FACULTY OF BIOSCIENCE ENGINEERING

USE OF COLD ATMOSPHERIC PLASMA TO INACTIVATE FOOD ASSOCIATED BACTERIA IN FOOD AND WATER SYSTEMS

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Master's Dissertation submitted to Ghent University in partial fulfilment of the requirements for the degree of Master of Science in Food Technology

Academic Year: 2022-2023

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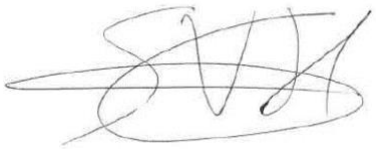
The promoter,



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As I write the final page of my master's thesis, marking the culmination of almost a year of hard work, it is impossible not to acknowledge the invaluable support and guidance I've received from certain individuals who deserve far more than mere words in this document.

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Negar Rahdar, August 2023

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List of Abbreviations

BHI	Brain Heart Infusion
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
XLD(A)	Xylose Lysine Deoxycholate (agar)
PALCAM	Polymyxin Acriflavin LiCl Ceftazidime Esculin Mannitol
PBS	Phosphate Buffered Saline
CP	Cold Plasma
CAP	Cold Atmospheric Plasma
DBD	Dielectric Barrier Discharge
CFU	Colony Forming Units
WHO	World Health Organization
EFSA	European Food Safety Authority
EU	European Union
L. monocytogenes	*Listeria* monocytogenes
E. coli O157:H7	*Escherichia coli* O157:H7
S. Typhimurium	*Salmonella* Typhimurium
UV	Ultra-violet
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
RONS	Reactive Oxygen and Nitrogen Species
PAW	Plasma-activated water
ATCC	American Type Culture Collection
KCTC	Korean Collection for Type Cultures
DI water	Deionized water
a_w	Water activity
EPS	Extracellular polymeric substance
kGy	Kilo Gray
STEC	Shiga toxin-producing *Escherichia coli*
MPN	Most Probable Number
F&V	Fruits and Vegetables
DNA	Deoxyribonucleic acid
ANOVA	Analysis of Variance

Abstract

Cold atmospheric plasma (CAP) is an emerging non-thermal technology for inactivating foodborne pathogens and has received significant research attention recently. This study evaluated the direct efficacy of plasma bubbles generated with a dielectric barrier discharge reactor (DBD) against different foodborne pathogens and their surrogates as well as some Gram-positives like *Micrococcus luteus* in water with micro-bubbling (PAW bubbles) for water disinfection purposes. Additionally, water-based decontamination of *Salmonella* species and *Listeria monocytogenes* inoculated on tomato surface and in the wash water was assessed. As the last part of this research, plasma ability in preventing cross-contamination was investigated. Throughout the experiments, two voltage values, 120 V and 150 V, were tested. Rapid removal of all pathogens occurred at both voltage levels achieving >3.5 log CFU/ml reduction for all pathogens, including various *L. monocytogenes* isolates, *E. coli* O157:H7, and three *S. enterica* serovars, within 20 and 10 seconds at 120 V and 150 V, respectively. *Micrococcus luteus* and surrogate strain of *E. coli* was found to be the most resistant strains in water with only 1.25- and 2.95-log reduction after 20 s. Tomato decontamination at 120 V led to around 2 log CFU/g reduction for *Salmonella* species and 2.36 for *L. monocytogenes* within 3 minutes treatment time. Higher voltage enabled shorter exposure times, yielding around 1.5 log CFU/g reduction for *Salmonella* and 3 log CFU/g reduction for *L. monocytogenes* within 1 minute. Pathogen levels in wash water were consistently reduced below detection limits in both cases. Inactivation was attributed to three reactive oxygen species: superoxide, hydroxyl radicals, and ozone which their critical role was demonstrated through a scavenger test. Cross-contamination was prevented in most cases, though minor contamination was detected in some cases after enrichment which was considered to be removed by further experimental design optimization.

Keywords: Cold plasma, Foodborne pathogen, Tomato, PAW bubbles, Cross-contamination, water disinfection, wash water

Introduction

Contaminated water from various origins can significantly contribute to the transmission of infectious diseases, underscoring the urgent need for robust water disinfection measures (Laroussi et al., 2002). Simultaneously, the rising consumption of fresh or minimally processed fruits and vegetables, recognized for their health benefits, has led to a surge in foodborne outbreaks linked to these products. Iwu & Okoh, (2019) reported that even in developed countries there are approximately 2 million deaths due to foodborne diseases annually. This surge has heightened concerns within the food industry regarding product safety (Rothwell et al., 2023). The research focused on fresh produce has highlighted the crucial role of maintaining the safety of wash water to effectively counter the risk of cross-contamination and ensure the safety of these products after harvesting. Cabrera-Díaz et al. (2022) reported that pathogens can adhere to the surface of fresh produce through two distinct mechanisms, first where they loosely attach to the surface and can be removed mostly through regular water rinsing (LA), while in second case pathogens strongly affix to the surface, requiring the use of sanitizers or further decontamination processes for effective removal (SA). This highlights that even after conventional washing, pathogens may persist in both wash water and on produce surfaces. Consequently, an effective approach is imperative to eliminate bacteria from both mediums. Commonly employed chemical sanitizers can effectively inactivate bacteria; however, some of these agents generate by-products or leave residual effects that can adversely impact both human and the environment (Perinban et al., 2022). Liao et al. (2017a) stated that plasma is a novel technology that, due to its flexibility for various purposes and limited negative consequences after utilization, has become a potent alternative to other processes.

The primary objectives of this study were as follows:

- Firstly, the study aimed to assess the kinetics of plasma inactivation for various bacterial strains in water and identify the most suitable operational parameters.
- Secondly, the research sought to investigate the effectiveness of a plasma bubble setup in facilitating disinfection and decontamination processes for reducing pathogens at ambient temperature using air as the medium.

- Ultimately, endeavors were made to design a realistic set of cross-contamination experiments to comprehensively evaluate the effect of plasma in this process.

A. State-of-the-art

1. Water Safety

Safe water stands as a pivotal objective within the framework of the Sustainable Development Goals, aiming for attainment by 2030. As outlined by the World Health Organization (WHO), the significance of safe water access cannot be underestimated, regardless of its application – be it for consumption, culinary endeavors, or other utilizations. Contaminated water holds the potential to serve as a gateway for the propagation of numerous illnesses, such as diarrhea which is the most common example that approximately led to 2.4 million deaths annually (Davison et al., 2005). All water sources including surface water, wastewater, and the water used for irrigation and food production, have been reported to serve as carriers for the transmission pathogenic *E. coli* particularly O157:H7 strain (Sommer et al., 2000). Polluted water can carry not only pathogenic bacteria but also insects capable of causing various diseases in humans, while also serving as a breeding ground for these harmful organisms. The malaria-carrying mosquito, for instance, is responsible for 1.2 million to 2.7 million deaths per year, while parasitic worms have led to schistosomiasis in over 220 million people in 2017 (Fazal-Ur-Rehman, 2019). The mentioned diseases are not the most serious ones but those that can fade away easily by proper water treatment and save the lives of people. The following diseases are associated with water quality and safety: cholera, Dracunculiasis, Typhoid fever, Hepatitis, Respiratory Tract Infection, and Kidney Damage (Fazal-Ur-Rehman, 2019). Waterborne diseases are still a big concern as they cause human death and need to be prevented to guarantee both human and food products' safety (Davison et al., 2005).

1.1. Irrigation water safety

Irrigation water and manure-amended agricultural soil are of high importance in preventing the introduction of various bacteria during pre-harvest steps, as they are of the common and most probable gateways for bacterial contamination. The choice of irrigation water depends on the

farm's geographical location and the availability of different water sources, including rainwater, deep or shallow groundwater, surface water, or treated wastewater (Iwu & Okoh, 2019). Generally, many water sources, particularly wastewater, contain high levels of impurities and microbial load that must be efficiently treated before being used for irrigation. The presence of pathogenic bacteria in contaminated water poses a significant risk of transmission to fresh produce (Ölmez, 2016). Van Der Linden et al. (2013) reported that spray irrigation poses a higher risk of contamination as it increases the direct contact between bacteria and the crop. Adefisoye & Okoh, (2017) conducted a one-year standard sampling of two wastewater effluents which was used for agricultural application afterwards in South Africa and observed a high bacterial count, approximately 5 logs CFU/100ml of *E. coli* and *Vibrio* species. This finding underscores the importance of disinfecting wastewater before its use in agricultural practices to ensure food safety and prevent potential health risks.

However, different studies claimed irrigation water regardless of disinfection processes still has high microbial load which emphasis the inefficiency of traditional methods and the need for advanced procedures. A study by Materon et al. (2007) found that the aerobic bacterial load in irrigation water of a cantaloupe farm was as high as 7.7 Log CFU.ml^{-1}, along with 5.5 Log CFU.ml^{-1} of total coliforms and 4.0 Log CFU ml^{-1} of fecal coliforms. Also, around 5.3 Log CFU.cm^{-2} of *Salmonella* and 4.6 Log CFU.ml^{-1} of *L. monocytogenes* entered the farm through irrigation water. Some studies show that *Listeria* can survive in water resources around 40 days if the water temperature is around 20 °C (Gartley et al., 2022). Therefore, in this research plasma bubbles were used to evaluate bacteria inactivation in water first and further applied to fresh produce, since it is impossible to ensure the safety of fresh produce without ensuring the safety of water.

1.2. Wash water safety

Traditionally, post-harvest washing has been a crucial step in reducing microbial load and preventing cross-contamination of fresh produce. However, recent research has highlighted concerns regarding the effectiveness of this process in achieving its intended goals (Murray et al., 2017). Due to the global water shortage in most places, potable water is no longer a feasible option for use in the washing process, furthermore, treated wastewater and used post-harvest

water have become alternative water sources for washing. López-Velasco et al. (2012) reported that the use of treated water in re-circulated systems, such as tanks, can lead to an increased organic matter level, becoming a critical point that may cause cross-contamination instead of preventing it. Adding antimicrobial agents to the water tank can reduce microbial load and the possibility of cross-contamination, Sodium hypochlorite, peroxyacetic acid, aqueous ClO_2 and ozone are among the most used sanitizers (Table 1) (López-Velasco et al., 2012). However, it is important to note that the U.S. Food and Drug Administration (FDA) has set regulations for the use of chlorine-containing compounds, with a maximum residual amount of 3mg/L in the wash water.

The inefficiency of the washing process, can be attributed to two main reasons identified by Weng et al. (2016) which are (i) the nature of pathogens attachment such as biofilm formation, López-Velasco et al. (2012) observed higher resistance to sanitizers in microorganisms embedded in particles compared to non-attached ones, and (ii) the presence of high organic loading in wash tanks, which interacts with sanitizers and further provides protection for pathogens by neutralizing their antimicrobial effect. For instance, free chlorine can readily react with inorganic matter in wash tanks resulting in the formation of byproducts that are less effective in pathogen reduction than free chlorine, this was the main reason for the *E. coli* O157:H7 outbreak with spinach, where the pathogen was introduced through irrigation water and then low levels of free chlorine in wash tanks could not efficiently reduce pathogens and cross-contamination occurred between different batches (Keith Warriner and Azadeh Namvar, 2013). Furthermore, the post-harvest washing step seems to be incapable of both reducing pathogens to an acceptable level and preventing cross-contamination as pathogens can disseminate during the washing process (Weng et al., 2016). More importantly an inadequate washing program for fruits and vegetables can consequently lead to microorganism internalization (López-Velasco et al., 2012).

