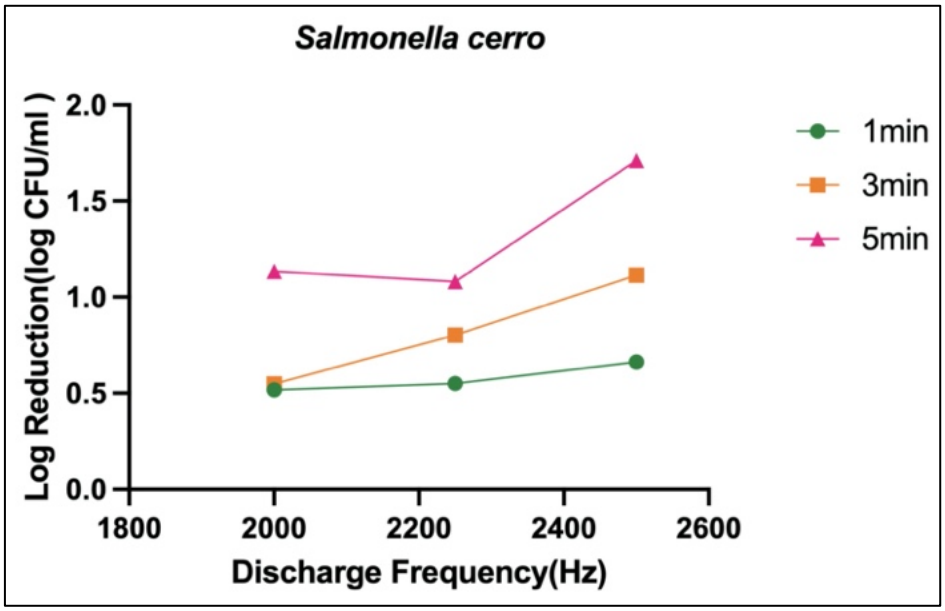
Figure 2.1: Plasma Leap System with Pin Reactor


Escherichia coli K-12
Log Reduction(log CFU/ml)
2.0
1.5
1.0
0.5
0.0
1800
2000
2200
2400
2600
Discharge Frequency(Hz)
1min
3min
5min

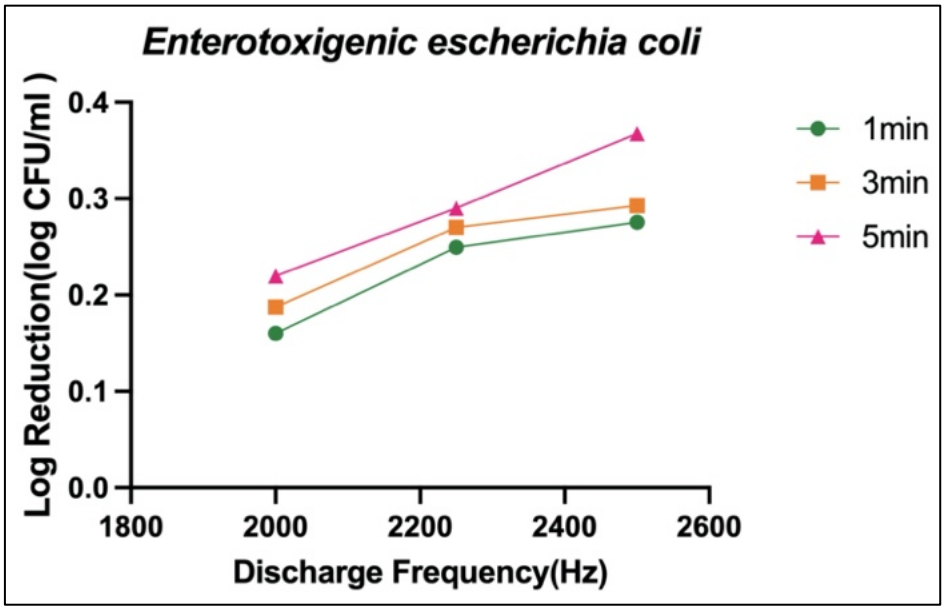
A


Salmonella dublin
Log Reduction(log CFU/ml)
2.0
1.5
1.0
0.5
0.0
1800
2000
2200
2400
2600
Discharge Frequency(Hz)
1min
3min
5min

B



C



D

Figure 2.2: Effect of Different Cold Plasma Treatment Time and Discharge Frequency on Bacterial Inactivation. A: *Escherichia coli K-12*; B: *Salmonella cerro*; C: *Salmonella dublin*; D: *Enterotoxigenic Escherichia coli*

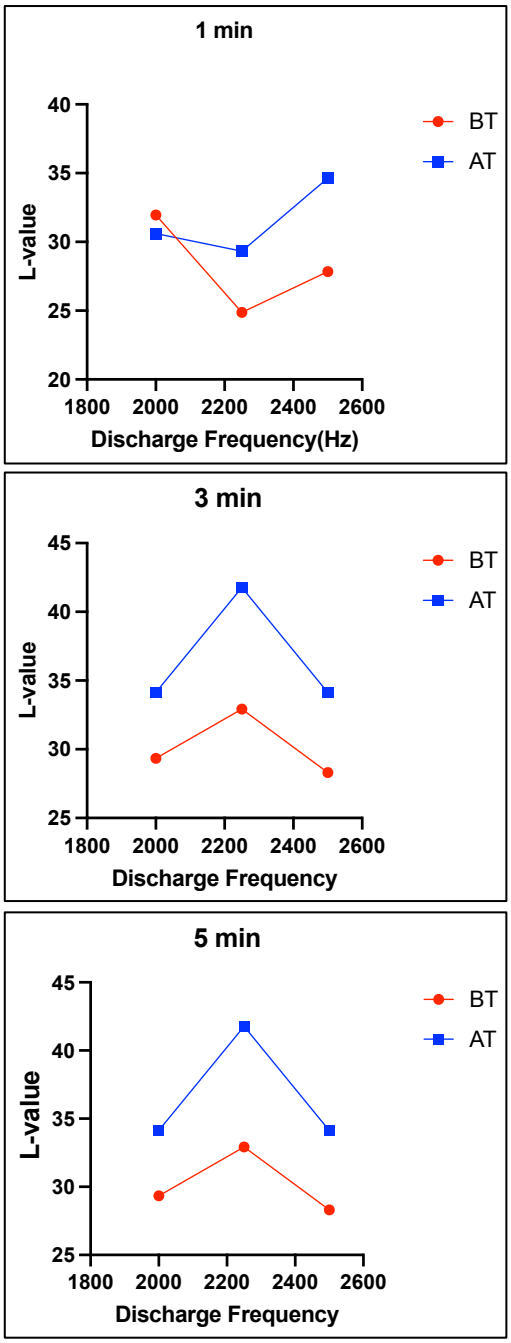
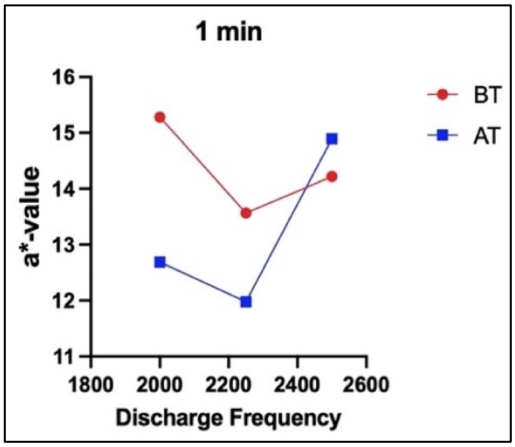
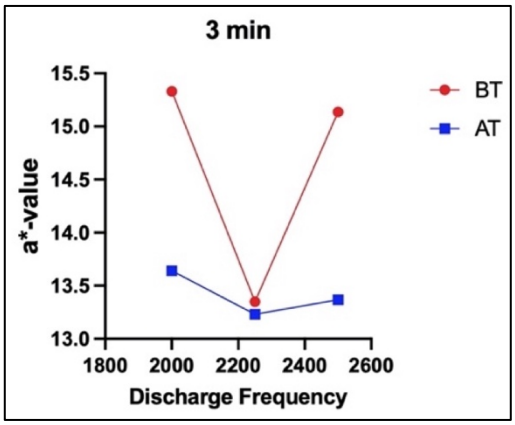


Figure 2.3: Effect of different cold plasma discharge frequency on beef surface color (L-value); A :1min, B: 3min, C: 5min



A



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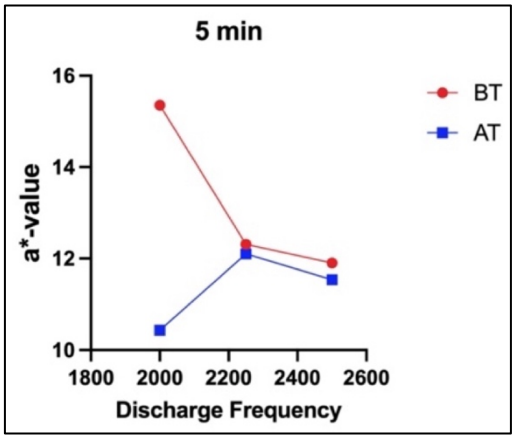
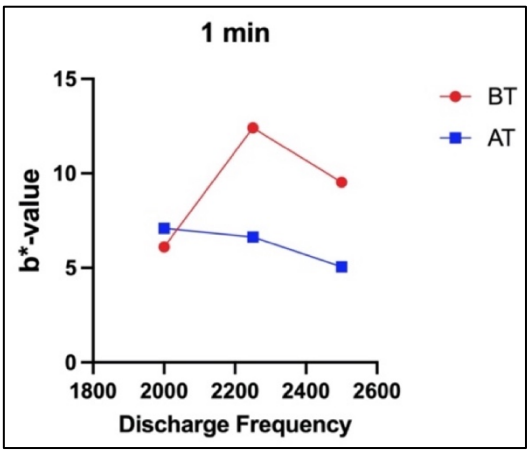
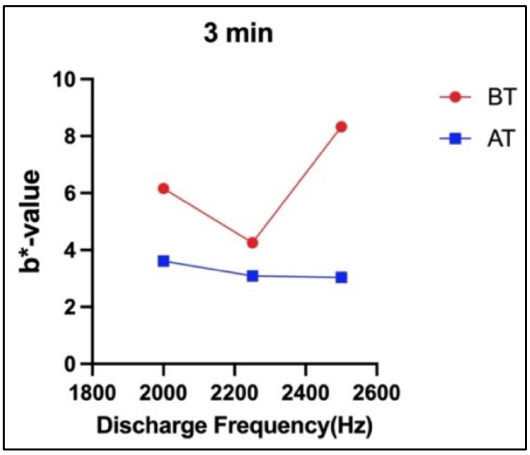


C

Figure 2.4: Effect of different cold plasma discharge frequency on beef surface color (a*-value); A :1min, B: 3min, C: 5min



A



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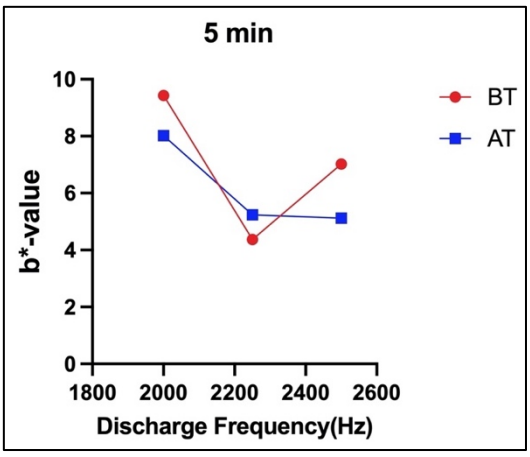
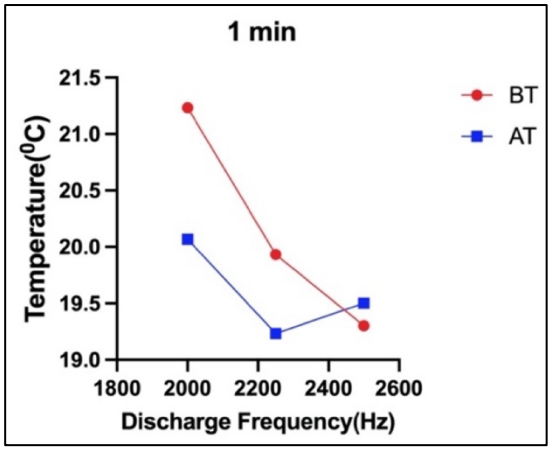
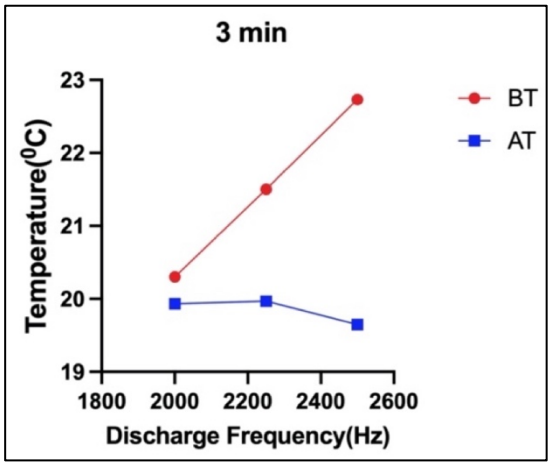