To achieve an efficient washing step, B. Zhou et al. (2014) proposed regular monitoring of the active sanitizer level in water tanks to ensure it remains above the designated concentration required for pathogen inactivation within the washing time so it cannot reach the other products and contaminate them. However, Gombas et al. (2017) argued that addressing washing inefficiency is more complex than solely maintaining sanitizer levels which is itself difficult to be

done. During the development of a validation protocol for the washing process Gombas et al. (2017) encountered several issues despite the underlying assumption of the wash tank be a static environment, which is dynamic as the microbial and organic loading is fluctuating constantly. The issues were:

(i) Antimicrobial efficiency should be determined. According to Van Haute et al. (2013) antimicrobial efficiency can be determined by measuring 'CT value' which is dependent on sanitizer concentration (ppm) and treatment time (min). This became an issue since setting a specific value for concentration as well as keeping it at that level during washing time is complicated.

(ii) How much log reduction is desired in the washing time? Murray et al. (2017) reported that a 2~5 log reduction is desired, however, the concentration of sanitizer needs to be set after considering both required pathogen inactivation and consumer health, so it should not exceed the level that might induce any adverse effect to consumers even in long runs.

(iii) How fast do the pathogens need to be inactivated? Pathogen transfer can be done in a fraction of second, therefore finding the proper kinetics is complex.

(iv) How to maintain the antimicrobial agent concentration in accepted level? Besides the presence of organic and inorganic matter that can significantly affect the active compound's level, Driss & Bouhelassa, (2014) found that water properties such as pH, temperature, turbidity and conductivity could also influence the sanitizer stability and required concentration.

Keith Warriner and Azadeh Namvar, (2013) proposed that as an alternative, using methods that can directly decontaminate the fresh produce or methods that can work in gaseous states like O_3 and plasma, could be a better option for preventing cross-contamination and eliminating washing-related contamination.

Table 1. Advantages and disadvantages of conventional methods for water disinfection (Kostya (ken) Ostrikov et al., 2020; Xu et al., 2020)

Disinfection method	Advantage	Disadvantage
Chlorination	Effective inactivation rate, cheap	By-product formation, reaction with organic matter
Ozone	Oxidizing and further reducing the organic matter, few by-products than Chlorination, minimal odor and taste change, rapid decomposition to oxygen	Expensive, on-site generation is required, which can cause lipid oxidation, human exposure restriction, low energy efficiency
UV	No residual effect, effective against chemicals as well, low cost of the equipment	Low penetration effect, limited range of equipment for solid disinfection

1.3. Wastewater safety

Based on WHO guidelines, if wastewater is intended to be used for fresh produce the concentration of fecal coliforms should not exceed the most probable number (MPN) of 100 $CFU.ml^{-1}$. When talking about waste treatment, Crini & Lichtfouse, (2019) proposed that water can be divided into four groups that require further processing, (i) rainwater, (ii) agriculture water, (iii) industrial wastewater further subdivided into wash effluent and process water, and (iv) domestic wastewater. Each group requires specific treatment due to the presence of different impurities and contaminants.

For industrial wastewater treatment, three main categories of processes are considered: physical, chemical, and biological (biosorption and bioprecipitation). Chemical methods, such as precipitation, coagulation, adsorption, and ion exchange, are commonly employed for metal extraction. These techniques are known for being easy to implement, cost-effective, and effective in reducing chemical oxygen demand (COD). Nevertheless, they do have some drawbacks, including high sludge production, the need for physicochemical inspection of effluent (particularly pH monitoring), and the requirement for a substantial amount of chemicals (Crini & Lichtfouse, 2019; Dutta et al., 2021). Azimi et al. (2017) reported that electrochemical treatment is also another chemical process for wastewater that can efficiently remove metals better than other counterparts, however, due to the high energy consumption and high capital cost it is not

widely used. Physical methods are primarily applied to separate solid particles and can be divided into filtration and sedimentation processes. Ahmed et al. (2021) stated that physical treatment will not change biochemical properties, and the main advantage of these methods is their simplicity and flexibility, and lower solid waste production compared to other methods. Wastewater can be treated with plasma as well, for this purpose, most commonly O_3 or O_2 gases had been used in DBD reactors since uniformity of the treatment is of high importance. Besides the DBD reactor, Corona discharge is also reported to be efficient for water treatment. The most important advantage of plasma in water treatment is its ability to be implemented in open space while other methods such as UV require a closed space (Ghernaout & Elboughdiri, 2020). The efficiency of plasma in wastewater treatment can be found in Table 2.

Table 2. Examples of plasma efficiency in wastewater treatment against different bacteria

Sample source	Microbial strain	Time	Log Reduction	Reference
Dairy and meat industry	*E. coli*, *Clostridium perfringens*	*5 minutes*	below the limit of detection	(Patange, Lu, et al., 2019)
		2 minutes	E. coli 49% reduction, Clostridium perfringens 29%	
	Vegetative and spore of *Bacillus megaterium*	*5 minutes*	vegetative cells below the limit of detection, spores ±1.9 log CFU/ml	
Seaweed processing plant	*E. coli*	*20 minutes*	below the limit of detection	(S. Ma et al., 2020)
Blackberry processing plant	Total bacteria count	*3 minutes*	0.41 log CFU/ml	(Mohamed et al., 2016)
Beetroot processing plant	Total bacteria count	*3 minutes*	2.24 log CFU/ml	

2. Fresh produce safety

Fruits and vegetables (F&V), play a crucial role in our daily diet as they serve as a primary source of essential micro- and macro-nutrients (Iwu & Okoh, 2019). Micro-nutrients, including minerals, vitamins, polyphenols, and carotenoids, contribute to various health benefits. On the other hand, macro-nutrients like carbohydrates and dietary fiber provide essential energy and aid in

maintaining a balanced diet. Recently people tended to consume more fresh produce than ever as they became aware of the health benefits of these products and due to their increased global availability of them (Ziuzina & Misra, 2016). However, since these products tend to be consumed raw or with minimal processing, the risk of passing on food pathogens to humans is high (Carstens et al., 2019). Therefore, as a result of increased consumption, the number of outbreaks originated from the F&V also raised in compared to the past. In addition to the whole F&V, fresh-cut packages of these products have gained much attention recently. However, ensuring the safety of these packs presents greater challenges compared to intact products since, in the case of pathogen presence, they can grow to very high numbers in the injured or cut areas of the produce (Ölmez, 2016). It is important to note that due to the high water activity (a_w) and sugar content of F&V, these products provide a good environment for bacteria to grow and therefore particular attention is required in order to ensure their safety (Q. Y. Han et al., 2023).

To assess the acceptability of a product, a food control procedure has been developed, which includes the checking safety, nutrition level, and quality levels. Food safety stands as the most crucial step in food control, directly impacting human health (Seroka & Wojciechowska-Solis, 2019). In other words, due to the rise in fresh produce production, these products adversely affect human health and need to be checked for pathogen presence to mitigate the detrimental effect. These products have caused outbreaks of foodborne pathogens originated from *L. monocytogenes*, *Salmonella* and *Escherichia coli* (*E. coli*) O157:H7 in the past few years. However, foodborne pathogens include not only bacteria but also viruses, molds, protozoa and yeasts. Hence, ensuring the safety of these products has become a critical concern in the food industry. Among fresh produce-related outbreaks leafy vegetables, sweet fruits like melons, and tomatoes have frequently been reported as the contaminated sources (Machado-Moreira et al., 2019; Murray et al., 2017). In the United States, most of foodborne outbreaks are linked to fresh produce, 2,006 outbreaks were recorded with *E. coli* O157:H7 from spinach and several Salmonellosis outbreaks were linked to fresh tomatoes (Iwu & Okoh, 2019).

According to Ölmez, (2016) the increased number of foodborne outbreaks can be attributed to two main factors: Firstly, the globalization of the food chain has led to more people having access to fresh produce. Consequently, if a batch of produce is contaminated, the potential for

widespread impact on public health is greater (Machado-Moreira et al., 2019). Furthermore, this globalization has made food safety management more complex. Secondly, changes in agricultural processes have shifted the focus towards increasing supply rather than prioritizing safety measures.

As fresh produce is grown on the open field, it can be contaminated through native bacteria in soil, water, animals and vegetation; also, pathogens can be introduced to the crops in pre- and post-harvest stages like water for irrigation and as pesticide carrier, soil and manure, air, washing, transportation, storage, field workers, sorting, harvesting equipment or packing (Nguyen-the & Carlin, 1994). In the case of post-harvest unit operations, those that can cause tissue damage, such as cutting or peeling, make the produce more susceptible to contamination due to the release of nutrient-rich intracellular exudates (Ölmez, 2016). Therefore, F&V need to be effectively decontaminated prior to distribution to the final consumers.

Based on European Food Safety Authority (EFSA) annual reports, between 2017 and 2021, there were 23,893 foodborne outbreaks in European Union (EU) countries which resulted in 200,893 human cases, 17,617 hospitalizations, and 198 deaths. The detail of outbreaks caused by the three focused pathogens are given in Table 3.

Table 3. Incidence of main pathogens outbreak, 2017-2021, in all EU countries.

Causative agent	Outbreaks	Human cases	Hospitalizations	Deaths	Share in total death (%)
Salmonella	5,777	45,063	9,178	35	16.36
L. monocytogenes	92	839	536	96	44.86
E. coli	289	2,861	351	4	1.87

The prevalence of *E. coli* O157:H7, *S*. Typhimurium and *L. monocytogenes* are commonly associated with salad vegetables and fruits like apples, tomatoes and watermelons (Mostafidi et al., 2020).

A brief explanation of these pathogens will be provided in the following parts.

2.1. Foodborne pathogens

As mentioned previously, foodborne pathogens include not only pathogenic bacteria but also other microorganisms that can have adverse effects on human health, such as Hepatitis, which can occur after ingesting contaminated food or water containing the hepatitis A virus. In this project the focus is on three bacterial species because of their high contribution to outbreaks.

2.1.1. Salmonella

Salmonella is a type of bacteria that is rod-shaped, Gram-negative, and does not form spores. It is facultatively anaerobic and belongs to the *Enterobacteriaceae* family. *Salmonella* is divided into two species: *Salmonella enterica (S. enterica)* and *Salmonella bongori (S. bongori)* (Thomas Hammak, 2017). *S. enterica* is further categorized into six subspecies, and each subspecies contains different serovars, identifiable by specific flagellar and lipopolysaccharide structures. For instance, *S.* Typhimurium, *S*. Poona, and *S*. Thompson are some of the serovars used in this thesis (Coburn et al., 2007). *S. enterica* can be transmitted through contaminated food or water ingestion or through direct contact with infected individuals or animals, making it a zoonotic pathogen. After ingestion, the bacteria invade the intestinal cells in the colon and may further penetrate the small intestine's epithelium, however, in case of severe infection, the bacteria can spread to various body parts, such as blood stream and nervous system, leading to life-threatening conditions (Albrecht, 2023-c). The illness caused by *Salmonella* is known as enteric Salmonellosis, characterized by symptoms like fever, abdominal cramps, diarrhea, and vomiting (Knodler & Elfenbein, 2019). These symptoms usually manifest between 6 to 72 hours after exposure and can vary in severity based on the host's characteristics. Children under 5 years old, individuals over 65 years old, and immunosuppressed adults are particularly vulnerable to the infection. *S.* Typhimurium, a specific serovar, thrives in a pH range of 3.8 to 9.5, with an optimum pH of 7 to 7.5. The bacteria require a minimum a_w of 0.94 for growth and thrive best at a temperature of 37°C (Knodler & Elfenbein, 2019). *Salmonella* is widespread in nature, surviving in pond-water sediments, contaminating irrigation water, and colonizing the intestinal tracts of vertebrates (Fernández & Thompson, 2012). Apart from dairy, eggs, poultry, and meat, fruits and

vegetables such as tomatoes and cantaloupe have been identified as sources of *Salmonella* outbreaks (Hammak, 2017).