C

Figure 2.5: Effect of different cold plasma discharge frequency on beef surface color (b*-value); A :1min, B: 3min, C: 5min



A



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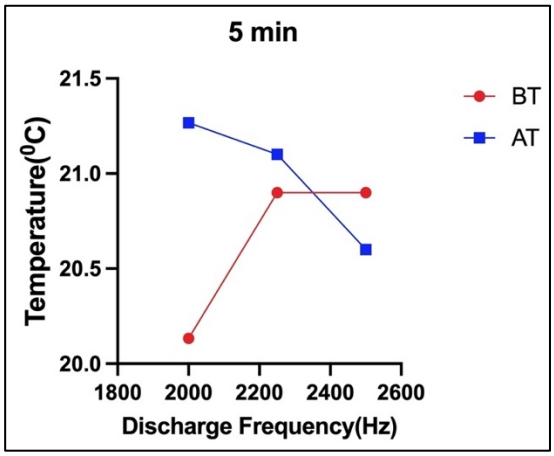
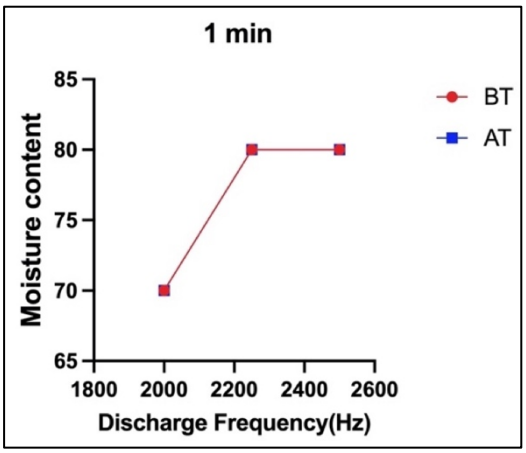
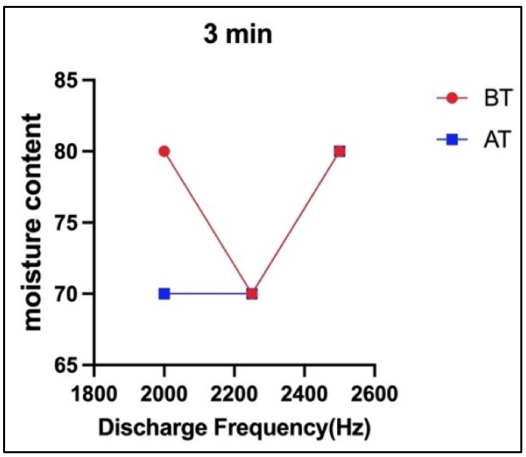


C

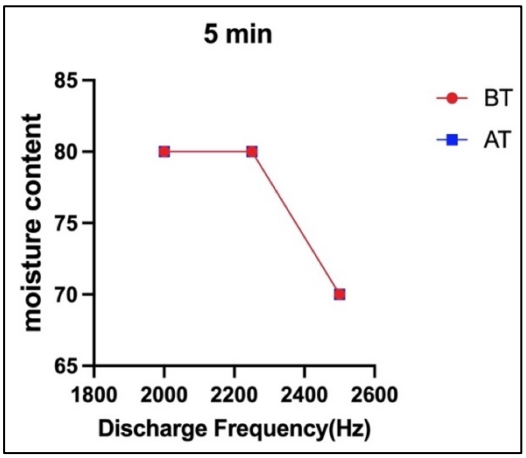
Figure 2.6: Effect of different cold plasma discharge frequency on beef temperature A. 1 min; B. 3 min: C. 5 min



A



B



C

Figure 2.7: Effect of different cold plasma discharge frequency on beef moisture content

A. 1 min, B. 3 min, C. 5 min

CHAPTER 03: PLASMA ACTIVATED WATER FOR INACTIVATION OF ESCHERICHIA COLI K-12

3.1 ABSTRACT

Plasma-activated water (PAW) is an antimicrobial technology that uses an ionized gas mixture to create a cocktail of reactive species in water. The PAW resulting from the air contains a variety of reactive oxygen and nitrogen species (RONS) that are linked to its antibacterial activity. In this study, the antimicrobial efficacy of a novel plasma-activated water (PAW) reactor, referred to as Plasma Leap water Reactor, was investigated against Escherichia coli, a common organism. The PAW generated from the reactor contains a variety of reactive oxygen and nitrogen species (RONS) that are linked to its antibacterial activity. The factors that can affect plasma activity, including plasma generating conditions, reactor design, and the contribution of each species, were considered in the study. The efficacy of the PAW reactor was tested in distilled water with five different discharge frequencies in the range of 500Hz to 1500Hz and six different treatment times between 2 seconds to 60 seconds. The results showed that the PAW generated with the Plasma Leap water reactor was able to completely inactivate all bacteria (6 log) within 5 seconds of treatment under all conditions tested. Further testing was performed at different temperatures, including room temperature (20 ^{0}C), 16 ^{0}C, and 4 ^{0}C, to demonstrate the use of plasma leap water reactors in high-risk environments requiring complete sterilization. The reactive species in each of the PAW samples were also quantified with ozone concentration based on the different discharge frequencies and treatment times. This study

highlights the significance of discharge frequencies, treatment times, and testing temperatures on the production of reactive species and the antimicrobial efficiency of the Plasma Leap Water Reactor. The results demonstrate the effectiveness of PAW, generated using oxygen as the feed gas, for use in food decontamination processes.