2.1.2. L. monocytogenes

Listeria is a genus of Gram-positive, psychrotrophic, non-spore-forming, rod-shaped with rounded ends and facultatively anaerobic belonging to the family of *Listeriaceae* (Patil et al., 2010). It has 17 different species *and L. monocytogenes* is one of those. This species can cause Listeriosis infection which count as one of the serious infections with a mortality rate of 30% of the population at risk and it happens after ingestion of contaminated food (Calvo et al., 2016). For this disease pregnant women, infants, and individuals aged 65 are among the high-risk group (Gray1 & Killinger, 1966). Listeriosis can cause different symptoms based on the person and the body part which is affected. It can cause gastrointestinal symptoms, however, when it became invasive after spreading beyond the gut it can cause more severe and serious symptoms such as meningitis and encephalitis (Chen, 2017). "In the case of pregnant women, invasive infection can lead to miscarriage, stillbirth, premature delivery, or life-threatening infection of the child (Centers for Disease Control and Prevention, 2018)". Numerous food items have been linked to *L. monocytogenes* contamination, given its ability to thrive in cold environments. This includes products like fresh vegetables, ice cream, chocolate milk, and seafood, all of which can be stored in refrigerated temperatures (Albrecht, 2023-b).

2.1.3. *E. coli* O157:H7

Escherichia coli (E. coli) is a natural part of the beneficial bacteria found in the human gut. However, certain groups of *E. coli* are considered harmful and can cause health problems in humans. One of the most important pathogenic groups is called enterohemorrhagic *E. coli* (EHEC), and *E.* coli O157:H7 is a notable member of this group (Chen, 2017). EHEC strains are Gram-negative, rod-shaped bacteria with various serotypes, but *E. coli* O157:H7 is the most concerning as it can lead to illness. Infections usually occur when contaminated food or water is ingested. In severe cases, it can result in bloody diarrhea, kidney failure, and even death. While some cases may only cause mild watery diarrhea, others can progress to Hemolytic Uremic Syndrome (HUS) with a mortality rate of 3-5%. Outbreaks have been associated with various food sources, including ground meat, water, unpasteurized fruit juice, lettuce, spinach, and sprouts. Children

and immunosuppressed individuals are particularly vulnerable to these infections. The optimum growth temperature is 37°C, minimum a_w and pH for growth is 0.90 and 3.6 respectively (Albrecht, 2023-a).

2.2. Common fresh produce decontamination methods

Traditional technologies for assuring safety were limited to the use of heat such as pasteurization, sterilization and boiling. However, those methods had an impact on sensorial and nutritional properties in a negative way that reduce product acceptability by consumers (Aktop et al., 2023). Besides thermal processing, two major categories of disinfection methods exist which are milder, namely chemical and non-thermal physical, of which chlorine (chlorine dioxide), ozone (O_3), H_2O_2, essential oils, organic acids, and electrolyzed water fall into the chemical category, while ultraviolet (UV), ultrasound, irradiation and cold plasma (CP) fall into the latter category (Deng et al., 2020).

2.2.1. Ultra-violet (UV)

UV radiation can be categorized into UV-A, UV-B, and UV-C based on wavelength, with UV-C (200-280nm) being predominantly used for disinfection purposes. Traditionally, UV radiation has been employed to disinfect air and water by damaging the DNA/RNA of microorganisms through the formation of nucleotide dimers (Deng et al., 2020). UV radiation can be utilized for various food products, including liquid food and beverages, dairy products, as well as fruits and vegetables (Singh et al., 2021). This method offers advantages such as simplicity, speed, ease of implementation, and cost-effectiveness. Furthermore, UV treatment does not leave any residual substances on the products. However, one limitation of this method is the potential alteration of the structure of dissolved organic matter (DOM), which can lead to taste and odor issues through biofilm formation. Biofilms may be formed by bacteria that consume modified DOM as a food source. The penetration capability of UV radiation is relatively low, with a maximum depth of approximately 1 mm in transparent samples without significant energy loss. Ochoa-Velasco et al. (2018) reported This limitation can be mitigated to some extent by applying sufficient UV doses for extended periods; however, it is generally recommended to use UV radiation for surface decontamination or treatment of liquid samples. UV radiation has demonstrated potential for

extending the shelf life of food products as a green technology (Singh et al., 2021). In terms of its impact on quality attributes, Unluturk & Atilgan (2015) reported that UV exposure resulted in changes in ascorbic acid content and color values, with increased L* values and decreased a* and b* values, while pH and total soluble solids remained unchanged. UV radiation is effective not only against pathogenic bacteria but also against yeasts and bacteria, although yeasts are comparatively more resistant due to their larger size and the presence of a nuclear membrane that provides DNA protection (Deng et al., 2020). It is important to note that UV radiation is primarily effective against microbial contaminants and may not be effective for reducing other types of contamination, such as chemical pollutants, requiring additional measures if such contamination is a concern.

2.2.2. Ozone

As a powerful oxidizing agent, the mechanism of O_3 disinfecting is reacting with cellular components such as the membrane's unsaturated fatty acids or proteins and gradually oxidizing them (Kaavya et al., 2021). Ozone oxidizes sulfhydryl groups in proteins and similar structures like enzymes and amino acids, leading to the structural decomposition of those compounds. In the case of unsaturated fatty acids, ozone can reform them into peroxides that cause the leakage of cell constituents and ultimately result in cell death (Niveditha et al., 2021). Like UV radiation, ozone is eco-friendly and dissipates quickly without leaving any residues. Pandiselvam et al. (2019) point out that around 50% of O_3 will decomposes after 20 minutes. One advantage of ozone over UV radiation is its high penetration capacity, making it suitable for treating voluminous samples. Ozone can be applied in both gaseous and aqueous modes, making it applicable to various products. However, it presents some challenges such as its low solubility, which requires on-site generation and results in high capital costs. Additionally, the formation of intermediate compounds, such as aldehydes, that cannot react with ozone poses a significant challenge (J. Wang & Chen, 2020). Requiring specific conditions for each product based on their physicochemical characteristics, make it a complicated method in compared to others like UV (Pandiselvam et al., 2019). For example, foods with higher pH require longer treatment times or higher ozone concentrations, as ozone stability is lower in alkaline environments. Excessive ozone exposure can induce oxidation, leading to the destruction or alteration of color and aroma.

Additionally, it can negatively impact human health, especially concerning the respiratory system (Deng et al., 2020). Murray et al. (2017) reported that O_3 is a corrosive agent which can extensively corrode metal surfaces. Color can be affected by the reaction of ozone with carotenoids, as reported by, Bridges et al. (2018) who observed bleaching of tomatoes after applying 1.71 µg of ozone per gram.

2.2.3. Irradiation

Irradiation also showed its potential in decontaminating fruit and leafy vegetables. Omac et al. (2017) reported that after treating contaminated leafy green vegetables such as spinach, *Listeria* populations were reduced by 65%, which make the product safe to consume. Shayanfar et al. (2017) observed 4 logs reduction in the Shiga-toxin STEC population in strawberries after irradiation with low doses of around 1 kilo Gray (kGy). Even though this method is effective in reducing and inactivating pathogens, some drawbacks restrict the common use of it. Murray et al. (2017) reported that other than consumer acceptance which is still a burden for irradiated products, other disadvantages such as ripening retardation and viruses and endospore resistance to it, are present.

2.2.4. Chlorine

The mechanism of microorganism inactivation by chlorine involves increasing membrane permeability, leading to leakage and subsequent cell death (Praeger et al., 2018), however, this is not the only mode of action for chlorine. Other possible mechanisms include the suppression of protein synthesis (Artes et al.,2009) or a mechanism similar to photocatalytic disinfection (Van Haute et al., 2013). One significant disadvantage of chlorine treatment is the long duration required to achieve the desired reduction in microbial populations. For example, after 5 hours of experimentation with a chlorine dose of 1.71 mg ClO_2/g produce, populations of *E. coli*, *S. enterica*, and *L. monocytogenes* on beefsteak tomatoes only reduced by 1.6, 1.1, and 1.1 log CFU/g, respectively. The same dose and contact time resulted in lower reductions for baby-cut carrots, with values of 1.2, 0.5, and 0.8 log CFU/g. (Bridges et al., 2018). Chlorine treatment can generate by-products, with trihalomethanes (THMs) being the most common, including chloroform. Government regulations impose limits on the concentration of THMs in fresh

produce due to their potential adverse health effects in long-term exposure. Chlorine is not effective against bacterial spores, which are potential risks on the surface of fresh produce (Al-Abri et al., 2019). Moreover, a small and safe dose of chlorine typically cannot achieve a reduction in microbial load greater than 1 to 2 logs, which may be sufficient for spoilage control but not for ensuring safety due to the low-infective dose pathogen presence. Additionally, chlorine is corrosive and may not be suitable for use with all containers and equipment. As an alternative to chlorine, chlorine dioxide has been investigated and shown to be more efficient due to its nearly 2.5 times greater oxidizing capacity and its lower corrosiveness compared to chlorine and O_3. Chlorine dioxide can kill spores, unlike chlorine, and does not produce carcinogenic by-products (Praeger et al., 2018). However, there are still other challenges associated with chlorine dioxide present that need to be addressed including long application times (around 2 hours), changes in aroma, and reversible damage to some organisms, which may allow them to grow again during shelf-life. Both chlorine and chlorine dioxide are thermally unstable and pose explosion risks in the workplace (Deng et al., 2020). But till now chlorination is still the easiest and cheapest method that is commonly used in the industry.

Fruit and vegetable decontamination can also be achieved with peracetic acid which acts like sodium hypochlorite and its activity is related to reactive oxygen radicals' generation (Chinchkar et al., 2022), Bromine which is usually used in combination with chlorine, and Advanced oxidation (Freese & Nozaic, 2004).

3. Plasma technology

3.1. Definition and classification

The term "plasma" was first introduced by Langmuir in 1928 to describe a gas that contains an almost equal proportion of electrons and ions (Gururani et al., n.d.). Plasma, the fourth state of matter alongside gas, liquid and solid, can be created as ionized gas when electrons are removed due to high energy induction. In other words, when a gas is excited, electrons gain more energy and become free. When exposed to a powerful electric field, these free electrons can act as centers for other electrons to absorb and interact with. This cluster of electrons then further

interacts with gas atoms through collisional processes, resulting in plasma generation. The strength of the electric field is a key parameter in plasma production. The stronger the field, the more concentrated and denser the plasma will be. Consequently, plasma is a gaseous mixture of free radicals, free electrons and charged ions (Scally et al., 2023). The efficacy of plasma depends on the type of gas used in the generation process.

Plasma can be classified into two main categories: thermal and non-thermal. In thermal plasma, also known as equilibrium plasma, the temperature and energy of all species, including ions, electrons and radicals are the same, indicating that all constituents are in thermal equilibrium (Scholtz et al., 2015). This type of plasma generally has a high collision frequency and an overall temperature on the order of 10^4 K. Additionally, unlike non-thermal plasma, which has a low ionization rate, the degree of gas ionization in thermal plasma is more or less equal to 100%. On the other hand, non-thermal or non-equilibrium plasma has an electron temperature around 10^4 K while ions and heavy species have nearly room temperatures (Surowsky et al., 2015; Hong et al., 2021).

Non-thermal plasma can be further divided into two groups: non-equilibrium, also known as cold plasma (CP) with temperature <60 ˚C, and quasi-equilibrium with a temperature between 100 and 150 ˚C (Kostya (ken) Ostrikov et al., 2020). Atmospheric pressure or lower pressure can be used in CP generation that the former one is of high interest because it has mild conditions for food application and does not require extreme conditions. When air is used as the CP medium the setup will be called Cold Atmospheric Plasma (CAP) which can be utilized in room temperature (Hong et al., (2021). CAP is gaining attention due to low energy consumption and not having any chemical residuals after exposure (Hemmati et al., 2021). CAP can be generated using various devices and setups such as electric discharge, corona discharge, dielectric barrier discharges (DBD), microwaves (only setting without electrodes), radio frequency waves, plasma jets or gliding arc discharge. DBD Plasma reactors and plasma jets have gained significant attention due to their simple setup and convenient usage.

3.2. Applications of CAP

CAP is still an emerging technology that need to be studied more for all the capabilities. Till now the usage of non-thermal plasma technology was studied the most in microbial inactivation and safety improvement and proved to be capable of not only reducing microbial populations but also eliminates other pollutants such as pesticides and hazardous compounds that contaminate water and foods due to industrialization. Additionally, CP not only increases safety by reducing and inactivating food-borne pathogens but also is able to improve quality by inactivating enzymes that may cause off-flavour and discoloration (Misra et al., 2016). This technology can be used prior to washing to disinfect wash water and make it safe for further processes and can also be applied after the washing process to disinfect wastewater. Plasma has demonstrated its capability to inactivate yeast, mold, viruses, spores, and biofilms on food products, particularly heat-sensitive ones, unlike traditional thermal processes. Moreover, it can be applied for packaging and equipment decontamination (Costello et al., 2021). Plasma can also be used for functionality enhancement, allergens alleviation, grain germination enhancement, and oil hydrogenation to produce trans-free products that help to reduce the possibility of heart disease (Chizoba Ekezie et al., 2017). Additionally, it can be utilized in fields beyond the food industry, such as medical and biomedical purposes, wound healing, cancer treatment and other innovative applications (Costello et al., 2021; Chen et al., 2016).

In the food industry, besides products safety from the microbial point pesticide residuals are a big concern as well. Different studies had been conducted to verify CAP potential in pesticide degradation, data can be found in Table 3. Most commonly organophosphorus compounds like parathion degradation can occur due to oxidation and S–C, S–P, C=C and S=P bonds breakage as well as losing functional group by RONS. All these bonds breaking down will lead to intermediate amines and aromatic acids and eventually, pesticide disintegration, high ORP and low acidity will also accelerate these reactions (Q. Y. Han et al., 2023). In the case of phoxim S=P and N–O bonds will cleave and produce P=O double bond and diethyl hydrogen phosphate respectively. For chlorothalonil C and Cl bonds will be attacked by radicals and further cleave so that the

compound structure is destroyed (Ali et al., 2021). Another possible pathway for degradation is the effect of H_2O_2 to form hydroxylated products, H_2O_2 can directly attack the benzene ring of pesticides.

Table 4. Effect of plasma on pesticide degradation

	Pesticide	Reduction (%) after plasma exposure	Reference
grape	phoxim	73.60	(Zheng et al., 2019)
	carbonyl	83	
	chlorpyrifos	79	
tomato	thiram	79.47	(Ali et al., 2021)
	chlorothalonil	85.30	
strawberry	carbonyl	73	(Q. Y. Han et al., 2023)
	chlorpyrifos	69	

3.3. Different setup configuration

3.3.1. DBD reactor

A DBD reactor (Figure 1) consists of two metal electrodes: one ground electrode and one high-voltage electrode. These electrodes are coated and separated by dielectric layers typically made of glass, quartz, or mica. When a high potential difference is applied between the electrodes, the dielectric material or gas between them loses its dielectric properties and acts as a conductor, resulting in the generation of numerous micro-discharges (Katsigiannis et al., 2021). Furthermore, to sustain the formation of plasma, the applied voltage must exceed the break-down voltage. The break-down voltage (V_b) depends on factors such as product pressure, inter-electrode distance, and gas composition, which can be explained and demonstrated by Paschen's law and curve. The flexibility of this setup allows for different configurations based on sample conditions and specific applications, enabling the alteration and improvement of plasma discharge (Scally et al., 2023; Das et al., 2018). Additionally, the setup can incorporate a bubble column or reactor, where plasma bubbles can be utilized for disinfection, leading to more significant effects. Bubble properties play a crucial role in their efficiency and application. For example, larger bubbles are more practical when homogeneity is essential, while smaller bubbles induce faster flow and

improved mass transfer (Wright et al., 2019). DBD is suitable for homogenous disinfection of large surfaces, and it offers advantages such as the ability to use a wide range of gases and flow rates (Nwabor et al., 2022). According to Aktop et al. (2023) proper and efficient settings for this reactor are provided in Table 5.

Table 5. recommended values for DBD reactor application

Parameter	value	unit
Gas pressure	10^4 — 10^6	Pa
Frequency	0.05 — 500	kHz
Voltage	10	kV

3.3.2. Plasma jet

In a plasma jet reactor (Figure 1), two concentric electrodes are placed in a manner where the inner one has a high voltage ranging from 100 to 250 V and a high frequency of 13.56 MHz to induce gas ionization. The outer electrode serves as a ground electrode and is either covered with an inner insulating ceramic layer or has two quartz tubes. The electrodes are typically separated by a gap in the magnitude of millimeters. The designated gas flows inside the high-voltage electrode, and plasma discharges are transferred to the sample placed below the device through a nozzle (Misra et al., 2016). To generate the plasma flame, noble gases mostly helium, are commonly used at high velocity and flow rates enabling this setting for direct application and penetration into narrow spaces (Aktop et al., 2023). Compared to the DBD setup, the plasma jet configuration is more expensive.

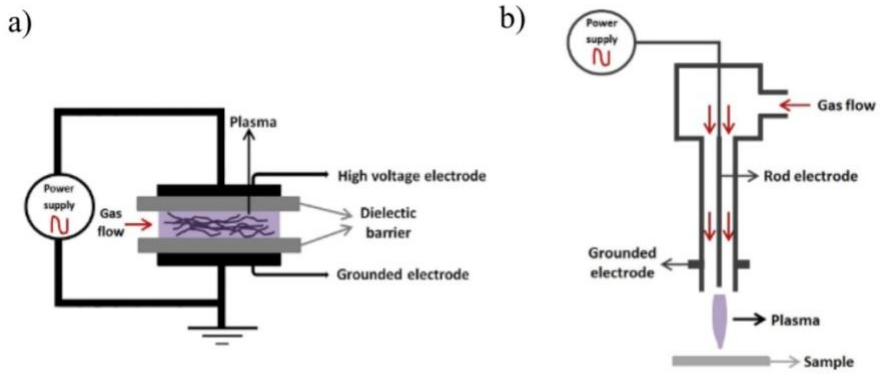

Figure 1. a) DBD reactor, b) Plasma jet reactor configuration (Aktop et al., 2023)

3.4. Inactivation mechanism

When the process of plasma generation is done and ionized gas has been produced due to collision of electrons, atoms and molecules, plasma will include UV photons and chemical species that are responsible for the inactivation effect. Chemical species include (i) free radicals, (ii) hydrogen peroxide (H_2O_2), (iii) reactive oxygen species (ROS), (iv) reactive nitrogen species (RNS), and (v) charged particles such as free electrons and ions (J. Y. Han et al., 2023a). Each of these components can inactivate bacterial cells individually, however, the synergistic effect of them might improve the plasma's bactericidal effect. Table 6 provides a list of RONS.

Table 6. Different reactive species in plasma that causing main damage to microorganisms (Kostya (ken) Ostrikov et al., 2020)

		species name	***chemical formula***			***species name***	***chemical formula***
ROS	non-radicals	*Hydrogen peroxide*	H_2O_2	RNS	non-radicals	*Peroxynitrite*	$ONOO^-$
		Ozone	O_3			*Ammonium ions*	NH^+_4
	radicals	*Hydroxyl*	$\cdot OH$			*Nitrate*	NO_3^-
		Singlet oxygen	$^1O_2$			*Peroxynitric*	$OONO_2^-$
		Superoxide	O^-_2			*Nitrite*	NO_2^-
		Hydroperoxyl	$HOO\cdot$		radicals	*Nitric oxide*	$NO\cdot$

Regarding the inactivating effect of UV, contradictory findings have been reported. Vleugels et al. 2005 suggest that UV is less effective compared to other plasma particles as it is readily absorbed by the plasma-producing gas atoms. Conversely, Nwabor et al. 2022 found that wavelength of UV produced by plasma is in the range of 100-380 nm (UV-C range) that can cause cell damage by altering or damaging DNA, thus interfering with replication and transcription processes.

Inactivation by chemical species can occur through various mechanisms, including (i) DNA damage, (ii) cell membrane damage, and (iii) changes in the functionality and structure of macromolecules such as proteins, fats, and carbohydrates (Bourke et al., 2018).

Oxidative damage to lipids can disrupt mass transfer across the cell membrane, disturb cell balance, and potentially allow charged particles to enter the cell, leading to a reduction in internal pH (Xu et al., 2021). Also, RNS upon entering the intracellular space, primarily contribute to pH drop. The type and concentration of RONS depend on plasma voltage, gas type, treatment time, and humidity, where as an illustration, higher humidity levels result in more hydroxyl groups and H_2O_2.

Free radicals commonly exert their effects through DNA breakage or alteration in DNA bases. Charged particles, such as ions or electrons, can accumulate on the cell membrane surface, disrupting the balance of electrostatic forces and causing membrane rupture and integrity loss. This phenomenon, known as ion bombardment, occurs when charged particles are accelerated toward their target due to electric field energy introduced by the plasma (Aktop et al., 2023).

ROS specifically target polyunsaturated fatty acids (PUFAs) in the membrane. By compromising the double bonds in PUFAs, hydroxyl and hydroperoxyl radicals can more easily attack them, creating fatty acid radicals. Consequently, the C-H bond adjacent to the double bonds becomes weaker, rendering it more susceptible to ROS activity. Fatty acid radicals eventually transform into lipid hyperoxides, leading to membrane permeability (Liao et al., 2017b). As a result of increased permeability, protons generated by plasma can enter the cytoplasm through membrane pores, resulting in cell death (Han et al., 2023).

Another possibility is that RONS can disrupt peptidoglycan bonds in the cell walls, resulting in the removal of intracellular components and the lysis of the cells. In addition, the present electric field can alter cell morphology and cause damage to the cells (Liao et al., 2022).

Reactive species divided into short-lived and long-lived species. Hydroxyl radicals, superoxide anion and singlet oxygen are among the short-lived particles which are believed to play an important role in direct plasma treatment in aqueous environments (Xu et al., 2020). Hydroxyl radicals are the strongest oxidizers with a potential of 2.85 V and are the most reactive radicals, responsible for the destruction of peptide bonds and further proteolysis, they have a short half-life around 2 ns (Xu et al., 2020). Additionally, they are initiator of lipid peroxidation when they formed due to the interaction of superoxide anion and H_2O_2 (Linley et al., 2012). The use of H_2O_2 for surface disinfection relies on its biocidal and oxidizing properties. H_2O_2 can oxidize -SH groups in proteins, DNA, and lipids. Deformation and destruction of lipids and proteins may result in membrane depolarization. The mode of action of H_2O_2 against *E. coli* depends on its concentration; low concentrations cause significant DNA damage, while high concentrations cause cell damage through oxidative damage to other intracellular components (Linley et al., 2012). Liao et al. (2022) reported that superoxide anions which can be formed by the deprotonation hydroperoxyl radicals is the key particle in viruses' inactivation, has an approximate half-life of 1 µs and can destroy Fe-S clusters in enzymes and disrupt their function. Xu et al. (2020) reported that in some cases superoxide anion may not directly affect the bacteria but can act as a precursor for ·OH radicals, moreover, in acidic environment where the pH<4.7 it can convert to HOO· radicals which can penetrate cells more easily. Zhang et al. (2013) indicate that plasma can also affect bacterial pigments like staphyloxanthin, an antioxidative carotenoid that protects *Staphylococcus aureus*, but it can be destroyed by plasma exposure. A simple method to assess this is by observing a color change in the inoculum from goldish yellow to white following treatment. Kostya (ken) Ostrikov et al. (2020) reported that singlet oxygen with a half-life of 4 µs and an oxidizing potential of 0.65 V is also a key particle in plasma inactivation effect which can cause noncatalytic lipid oxidation and will form only one lipid hydroperoxide.