Keywords: Cold Plasma, Cold Plasma Activated Water, Plasma Leap Bubble Water Reactor, Reactive Species, Food Safety, Ozone, Bacterial Inactivation

3.2 INTRODUCTION

In recent years, antimicrobial technologies have garnered significant attention in various industries, such as medical, food processing, and the water industry, where the growth of microbes on surfaces or within products can lead to significant problems. In particular, plasma technology has emerged as a promising and economically viable solution for advanced oxidation processes in wastewater treatment. Plasma-activated water (PAW) is generated by exposing water to plasma, resulting in the formation of a cloud of plasma above or through the water using cold plasma or non-thermal plasma (Zhao et al., 2020). This process leads to the creation of a unique cocktail of biochemical reactive species in the water, referred to as Plasma Activated Water. In the food industry, cold plasma has been tested as an alternative non-thermal treatment process (Varilla et al., 2020a). The inactivation of microbes is believed to be due to the presence of various reactive species produced by cold plasma, such as free radicals (such as reactive oxygen species (ROS) and reactive nitrogen species (RNS)), positive and negative ions, and ultraviolet radiation (UV) (Liao, Su, et al., 2018).

Plasma is known as the fourth state of matter and is comprised of positive and negative ions, electrons, excited and neutral atoms, free radicals, ground and excited state molecules, and ultraviolet photons (Thirumdas et al., 2018). The ionization of a neutral gas with sufficient energy leads to a plasma treatment, which results in the generation of reactive gaseous species. In air, the discharge process results in the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), also known as reactive oxygen and nitrogen species or RONSs. The primary species generated during this process include H, O, OH, N, NO, and other ions, as well as secondary species such as H2O2, O3, NOx, HNO2, HNO3, and ONOOH (Gao et al., 2022). The antimicrobial effect of direct plasma treatment on bacteria is believed to result from a combination of physical and chemical factors (P. Bourke et al., 2017).

In this context, this study aimed to evaluate the efficacy of a novel plasma activated water (PAW) reactor called the Plasma Leap Water Reactor against the common bacterium Escherichia coli. The study examined the effect of different discharge frequencies and treatment times on the production of reactive species and antimicrobial efficiency of the Plasma Leap Water Reactor in distilled water.

3.3 OBJECTIVES

i. To determine appropriate plasma technique and condition for Plasma Activated Water generation using plasma leap bubble reactor
ii. To analyze the appropriate Escherichia coli inactivation using different discharge frequencies and different treatment time of bubble reactor.
iii. To analyze physicochemical properties of PAW varied with different discharge frequencies and different treatment times.

3.4 MATERIALS AND METHODS

3.4.1Plasma Leap Bubble Water Reactor

The Plasma Leap 100 system, which was purchased from Plasma Leap Technologies in Sydney, Australia, includes both a plasma leap pin reactor and a water bubble reactor. In this study, the focus was primarily on the water bubble reactor. The system is divided into several major components, including the High Voltage Power Supply Unit, the Controller, the Water Reactor, the Rotameter, and the electrodes. The High Voltage Power Supply Unit provided high voltage pulses of up to 80kV, with repetitive pulse frequencies ranging from 100Hz to 3000Hz and output power from 50W to 400W. The Controller was equipped with a digital touch screen and allowed for control of parameters during operation. The Water Reactor was made of stainless steel and was equipped with acrylic spacers, PTFE T-fittings, and holes for plasma to escape into the water. The airflow rate was controlled using a rotameter attached to a Teflon fitting on the outside tubes leading to the gas source. The ground electrode was attached to a stainless-steel ground probe, while one electrode was attached to the stainless-steel bubble reactor. In this study, different discharge frequencies and treatment times were used to determine the inactivation of *Escherichia coli K12*. The discharge frequencies were 500Hz, 750Hz, 1000Hz, 1250Hz, and 1500Hz, and the treatment times were 2sec, 5sec, 10sec, 15sec, 30sec, and 60sec. The airflow rate was constantly maintained at 2mg/L, and compressed oxygen was used as the gas source in the bubble water reactor.

3.4.2 Bacterial Culture

A culture stock of *Escherichia coli K-12* was obtained from the Biology and Microbiology Department at South Dakota State University. The stock culture was maintained at -80°C in glycerol and used as a source to prepare inoculums. The *Escherichia coli K-12* bacterium was grown in 20 mL of Luria Broth for 16 to 24 hours at 37°C with continuous shaking at 150 rpm. The cultures were harvested by centrifuging them at 3000 rpm for 10 minutes at 4°C and rinsing them in PBS to obtain a concentration of 1 x 10^8 CFU per ml. The final inoculum was stored at 4°C and used within four hours. After back-plating, the final concentration was verified. The serial dilution was performed by transferring 0.1 mL of the E. coli culture to 0.9 mL of PBS after 16 hours of incubation. The concentration of Escherichia coli and the purity of the strain were determined using the plate count method with Luria Agar as the selected medium for the culture. After serial dilution and spread plate method, all plates were incubated at 37°C for 24 hours to grow colonies and count them. Typically, E. coli colonies appear beige in color with a shiny texture. The colony-forming units per 1 mL were calculated using the appropriate equation.

$$\frac{\text{CFU}}{\text{ml}} = \frac{Number of\ colonies\ X\ Dilution\ Factor}{Volume\ of\ culture\ plates} \quad \text{----------3.1}$$

3.4.3 Bacteria Inactivation Using Plasma Activate Water (PAW)

In order to optimize the inhibition assay, non-pathogenic bacteria were used (Xiang, Kang, et al., 2018). The experiments were conducted using the Leap 100 water reactor and five different discharge frequencies (500Hz, 750Hz, 1000Hz, 1250Hz, and 1500Hz) in distilled water. A 2 standard liters per minute oxygen source was provided to the reactors during plasma treatments, which were performed inside a fume hood. An aliquot of 1ml of the inoculum was added to 99mL of distilled water in a 125mL Glass Schott bottle in the plasma leap water reactor. The typical settings for the Plasma Leap 100 water reactor were 200 V, 100µs duty cycle, and 60 kHz resonance frequency, while the treatment times varied from 60s, 30s, 15s, 10s, 5s, 2s. For the control, the same volume of water and inoculum were used in the same reactors without the power source being on for a specific time duration. The bacteria were serially diluted with milli-Q water and enumerated by spreading the plates in 96-well plates. The experiments were repeated three times for each condition.