Aside from oxidative stress, the production of RONS can also cause physical stress, such as a decline in pH and an increase in conductivity and ORP that could indirectly affect bacteria. Consequently, the chemical state of the cell surface, membrane integrity, as well as the internal components and structure of the cell were damaged (Zhang et al., 2013).

3.5. Factors influencing plasma effect

Several crucial factors influence the efficacy of plasma inactivation, including plasma reactor type and configuration, plasma power setting (voltage and frequency), mode of exposure (direct and indirect), food matrix properties such as surface roughness or porousness, strain attributes, and the type and velocity of gas used to generate plasma. For instance, higher voltage and O_2 content can enhance plasma inactivation efficacy by increasing the concentration of ROS, particularly hydroxyl radicals and O_3 (Liao et al., 2022). Additionally, different intrinsic characteristics of bacterial strains result in varying resistance responses to plasma treatment. Mai-Prochnow et al. (2016) observed that both biofilms and planktonic form of Gram-positive bacteria exhibited higher resistance after 10 minutes of exposure to a plasma jet setup, than Gram-negative bacteria. This difference in response can be attributed to factors such as spore formation ability or variations in cell wall thickness. However, it is an oversimplification to expect all Gram-positive bacteria to be more resistant solely due to thicker cell walls (Van Haute et al., 2013). Contradictory findings regarding the effect of bacterial cell type exist, with some studies suggesting greater resistance in Gram-positive cells, while others demonstrate no significant difference in sensitivity. Lunov et al. (2016) observed Gram-negative bacteria were more susceptible due to their cell wall thickness, however, Hoon Park et al. (2015) argued that Gram-negative cell wall, though thinner, is more complex containing lipopolysaccharides and an outer membrane, while plasma can lead to oxidation of peptidoglycan, which is more abundant in Gram-positive cell walls, making them more sensitive.

Bacterial spores have been found to exhibit greater resistance to plasma inactivation. They can be inactivated through the carving effect of O_2 atoms and reactive radicals or by damaging key germination proteins (Hertwig et al., 2018). The exact mechanism behind spore resistance is not yet fully understood but could be related to DNA saturation with small acid-soluble proteins (SASP) and the spore coat, which consists of two layers and contains a large amount of spore proteins. These factors reduce permeability and contribute to limiting the effect of O_3. Another contributing factor could be the spore's ability to form dipicolinic acid (DPA), which helps resist the effects of UV radiation (Hertwig et al., 2017).

The effectiveness of plasma inactivation can also depend on the bacterial growth stage. For instance, bacteria in the stationary phase are much more sensitive to CP treatment compared to those in the exponential phase (Lunov et al., 2016). Additionally, pH can play a role, as demonstrated by *Bacillus cereus*, which showed a 4.7-log reduction at pH 5 but only a 2.1-log reduction at pH 7 (Chizoba Ekezie et al., 2017)

3.6. Plasma-activated water (PAW)

When water is exposed to plasma for a specific duration and then either stored or immediately used for washing, immersing, or soaking fresh produce we call it PAW. PAW exhibits potent antimicrobial potential even after storage. There are two methods for generating PAW: 1) direct contact between plasma and water, and 2) plasma production over the water. The reaction between water molecules and plasma reactive species leads to the formation of more radicals compared to cold atmospheric plasma in a gaseous state. This increased radical formation plays a significant role in controlling and inactivating microbial populations. PAW is characterized by its acidic environment, higher ORP, conductivity and RONS content. Table 7 presents the properties of water after 10 and 30 minutes of plasma exposure. PAW consists of both short-lived and long-lived components, with the second group containing the majority of RNS, O_3, and H_2O_2 (Lim et al., 2021). The intensity of reactive species in PAW depends on factors such as storage time, water source and the plasma generation method (H. Wang et al., 2022). Radicals, H_2O_2 and ROS are formed through the following reactions:

$$H_2O + e^- \rightarrow OH\cdot + H\cdot + e^-$$

$$2H_2O \rightarrow H_2O_2 + H_2$$

$$OH\cdot + OH\cdot \rightarrow H_2O_2$$

$$H_2O_2 + e^- \rightarrow 2OH\cdot$$

Q. Wang & Salvi (2021) found that storage temperature is a crucial factor in addition to storage time for PAW. PAW stored at 4 ˚C exhibits higher concentrations of RNS and better inactivation activity compared to PAW stored at 22 ˚C, because lower temperatures help to maintain reactive species, enhancing PAW efficacy. Lim et al. (2021) found that PAW can remain effective with same efficiency as the production moment for up to one hour after it has been produced while

Shen et al. (2016) and Frías et al. (2020) claimed that antibacterial effect of PAW can persist up to 30 days, particularly when stored at low temperatures. The antimicrobial effect of stored PAW is solely attributed to RONS as the effect of UV, free electrons and electromagnetic field are present only during plasma discharge (R. Zhou et al., 2018). In the presence of PAW, bacteria experience changes in morphology, resulting in irregular, flattened, and distorted cells, which facilitates their inactivation (Ma et al., 2016). However, studies evaluating fresh produce samples with transmission electron microscopy (TEM) have shown that PAW does not alter the morphology of their cells.

Lin et al. (2019) found while the cuticle top layers of eggshells fade after chlorine treatment, PAW exposure does not lead to any morphological changes in eggshells. Additionally, Frías et al. (2020) reported that PAW immersion does not significantly alter the quality attributes of tofu. Water holding capacity and total polyphenol content remain unchanged, while texture properties are enhanced even after storage.

PAW has also demonstrated efficacy in treating sensitive fruits such as strawberries and fresh-cut fruit pieces like apple pieces without altering their firmness, pH, color, and antioxidant concentration (Luu et al., 2021). Moreover, PAW has the potential to promote plant growth and enhance germination under drought conditions (Thirumdas et al., 2018). Li et al. (2019) observed that PAW is effective in biofilm removal, reducing *E. faecalis* biofilms and downregulating the expression of quorum sensing-related virulence genes after PAW exposure. Additionally, they found that PAW can have an inhibitory effect on planktonic bacteria after 45 seconds of exposure.

Table 7. Changes in water characteristics after plasma exposure (Zhou et al., 2018)

	Control	10 min	30 min	unit
Conductivity	250	560	724	$\mu s.cm^{-1}$
ORP	24	580	650	mV
pH	7	3.8	3.1	

Table 8. Effect of PAW microbial inactivation on different products

Fresh produce	Strain	PAW treatment time (min)	Reduction (Log CFU/gr)	Reference
Mushroom	*E. coli*	10	1.3	(Zhao et al., 2021)
Tomato	*S.* Typhimurium	1	4.0	(Hou et al., 2021)
Lettuce	*L. innocua*	5	2.4	
Fresh-cut radicchio	*L. monocytogenes*	60	2.5	(Berardinelli et al., 2016)
Fresh-cut potato	*E. coli*	10	3.7	(Lim et al., 2021)

3.7. Bacterial response to plasma-induced stress

Bacteria respond to plasma-induced stresses through various mechanisms, including the activation of sigma factors, DNA and protein repair systems, metal homeostasis, mutation, and oxidant detoxification enzymes.

3.7.1. Sigma factors

Sigma factors have a vital function in gene and protein transcription and can be categorized into two groups: σ^{54} and σ^{70} (Liao et al., 2022). The σ^{70} group is further divided into four subgroups. The fourth subgroup responds to external environmental signals, the third subgroup selectively regulates gene expression under extreme conditions like high temperature, and the first subgroup is the primary factor necessary for bacterial growth. Comparison between *L. monocytogenes* wild type and knockout mutants of sigB, rsbR, prfA, gadD and Imo0799 revealed that sigB and prfA are essential for stress resistance, such as oxidative stress and biofilm formation respectively as they were overexpressed after plasma exposure. sigB mutants were found to be highly sensitive compared to other mutants with a reduction below the detection limit after 3 minutes of exposure, while the other mutants only showed a 2.5-2.7 log CFU reduction. There was no significant difference in biofilm levels between the wild type and the prfA mutant, but after 1 minute of plasma exposure, the prfA biofilm showed twice the reduction compared to the wild type, with a similar effect on metabolic activit. The metabolic activity of

the two types declined at different rates after 1 minute, and the most significant difference was observed at 3 minutes, where the wild-type metabolic activity remained at around 60% same as one minute, while the prfA knockout mutant decreased to 30%. The biological response of Staphylococcus aureus to plasma treatment has also been studied, as it is an important foodborne pathogen. Short exposures to plasma result in the upregulation of stress and transporter proteins, as well as antioxidants. After a long exposure of 3 minutes, biological processes such as DNA repair are affected. Three time points (1, 3, 5 minutes) were assessed to evaluate the effects of plasma on gene and protein regulation. Table 9 shows the upregulation of different genes in response to plasma-induced stress. (Cui et al., 2021; Liao et al., 2022; Liew et al., 2023; Lu et al., 2022; Patange, O'Byrne, et al., 2019).

Cui et al. (2021) found that the sigB (σ^B) expression, which is vital for initial biofilm adhesion to surfaces, in *L. monocytogenes* increased by 3.43 times after 220 seconds of plasma exposure. σ^B activity is primarily regulated by a series of Rsb proteins encoded by the rsb. Biofilm was formed as a result of this upregulation, but by using cold nitrogen plasma, the biofilm was destroyed, and the surface of the product was disinfected. Biofilms are aggregates of bacterial cells, proteins, and extracellular polymeric substances (EPS) that adhere to food surfaces and are more resistant to disinfection processes. Cold plasma has been investigated and shown to effectively decompose biofilms by eliminating EPS and killing bacteria. CP mechanism can be through ROS generation that enter the bacteria cells after disrupting the membrane and induce oxidative stress as well as inhibiting quorum sensing related genes and virulence genes. Morphological changes in biofilms after plasma treatment can be observed in Figure 3 using techniques such as confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM), and atomic force microscopy (AFM).

3.7.2. Metal homeostasis

Internal metal ions can play a role in bacterial physiological activities such as respiration and are essential cofactors for various enzymes like catalase, DNA polymerase and dehydrogenase. Yau et al. (2018) found that 16 proteins were affected by plasma treatment in *P. aeruginosa*, two of which were trigger factor (Tig) and bacterioferritin (BfrB) which are responsible for cell protection. BfrB is an iron storage protein with a ferroxidase centre composed of histidine and glutamine

acids. It oxidizes Fe^{2+} into Fe^{3+} which can be used by bacteria. Additionally, BfrB enhances the effectiveness of the defense system under oxidative stress. After plasma exposure, BfrB was upregulated by more than 3.3 and bfrB mutants were found to be much more sensitive to CP.

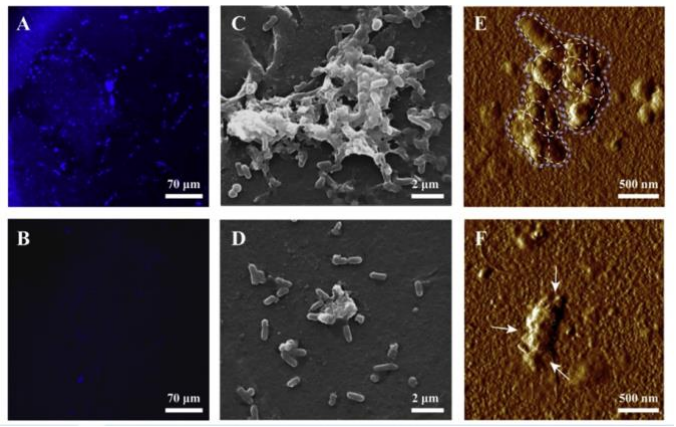

Figure 2. CLSM images of L. monocytogenes biofilm structures before (A) and after (B); SEM biofilm structures before (C) and after (D); AFM biofilm structures before (E) and after (F) plasma treatment (Cui et al., 2021)

Table 9. Log_2 FC (log fold change use to demonstrate gene expression changes) upregulation of different biological response of Staphylococcus aureus after plasma exposure (Liew et al., 2023)

biological response		**Upregulation (log_2)** Time (min)		
		1	**3**	**5**
Stress proteins	CsbD	1.02	-	-
	AmaP	0.9	-	0.62
Antioxidants	organic hydroperoxide resistance protein	0.96	-	-
Nitrosative stress	NarK/NarS	1.53	1.17	1.1
	nitrate reductase	1.26	1.15	1.03
DNA repairing	UvrB/UvrC	-	0.9	-
	YbaB/EbfC	-	0.57	0.56
	endonuclease III	-	1.18	0.76
Gene function	DnaD domain-containing protein	-	0.78	-

3.8. CAP advantages and disadvantages

The most important advantage of CAP is its short treatment time compared to existing and traditionally used methods. Kostya (ken) Ostrikov et al. (2020) reported other plasma advantages, such as being eco-friendly with low impact on the environment, not using any chemical compounds, enabling mild reaction due to its ability to work at low temperature. Additionally, it is easy to perform and cost-effective.

However, like any other process, plasma has some drawbacks. For instance, there is a possibility of electrode corrosion, safety regulation needs to be implemented when using very high voltages, proximity of the sample to the plasma-producing probe is necessary, and in case of high-lipid food products there is a risk of lipid autooxidation which can consequently lead to off-flavors (Kostya (ken) Ostrikov et al., 2020; Misra et al., 2016; Patange et al., 2018; Surowsky et al., 2015). Additionally, the applicability of CP is still in a laboratory scale and more research needs to be done to evaluate its effect on food quality attributes. Despite these challenges, ongoing research and advancements in cold plasma technology offer promising opportunities for addressing these issues and optimizing its application. Furthermore, evaluation of each specific application and consideration of the benefits and limitations should be done before any decision-making.

B. Materials and Methods

In this project, all procedures were performed under sterile conditions using a biological biosafety cabinet. Deionized water (DI) was used for all preparations, including media and the buffer saline solution.

Experimental design

An experimental plan was developed to investigate the potential of PAW bubbles in reducing populations of bacterial strains. Different time points were tested, including 5, 7, 10, 20 seconds for water disinfection, and 1 and 3 minutes for tomato decontamination.

The experiments were divided into three parts. The first part focused on water disinfection, while the second part was about tomato decontamination. The third part aims to check the potential of PAW bubbles in preventing cross-contamination between good and inoculated tomatoes.

In the first phase of experiments, various bacterial strains listed in Table 10 were used to assess the effectiveness of PAW-bubbles in water disinfection and to compare the effect of PAW-bubbles on different bacterial strains. Initially *E. coli* O157:H7 ATCC 42888 was used as a reference bacterium to determine the optimal plasma setting (discharge voltage, resonance frequency and duty cycle) that would result in a measurable reduction in the shortest period. This setting was then applied to all other bacterial strains.

Plasma setup

All experiments were conducted using the DBD plasma device model Leap100 (PlasmaLeap Technologies, Sydney, Australia). Air was supplied by an air compressor. The set-up includes:

- High voltage source
- Plasma power supply capable of providing high voltage output (up to 80 kV, p-p), based on an input voltage in the range of 50 to 325 V, and a frequency of 100–3000 Hz
- DBD reactor with a current probe and a plasma bubble reactor probe that produced and inserted plasma-generated particles through bubbles into the water
- Air compressor

- Rotameter (gas flow meter)

The bubble reactor was a new design by PlasmaLeap, consisting of an inner quartz tube (5.0 and 8.0 mm corresponding to its internal and external diameters) with a tightly inserted high-voltage stainless-steel electrode rod, and an outer dielectric tube (12.0 mm of internal diameter). At the bottom of the outer tube, there is a porous layer with several small holes which the gas will pass through and generate a lot of small bubbles. A schematic view of the PAW-bubbles system is shown in Figure 3.

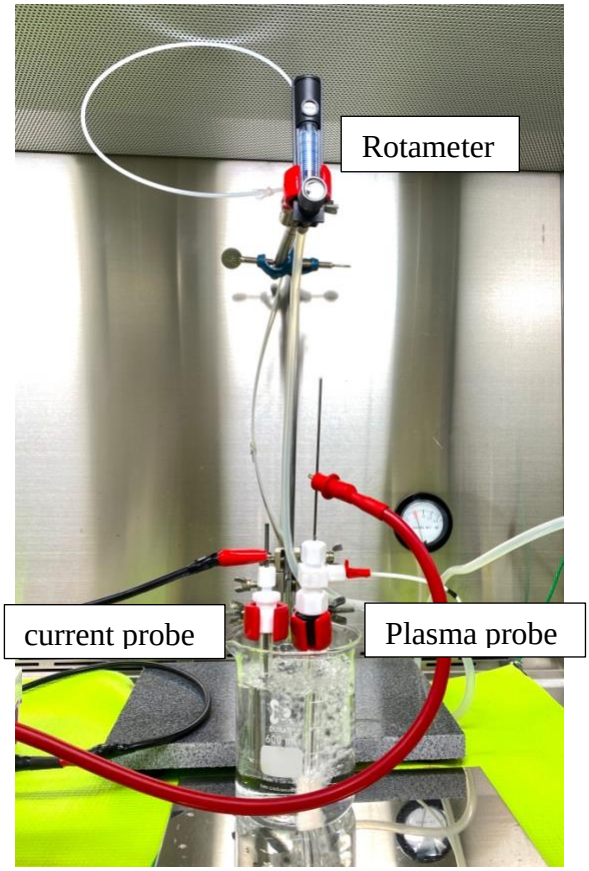

Figure 3. Plasma setup

1. Inoculum preparation

All bacterial strains were stored in Bacterial Freezing Tube containing 3 mm glass beads and 225 µl of 30% glycerol at -80 ˚C. A pre-culture was prepared by transferring one bead from stock to a TSA plate (MBcell, Seoul, Korea) and incubating at 35 ˚C for 18-24 hours. The next day, a single colony was picked and transferred to 30 ml TSB or in case of *Listeria* species BHI broth (Becton,

Dickinson and Company, Le Pont de Claix, France). The culture was then incubated in a shaking incubator set at 120 rpm and 35 ˚C overnight. Cultured bacteria were harvested by centrifugation at 4,000 rpm for 10 minutes at 4˚C. The main culture was prepared after washing the pellets two times. Each washing step included discarding the supernatant and resuspending the pellet in 30 ml 1X PBS (HanLAB, FBK, Korea). The main culture was further stored at 4 ˚C overnight. The cell concentration of the main culture was approximately 9 log CFU/ml.

2. Water disinfection

To initiate the water disinfection experiments, 5 mL of inoculum was transferred to a beaker containing 500 mL DI water to obtain a concentration of 7 log CFU/ml. Plasma probes were placed into the beaker in a manner that ensured they were fully submerged in the water, allowing plasma bubbles to directly interact with the water. Subsequently, an air compressor was activated to generate an airflow rate of 1 L/min. Microbial samples were collected immediately after 5-, 7-, 10-, and 20-second contact times in a 15 ml Falcon tube. 23 µl sodium thiosulfate ($Na_2S_2O_3$) were added to the sample taken to stop the possible activity of plasma-generated radicals that might still be present. These were then serially diluted 10-fold up to 10^{-5} in 1X PBS. The diluted samples were spot plated on dried TSA plates (0.6% yeast extract was added to TSA plates (TSA-YE) for Gram-positive strains). Spot plating was examined and found to be comparable to spread plating method (results in Appendix). The plates were incubated at 35 ˚C for 24 hours, and those a with valid bacterial count ranges were considered for CFU computation. The inoculum suspension treated with only air bubbles served as the control. All experiments were repeated at least three times for statistical analysis. The plasma settings used for disinfection can be found in Table 11.

3. Tomato inoculation

Fresh cherry tomatoes were purchased from a local market in Incheon, Republic of Korea. Upon purchase, the tomatoes were washed with tap water to remove dust and dirt, and the calyx was removed. Any tomatoes exhibiting discoloration, surface damage, or soft tissue were discarded, and only healthy tomatoes were used for further experiments. For tomato inoculation, nine cherry tomatoes weighing approximately 10 g each were immersed in 500 ml 1X PBS containing

5 ml of the main inoculum (part 3) for 15 min. Afterwards, the bacterial suspension was carefully drained, and the cherry tomatoes were air-dried for 1 hour inside a biosafety cabinet at room temperature. The inoculated tomatoes were then transferred to a sampling bag using sterile chopsticks and placed in a plastic container. The container was stored overnight in a cool room set at 15 ˚C to allow bacteria cells to adhere to the tomato surface (Bolten et al., 2020).

4. Tomato decontamination

For decontamination experiments, three inoculated tomatoes were placed in a beaker with 500 ml DI using sterile chopsticks. The beaker was then fitted into the plasma setup with the same power setting as the water experiment. Immediately after the PAW-bubbles treatment time (1 and 3 minutes) the tomatoes were transferred to a sampling bag, and a 5 ml water sample was collected into a sterile conical tube containing 12.5 µl 10% sodium thiosulfate. The water sample was then serially diluted up to 10^{-4} using 1X PBS. Subsequently, 20µl of each dilution was spot plated on either XLDA for *Salmonella* or PALCAM plates for *Listeria*. Plating was done in duplicates. Additionally, 150 ml 0.1% buffered peptone water and 1.125 ml 10% sodium thiosulfate were added to the tomatoes in sampling bags. Afterwards, the tomatoes were hand massaged for 1 minute without destroying the texture, 1 ml of the sampling bag solution was diluted up to 10^{-4} using 1X PBS and spot plated in either XLDA for *Salmonella* or PALCAM plates for *Listeria*. This process was initially conducted with only air bubbles to establish control sets for assessing the amount of bacteria entered the DI from tomato surface and then repeated with PAW bubbles to evaluate the plasma effect in reducing bacteria in DI water and on the tomato surface. Three inoculated tomatoes were directly transferred to a sterile sampling bag, massaged, diluted and spot-plated to measure the attachment of bacteria to the tomato surface. All plates were incubated at 35 ˚C, with XLDA plates incubated for 24 hours and PALCAM plates for 48 hours. The experiments were repeated at least six times for statistical analysis.

5. Cross-contamination experiment

In this experiment, the potential of PAW bubbles in preventing water-mediated cross-contamination was investigated. One inoculated tomato and one non-inoculated tomato were used, which were marked with a waterproof marker. First, the tomatoes were placed in separate

mesh bags using sterile chopsticks. Then, the meshed tomatoes were placed in a beaker containing 500 ml of DI water in a plasma setup with approximately 3 cm distance from each other. Simultaneously, the meshes were pulled out from the beaker after the plasma treatment using threads connected to them. Water samples were also collected after treatment. Next, the tomatoes were transferred to separate sampling bags. In the bag containing the non-inoculated tomato, 50 ml of BHI broth was added for enrichment. The bags with good tomatoes were then incubated overnight at 35 ˚C for *L. monocytogenes* and 42 ˚C for *S. enterica* strains. The following day, 0.1 ml of the enriched tomato-BHI was collected from each bag and spot plated on selective media for *Salmonella* or *Listeria* detection indicating cross-contamination between the tomatoes. Streaking was done for non-inoculated tomatoes later to have a comparison of two techniques and be able to check and verify the colonies. These plates were incubated at 35 ˚C, XLDA for 24 hours and PALCAM plates for 48 hours. For control, the same process was repeated using only air bubbles. Upon pulling out the tomatoes from the beaker a water sample for microbial analysis was taken, 10-fold diluted in 1X PBS up to 10^{-2} and then drop plated.