3.4.4 Cold Plasma Treatment of The Distilled Water Samples

The experiment was conducted using plasma leap bubble water reactors placed in a 125 ml Glass Schott bottle filled with 99 ml of distilled water and 1 ml of bacteria culture. The main component of the bubble water reactor was a quartz tube with a length of 175 mm, an outside diameter of 10 mm, and a wall thickness of 1.5 mm, with the last end sealed. Four holes with a diameter of 0.4 mm were positioned radially approximately 5 mm above

the sealed end. A stainless-steel rod with an outside diameter of 4 mm was inserted coaxially through the quartz tube, serving as the electrode. The reactive gas species generated in the plasma were transferred into the water using a conventional coaxial electrode configuration within the reactor.

3.4.5 Measurements of Plasma Activated Water Physiochemical Characteristics

In addition to measuring the pH and temperature of Plasma-Activated Water (PAW) produced by the Plasma LEAP Bubble Reactor, pH Measures the hydrogen ion concentration in a solution. Acidification results from the reactions taking place between chemical species formed in the plasma and water(Abidin et al., 2018). The concentrations of ozone (O3) were also measured over a period of 2 to 300 seconds. The ozone measurements were conducted using a multiparameter photometer from Hanna Instruments (H183399, Rhode Island, USA) and a colorimetric ozone kit. To assess the impact of temperature and pH on the efficacy of PAW, a Mettler Toledo pH meter was utilized to measure the pH of PAW and a thermometer was employed to measure the temperature after each treatment time and at specific discharge frequencies.

3.4.6 Microbial Analysis

Immediately after the activation of water using cold plasma, a microbial analysis was conducted. 1ml of plasma-activated water (PAW) was transferred to a sterile 2 ml conical tube. A serial dilution was prepared in a sterilized 96-well plate using a sterile 0.1ml plastic pipette and disposable, sterilized plastic tips under a laminar airflow hood in the Microbiology Laboratory. The preparation of up to the fifth or sixth dilution resulted in countable plates with colonies ranging from 30 to 300. A 0.1ml aliquot of the appropriate decimal dilution was spread onto selective agar plates to obtain colonies in the 30 to 300 range. The spread plates were incubated for 24 hours at 37°C. The microbial counts were expressed as colony-forming units per milliliter (log CFU/mL). The cold plasma treatment inactivated the microbes by approximately one log difference between the initial and post-treatment microbial counts.

Log reduction was calculated as follows:

$$\text{Log Reduction} = \text{Log } 10 \left[\frac{\text{Number of viable microorganisms before the treatment}}{\text{Number of viable microorganisms after the treatment}}\right] \text{----------3.2}$$

3.4.7 Statistical Analysis

In this study, all measurements were carried out in triplicate and the results were expressed as the mean and standard deviation (SD). The differences among the mean values were analyzed using analysis of variance (ANOVA) and were considered statistically significant at a significance level of $p < 0.05$.

3.5 RESULTS AND DISCUSSION

3.5.1 Effect of cold plasma exposure treatment time and discharge frequency for microbial inactivation Cold Plasma Activated Water

The effects of different discharge frequencies and treatment times of cold plasma treatment on *Escherichia coli K-12* colony-forming units (CFUs) were measured using distilled water and the plasma leap bubble water reactor (Figure 3.1). Across all discharge frequencies, plasma-activated water (PAW) generated by the water reactor was most effective, completely reducing bacteria (6 log) within 5 seconds even at room temperature (20°C) for distilled water. The cold plasma treatment of Escherichia coli strains was tested based on several parameters. The log reduction rate increased when the cold plasma exposure time was increased from 2 seconds to 60 seconds for all five different discharge frequencies, such as 0.1627 to 2.1072 log CFU/mL in 500Hz discharge frequency, 0.3912-6.0 in 750Hz, 0.3010-6.0 in 1000Hz, 0.3437-6.0 log CFU/mL in 1250Hz and 2.1072-6.0 log CFU/mL in 1500Hz, respectively (Figure 3.2). Out of all the discharge frequencies, 1500Hz was the most effective and CFUs were reduced more rapidly at higher discharge frequencies, specifically 1500Hz. The treatment response also varied with the temperature of distilled water, such as 20°C, 16°C, and 4°C. In 16°C of bacteria-inoculated distilled water after cold plasma treatment, the log reduction was 0.07169 log CFU/mL - 6 log CFU/mL in 500Hz, 0.1064 log CFU/mL - 6 log CFU/mL in 750Hz, 1.7232 log CFU/mL - 6 log CFU/mL in 1000Hz, 0.6774 log CFU/mL - 6 log CFU/mL in 1250Hz, and 2.1398 log CFU/mL - 6 log CFU/mL in 1500Hz (Figure 3.2). In 4°C and 500Hz, the log reduction of *Escherichia coli K-12* with plasma-treated water was 1.58357 log CFU/mL - 6 log CFU/mL during 2 seconds to 60 seconds time, 1.7879 log CFU/mL - 6 log CFU/mL in

750Hz, 1.9838 log CFU/mL - 6 log CFU/mL in 1000Hz, 2.8176 log CFU/mL - 6 log CFU/mL in 1250Hz, and 2.8176 log CFU/mL - 6 log CFU/mL in 1500Hz (Figure 3.2). Even in a short time duration of 5 seconds, the highest log CFU/mL was reached at 1500Hz, which was the most effective discharge frequency among all five frequencies for all three different treatment times.

3.5.2 Effect of Temperature Difference of Cold Plasma Activated Water

The temperature of cold plasma activated water was measured using a thermometer and a thermocouple after treatment. The results, shown in Figure 3.5, indicated that the temperature was between room temperature (approximately 20 ^{0}C) and 30 ^{0}C when the treatment time was increased from 2 seconds to 30 seconds. After a treatment time of 300 seconds and a discharge frequency of 1500Hz, the temperature increased to 41.8 ^{0}C, while it was 22.1 ^{0}C at 500Hz, 28.4 ^{0}C at 750Hz, 39.1 ^{0}C at 1000Hz, and 41.0 ^{0}C at 1250Hz. However, the temperature changes were not deemed statistically significant.