Parameter estimation

The data obtained for each treatment were fitted to a novel inactivation model described by Geeraerd et al. (2000) that encompasses loglinear inactivation by assuming very low values for C_C. Furthermore, the C_C value was neglected, and the final model equation will be as follow:

$$N_{(t)} = (N_{(0)} - N_{res}).\exp(-k_{max}t)$$

Where $N_{(t)}$ (CFU/ml) represent the survived bacteria cell density at time t, $N_{(0)}$ (CFU/ml) is the initial inoculum cell density, N_{res} (CFU/ml) is the remained bacteria cell density after treatment and k_{max} (1/min) represents the inactivation rate. To find the total log reduction $\log(N_{res})$ was subtracted from $\log(N_{(0)})$ for each time point.

Statistical analysis

All experiments were performed at least 3 times for water disinfection and 6 times for tomato decontamination and cross-contamination. Values are expressed as mean ± Standard Deviation (SD) in all figures and reported as mean ± Standard Error of the Mean (SEM) in tables. One-way ANOVA followed by the Tukey test as the post-hoc analysis was used at a 95% confidence level

($p<0.05$) where appropriate to identify significant differences in log CFU reduction of each strain compared to each other and to the control.

- Throughout this thesis writing process, ChatGPT was the sole AI tool used solely to correct any potential grammatical errors.

Table 10. List of bacteria strains used in the experiments and their origin

Strain	Number	Isolation source
S. enterica serovar Typhimurium	ATCC 14028	Tissue from pools of heart and liver from 4-week-old chickens
S. enterica serovar Poona	ATCC BAA-3139	Fresh cucumber
S. enterica serovar Thompson	ATCC BAA-3141	water
E. coli O157:H7	ATCC 43888	Human feces
E. coli	KCTC 52645	Mouse intestine
L. monocytogenes	ATCC 15313	Rabbit
L. monocytogenes	ATCC BAA-3131	Food production environment
L. monocytogenes	ATCC BAA-3132	Food
L. monocytogenes	ATCC BAA-3134	Cheese
L. monocytogenes	ATCC BAA-3153	Chocolate milk
L. innocua	KCTC 3586	Cow brain
L. grayi	KCTC 3581	Cornstalk and leaves
L. grayi	KCTC 3443	Rat feces
Micrococcus endophyticus	KCTC 19156	A plant in China
Micrococcus luteus	KCTC 29536	The soil of vegetable farm
Microbacterium ginsengiterrae	KCTC 19526	The soil of ginseng field

Table 11. Plasma power source setting for experiments

Voltage	120 – 150 V
Duty cycle	50 – 100 µsec
Frequency discharge	500 Hz
Frequency resonance	60 kHz
Air flow rate	1 L/min

Table 12. List of Medias for experiments and their properties

Media name	Abbreviation	Selectivity	Colony appearance
Brain Heart Infusion (broth)	BHI	Selective *(Listeria)*	
Tryptic Soy Agar	TSA	non-selective (used for *E. coli*, *Salmonella*)	shiny beige colonies
Tryptic Soy Agar + yeast extract	TSA-YE	non-selective (used for gram positives)	shiny beige colonies
Tryptic Soy Broth	TSB	non-selective	
Polymyxin Acriflavin Lithium-chloride Ceftazidime Esculin Mannitol	PALCAM	Selective *(Listeria)*	gray-green colonies with a black halo
Xylose Lysine Deoxycholate (agar)	XLD	Selective *(Salmonella)*	Colonies with black center

C. Results and Discussion

1. Finding the proper plasma setting

In the first part of experiments the effect of different plasma power settings against *E. coli* O157:H7 in DI water was evaluated. A higher power input, achievable by adjusting either the amplitude of the applied voltage or the frequency, can significantly increase the concentration of active species, thereby accelerating the inactivation process (Magureanu et al., 2021). The microbial reduction and reduction level were compared using different voltage and frequency values, as shown in Figure 4. Initially, the impact of two different resonance frequency values (60 kHz and 65 kHz) on bacteria inactivation was examined, while keeping the plasma settings at a voltage of 150 V and a duty cycle of 33 µsec. After a 10-second exposure, it was observed that 65 kHz inactivated *E. coli* O157:H7 to levels below the detection limit very fast that it was impossible to measure the reduction level at different time points. After checking the shorter treatment time with this setting, more than 4 logs reduction was observed just after 5-second exposure. In contrast, 60 kHz resulted in less than one log reduction within the same time frame. The former setting exhibited a very slow inactivation rate, as even after 20 seconds, the reduction in bacterial count was still less than one log. Considering the main purpose of this research, which aimed to

achieve bacterial inactivation in water where the reduction rate is measurable at least in three time points to be able to compare different strain resistance to each other, the first setting (65 kHz) was not deemed suitable for further experiments due to its unmeasurable reduction rate. Additionally, 65 kHz considering a high frequency could be not advised in long run because it can damage the device.

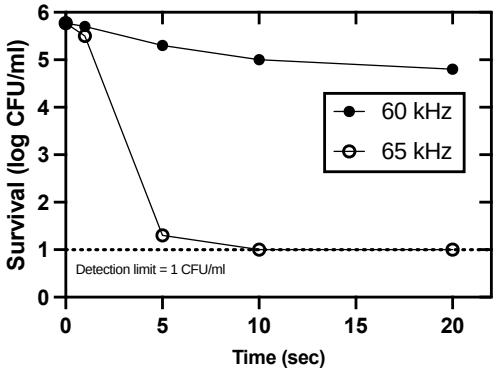

Figure 4. Effect of different resonance frequencies against E. coli O157:H7 survival in DI water after treatment (n=2)

In the next step, the resonance frequency was kept unchanged at 60 kHz, and various combinations of voltages (120 V and 150 V) and duty cycles (50 µsec and 100 µsec) were examined. The survival of *E. coli* O157:H7 with different settings can be found in Figure 5. Based on the data, it was observed that changing the plasma voltage had a greater effect on bacteria reduction compared to altering the duty cycle. According to Airoudj et al. (2011) plasma duty cycle represents the time that the plasma is actively ON within one cycle, since plasma production is not continuous throughout the whole cycle, furthermore, changing it can affect the delivered plasma power and higher values means plasma is actively ON for a longer duration. Consequently, an increased duty cycle can lead to more effective microbial inactivation. On the other hand, plasma voltage, as mentioned before, refers to the potential difference applied to the plasma source. Higher voltages can elevate ionization rate and electron density, thereby enhancing the reactivity of plasma particles. Lunov et al. (2016) also reported similar findings, demonstrating that 60 seconds of high voltage treatment resulted in complete inactivation of *E. coli* while using low voltage during the same time only caused the minor rupture of the *E. coli* cell wall,

furthermore, they concluded if low voltage is to be used, the treatment time should be extended. Similar findings were reported by Patange et al. (2018) as well, where they observed a greater reduction in *E. coli* by increasing voltage, 2.81-, 1.67-, and 0.89-log CFU/ml reduction was reported after 2 minutes of treatment with 80, 70, and 60 kV respectively.

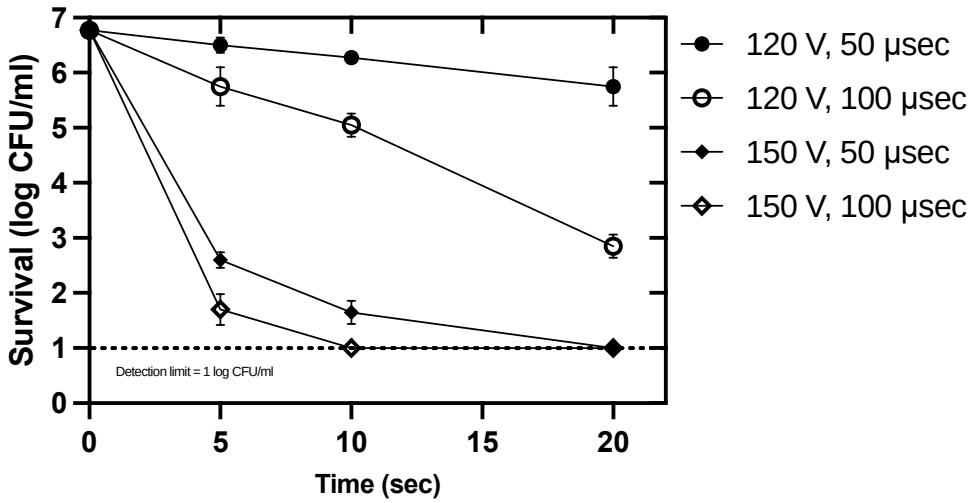

Figure 5. Mean survival of E. coli O157:H7 after plasma exposure with 4 different settings while frequency kept at 60 kHz (error bars represent SD) (n=3)

For the next experiments, 120 V and 100 µsec was selected as they had measurable reduction at each time point.

2. Water disinfection results

The effect of PAW bubbles against different bacterial strains in DI water was evaluated and the microbial reduction was compared in 5-, 7-, 10-, and 20-second exposure times (Table 13). The inactivation levels increased over time and 20 s of PAW bubbles treatment reduced all the pathogens to more than 3.5 logs CFU/ml. 20-sencond treatment of PAW bubbles resulted in reductions of 4.34 log for *E. coli* O157:H7, 3.62 log on average for *S. enterica* from different serovars, and an average of 4.61 logs for *L. monocytogenes* strains from different isolation sources. *Micrococcus luteus* demonstrated the highest resistance after 20 s with only 1.25 log reduction.

Table 13. The mean ± SEM reduction of all strains after 5-, 7-, 10-, and 20-second PAW bubbles treatment in DI water (red shades indicates higher reduction values starting from 1 and blue shades demonstrates reduction values lower than 1) (n=9)

Strain	Reduction (log CFU/ml)			
	Time (seconds)			
	5	7	10	20
E. coli 52645	0.05 ± 0.02	0.22 ± 0.07	0.36 ± 0.03	2.95 ± 0.10
E. coli O157:H7	0.30 ± 0.03	0.42 ± 0.05	1.10 ± 0.15	4.34 ± 0.26
S. enterica Typhimurium	0.53 ± 0.40	0.85 ± 0.50	1.78 ± 0.53	3.56 ± 0.26
S. enterica Poona	0.17 ± 0.04	0.22 ± 0.05	0.66 ± 0.06	3.21 ± 0.08
S. enterica Thompson	0.17 ± 0.04	0.21 ± 0.06	0.58 ± 0.12	4.11 ± 0.38
L. monocytogenes 15313	0.48 ± 0.19	1.30 ± 0.11	2.61 ± 0.04	5.02 ± 0.23
L. monocytogenes 3153	0.11 ± 0.05	0.22 ± 0.04	0.90 ± 0.15	4.72 ± 0.23
L. monocytogenes 3134	0.17 ± 0.09	0.30 ± 0.10	0.96 ± 0.16	4.82 ± 0.34
L. monocytogenes 3132	0.41 ± 0.29	0.34 ± 0.07	1.47 ± 0.25	4.53 ± 0.33
L. monocytogenes 3131	0.18 ± 0.03	0.29 ± 0.05	0.76 ± 0.08	3.89 ± 0.13
L. grayi 3443	1.08 ± 0.47	1.91 ± 0.82	2.64 ± 0.41	4.04 ± 0.16
L. grayi 3581	0.30 ± 0.09	0.71 ± 0.13	1.92 ± 0.36	4.19 ± 0.30
L. innocua	0.11 ± 0.05	0.29 ± 0.07	0.98 ± 0.18	4.88 ± 0.24
Microbacterium ginsengiterrae	3.18 ± 0.22	4.01 ± 0.25	4.78 ± 0.28	5.30 ± 0.03
Micrococcus luteus	0.16 ± 0.04	0.37 ± 0.06	0.62 ± 0.06	1.25 ± 0.07
Micrococcus endophyticus	0.88 ± 0.21	1.20 ± 0.31	2.18 ± 0.33	3.60 ± 0.16