3.5.3 Effect of pH Difference of Cold Plasma Activated Water

The results of the experiment showed that as the treatment time increased, the pH of the plasma-activated water (PAW) drastically decreased. This decrease in pH was attributed to the production of strong acids, such as hydrogen peroxide, nitric acid, and peroxynitrous acid, as a result of the plasma reaction (Thirumdas et al., 2018). Figure 3.4 demonstrates that the pH decreases as both treatment time and discharge frequency increase. For example, at a discharge frequency of 500Hz and a treatment time of 2 seconds, the pH was

7.36. However, when the discharge frequency was increased to 1500Hz and the treatment time was extended to 300 seconds, the pH dropped to 4.2. This data demonstrates the effect that treatment time and discharge frequency have on the pH of PAW, however it is not considered a significant difference.

3.5.4 Ozone Analysis of Cold Plasma Activated Water

The tests performed to evaluate the antibacterial activity of cold plasma activated water involved exposing ozone produced in PAW to different discharge frequencies over time. The results showed that the cold plasma activation was effective in generating ozone, as indicated by the increase in ozone concentration from 0 mg/L to 1.3 mg/L as treatment time increased from 2 seconds at 500Hz to 300 seconds at 1500Hz (Figure 3.3).

3.6 CONCLUSION

In this study, a comparison of the bactericidal performance of the novel plasma activated water (PAW) bubble reactor was conducted using different treatment times and discharge frequencies for distilled water. The results showed that the Plasma leap bubble reactor produced the most effective PAW, which was able to achieve a 6-log reduction of bacteria inactivation in distilled water within 5 seconds. Additionally, ozone was identified as a critical reactive species for antimicrobial activity. Given the widespread use of distilled water in various industrial processes, particularly in the food processing and medical industries, this study provides new insights into the potential of PAW as a sanitizing agent.

3.7 FIGURES

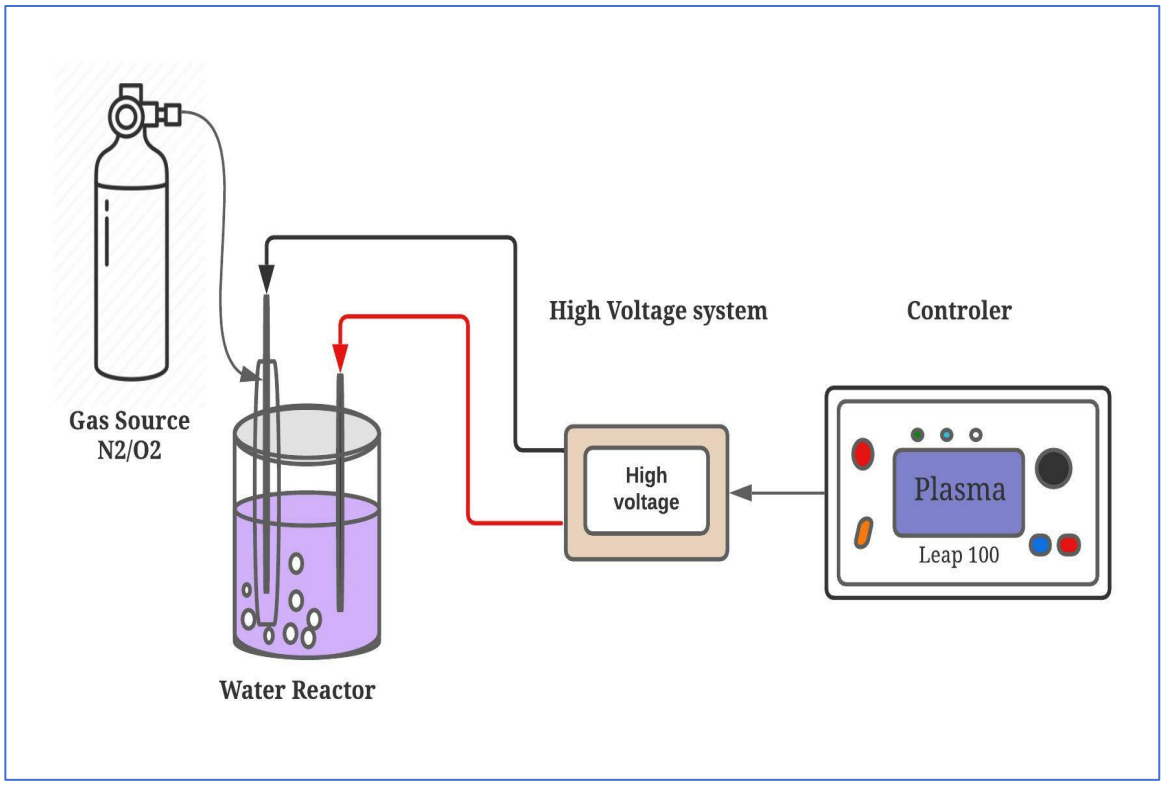


Figure 3.1: Schematic of the Plasma leap bubble reactor for PAW production

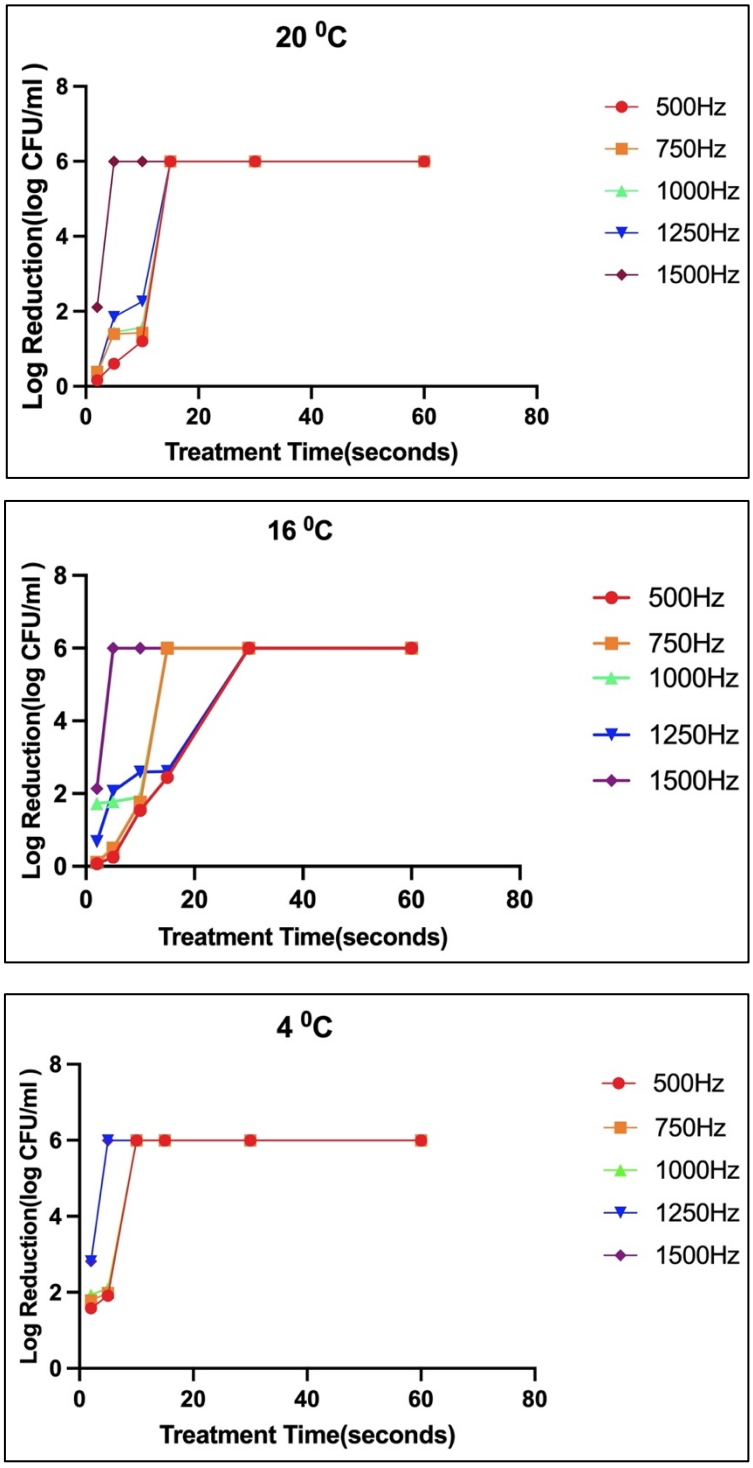


Figure 3.2: Log reduction of *Escherichia coli* K-12 in PAW at 20°C, 16°C, and 4°C

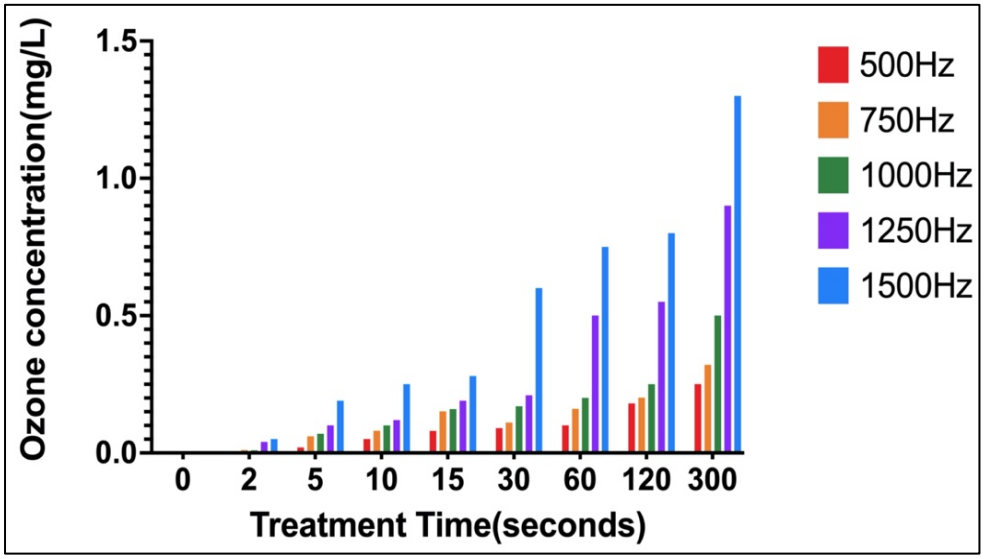


Figure 3.3: Ozone concentration of PAW in different treatment times and different discharge frequencies

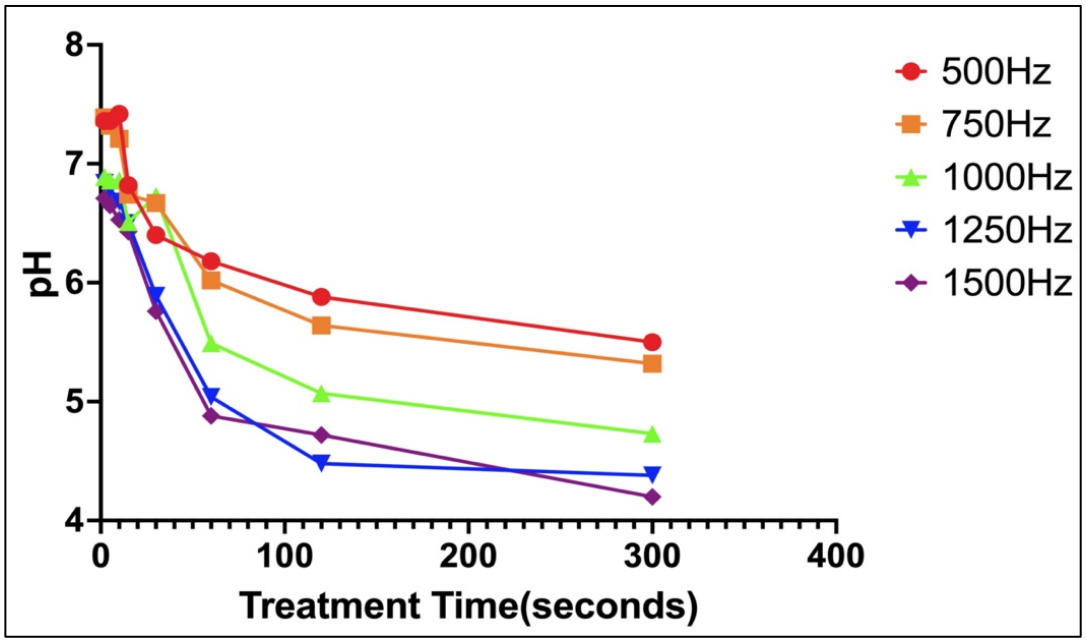


Figure 3.4: pH changes of PAW in different treatment times and different discharge frequencies

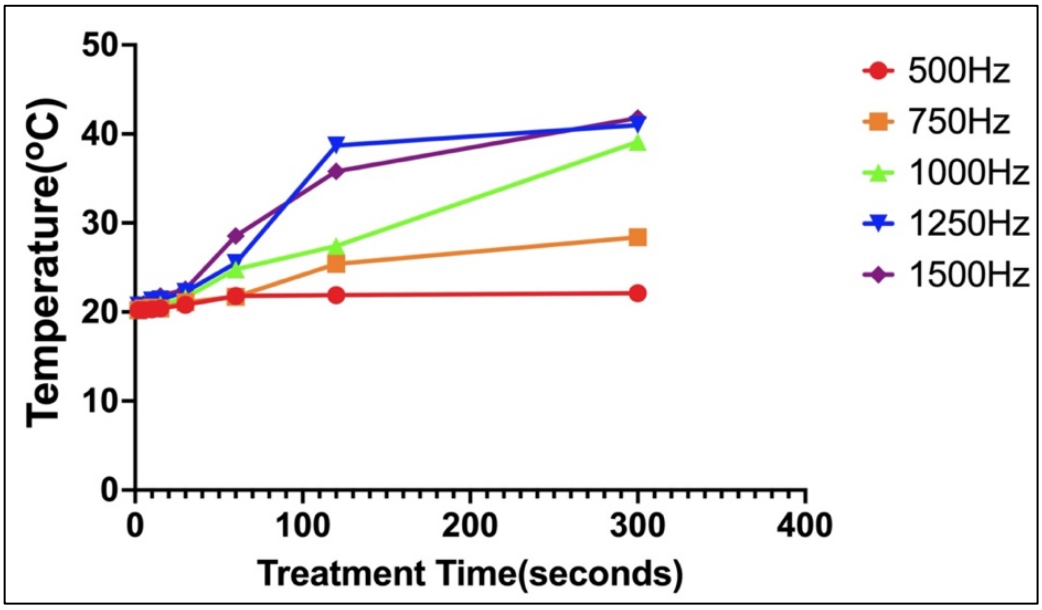


Figure 3.5: Temperature changes of PAW in different treatment times and different discharge frequencies

CHAPTER 04: OVERALL CONCLUSIONS

The results of the Plasma Leap Pin Reactor Study and the Plasma Leap Water/Bubble Reactor Study have been analyzed and the following conclusions can be drawn:

1. The impact of different treatment times (1 min, 3 mins, 5 mins) and discharge frequencies (2000 Hz, 2250 Hz, 2500 Hz) on beef surface color was evaluated. The L-values showed a slight increase, while the a* and b* values showed a slight decrease after the cold plasma treatment. However, no significant differences were observed.

2. The temperature of beef samples was measured before and immediately after the cold plasma treatment. The results revealed that all nine parameters were not significantly altered by the treatment, indicating that the majority of the temperature observations remained unchanged.

3. The moisture content analysis performed after 1- and 5-minute treatments for three different discharge frequencies did not reveal any significant differences. but it should be noted that the decrease in moisture content from 80 to 70, when the treatment was performed for 3 minutes at a frequency of 2000Hz, does not necessarily indicate a significant difference.

4. The results of the Plasma Leap Water/Bubble Reactor Study showed that the log reduction of Escherichia coli K-12 rate increased from 0.1627 log CFU/ml to 6 log CFU/ml as the exposure time to cold plasma was increased from 2 seconds to 60 seconds at five different discharge frequencies. The treatment response also varied with the temperature of the bacteria inoculated water, with a 1.0 log reduction observed in 2 seconds of treatment at 4 ^{0}C. The highest log reduction of 6 CFU/ml was reached in 5 seconds at a discharge frequency of 1500Hz, which was the most effective frequency among the five frequencies.

5. The temperature was found to be between room temperature (approximately 20 Celsius) and 30 Celsius when the treatment time was increased from 2 seconds to 30 seconds. After 300 seconds of treatment at a frequency of 1500Hz, the temperature increased to 41.8 Celsius, while it was 22.1 Celsius at 500Hz, 28.4 Celsius at 750Hz, 39.1 Celsius at 1000Hz, and 41.0 Celsius at 1250Hz. However, the changes in temperature were not statistically significant.

6. The pH of the water decreased as the treatment time and discharge frequency increased. For example, at 500Hz and 2 seconds, the pH was 7.36, while at 1500Hz and 300 seconds, the pH dropped to 4.2. As the treatment time increased, the pH of the water decreased drastically due to the presence of strong acids, but this change was not deemed statistically significant.

7. The antibacterial activity of cold plasma activated water was evaluated by exposing ozone produced in the water to different discharge frequencies over time. The results showed that the cold plasma activation was effective in generating ozone, as indicated by the increase in ozone concentration from 0 mg/L to 1.3 mg/L as the treatment time increased from 2 seconds at 500Hz to 300 seconds at 1500Hz.

CHAPTER 05: RECOMMENDATIONS FOR FUTURE STUDIES

In order to optimize the efficacy of the cold plasma leap process and address certain limitations, further research is necessary. The following suggestions are proposed to guide future studies:

1. Further investigation is required to gain a deeper understanding of the reaction mechanisms and potential negative impacts on the chemical and nutritional properties of food products in the context of the cold plasma leap pin reactor.

2. Comparative studies using different plasma sources and operating gases should be conducted to evaluate the amount of reactive species released and their impact on microbial efficacy in known food products.

3. In order to assess the potential applications of the cold plasma leap bubble reactor, additional studies are needed within the dairy and beverage industries.

4. Given the potential for cold plasma technology to improve food safety and product quality in the food processing industry, it is important to conduct research on its implementation on a larger scale and the optimal operating parameters.

CHAPTER 06: APPENDIX:

Table 2.1: Entries in the table represent P-value for Physical property analysis Before and After cold plasma treatment on Beef Surfaces.

Surface color	**P value**
L-value 1 min	0.2741
L-value 3 min	0.0895
L-value 5 min	0.0895
a*value 1 min	0.3125
a*value 3 min	0.1364
a*value 5 min	0.1998
b*value 1 min	0.1840
b*value 3 min	0.0655
b*value 5 min	0.6649
Moisture Content	**P value**
1 min	0.9999
3 min	0.5185
5 min	0.9999
Temperature	**P value**
1 min	0.4208
3 min	0.0793
5 min	0.3486

Table 3.1: Entries in the table represent P-value for Physiochemical property analysis Before and After cold plasma treatment of PAW

Physiochemical Characteristics	**P value**
PAW Ozone	0.0145
PAW pH	0.2022
PAW Temperature	0.2665

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