2.1. *E. coli*

The inactivation of pathogenic and non-pathogenic *E. coli* strains differed considerably with p-value < 0.05 (Figure 6). After 20 seconds of plasma exposure *E. coli* O157:H7 reduction was 4.34-while the reduction for surrogate was 2.95-log. The different inactivation values could be explained by the toxin production ability of *E. coli* O157:H7, which can interfere with its survival mechanism under stress induction. Other inactivation mechanisms demonstrated similar effects for *E. coli* reduction, with surrogates showing more resistance. Sommer et al. (2000) reported a similar finding where *E. coli* O157:H7 was more susceptible to UV compared to non-pathogenic strains. Gurtler et al. (2011) also observed higher resistance of a non-pathogenic surrogate after pulsed electric field exposure, with inactivation values of 2.86, 3.12 and 3.79 log CFU/ml in different temperatures (45, 50, 55 ˚C). In comparison, *E. coli* O157:H7 exhibited greater

susceptibility under the same conditions, with inactivation of 3.09, 4.08 and 4.71 log CFU/ml, respectively. Several studies focused on the inactivation mechanism of *E. coli* in water with different processes. S. J. Lee et al. (2018) reported that pulsed discharge plasma can achieve around 3 log reduction of *E. coli* in 60 s, however, using plasma with 0.9 ppm free chlorine can cause complete inactivation (>7 log reduction) in just 30 s. Cho et al. (2010) explained that the improved synergetic effect can be explained through the different working mechanism of each methods, as an illustration ozone and plasma which are more of oxidizing processes cause significant membrane damage while free chlorine inactivate bacteria through damaging intercellular components without damaging cell wall and membrane noticeably. Furthermore, using a highly oxidative process followed by a small dose of chlorine can serve as a good disinfection procedure, however, more studies is still needed to evaluate their synergetic effect.

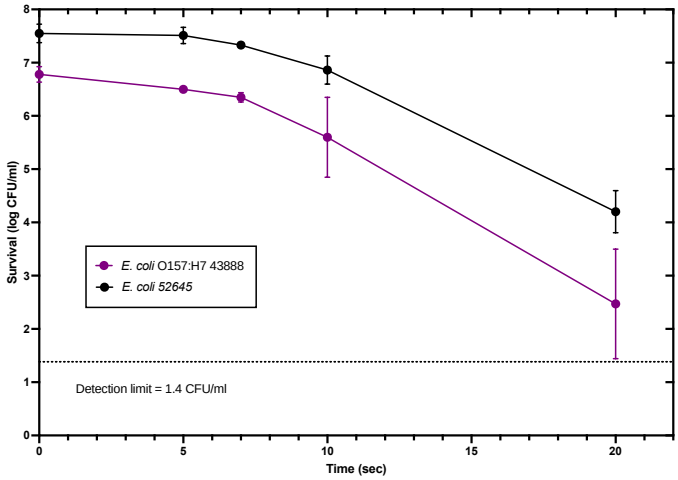

Figure 6. Mean ± SD survival of E. coli O157:H7, and non-pathogenic E. coli 52645 in DI water subjected to PAW bubbles (n=9)

2.2. *Listeria*

The survival rates of *L. monocytogenes* from different isolation sources and other *Listeria* strains (*L. grayi* and *L. innocua*) after PAW bubbles treatment are presented in Figure 7. After 20 seconds of plasma exposure, no significant difference in the mean reduction was observed among the different *L. monocytogenes* isolates with a p-value of 0.65. Pathogenic strains showed no significant difference with surrogates as well with a p-value of 0.70. Among all strains, *L.*

monocytogenes isolated from the food production environment exhibited the highest resistance with a reduction of 3.89-log after 20 seconds of treatment, while the rabbit isolate was the most sensitive one to plasma, showing a reduction of 5.02-log. Among the surrogates, *L. innocua* demonstrated the highest reduction (4.88 log) after plasma exposure, which was almost comparable to all isolates of the pathogenic strain and even more susceptible than some isolates. A similar finding was reported by Evrendilek & Balasubramaniam, (2011) where they observed that five minutes of high-pressure processing in yogurt drink resulted in a 4-log reduction in the *L. monocytogenes* population, while *L. innocua* was more susceptible and exhibited a 5-log reduction under the same treatment. However, some studies reported that even though *L. innocua* is close to *L. monocytogenes* it is more resistant when stress is applied. Patil et al. (2010) found that after both O_3 treatment and a combination of O_3 with organic acid treatment for orange juice, *L. innocua* was more resistant than one *L. monocytogenes* strain but more susceptible than the other strain. Furthermore, these findings indicate that different *Listeria* strains exhibit varying levels of resistance to plasma and other treatments, and it seems hard to exactly identify which one is more resistant.

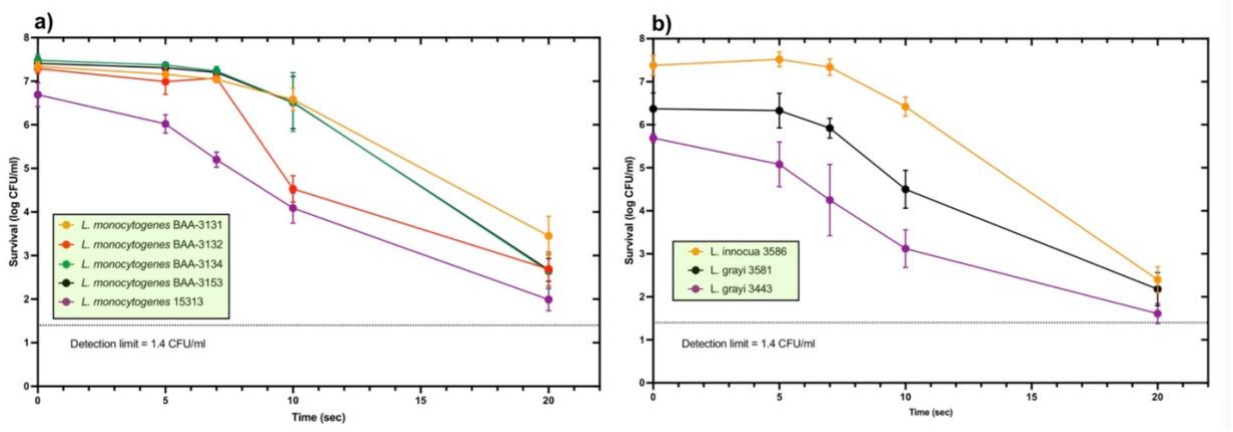

Figure 7. Mean ± SD survival of a) different isolates of L. monocytogenes, and b) non-pathogenic Listeria in DI water subjected to PAW bubbles (n=9)

2.3. *Salmonella*

The inactivation rates of *S. enterica* from different serovars after PAW bubbles treatment are presented in Figure 8. The reduction after 20 seconds of treatment was 3.56-log for *S. enterica* serovar Typhimurium, 4.11-log for serovar Thompson, and 3.21-log for serovar Poona. No

significant difference was observed in the mean reduction of different serovars after 20 seconds with a p-value of 0.79. A similar finding was reported by Fernández & Thompson, (2012) who investigated the inactivation of different *Salmonella* serovars using CAP treatment. Their study demonstrated that all Salmonella serovars were effectively inactivated in water within 24 seconds after treatment. Marsili et al. (2002) proposed that the *Salmonella* inactivation mechanism of plasma directly produced in liquid is mainly attributed to O_3 and free radicals. They further argued that air can be a more suitable alternative to nitrogen and other gases for plasma generation, as it contains oxygen, which can contribute to the formation of oxygen-based radicals. Moreover, using air is safer and more cost-effective than other gases, while also helping this technology earn the green technology label.

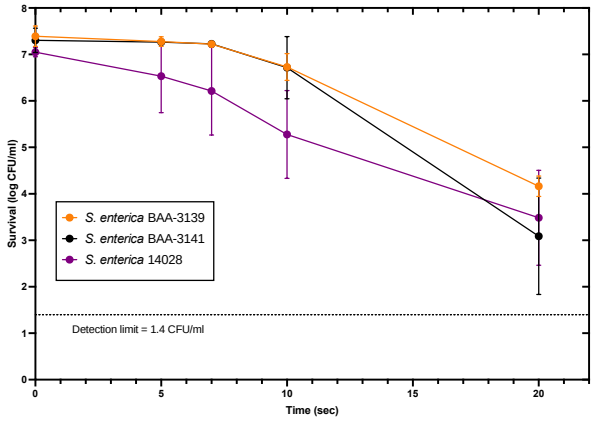

Figure 8. Mean ± SD survival of Salmonella enterica from different serovars in DI water subjected to PAW bubbles (n=9)

2.4. *Micrococcus* and *Microbacterium*

The inactivation rates of *Micrococcus luteus, Micrococcus endophyticus,* and *Microbacterium ginsengiterrae* after PAW bubbles treatment are presented in Figure 9. These Gram-positives were tested to provide a better comparison between Gram-positives and Gram-negatives. Additionally, *Micrococcus luteus* typically exhibits high resistance to other disinfection methods, and we wanted to further examine the plasma's efficacy against it. *Micrococcus* strains were more resistant than Microbacterium with 1.25- and 3.6-log reduction for *luteus* and *endophyticus* strain respectively. On the other hand, *Microbacterium ginsengiterrae* had a 5.30-log reduction

which was the highest among all the strains evaluated in this thesis. All three strains were significantly different with each other. After analyzing all *Listeria* strains with these three strains, *Micrococcus luteus* was significantly more resistant compared to other *Listeria* strains, however, other two bacteria strains had no significant difference with *Listeria.* Lai et al. (2016) reported that cold plasma is not effective in reducing *Micrococcus luteus* because this strain is one of the oxidizing agent-resistant Gram-positives. However, some studies claimed that plasma is effective against all Gram-positives including *Micrococcus* spp. and even spore-formers like *Bacillus* spp. As an illustration, Tanişli et al. (2016) reported that after treatment with DBD plasma reactor with neon gas at atmospheric pressure and room temperature with input voltage of 8kV *Micrococcus luteus* was effectively inactivated just like other bacteria such as *E. coli*, *S.* Typhimurium and *L. monocytogenes* within 60 seconds of exposure.

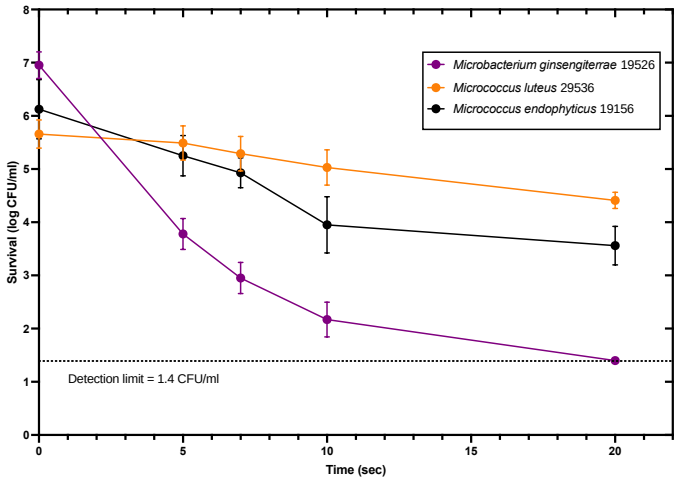

Figure 9. Mean ± SD survival of Micrococcus and Microbacterium in DI water subjected to PAW-bubbles (n=9)

Upon checking all the data for the reduction of *L. monocytogenes* (all isolates), *E. coli* O157:H7, and *S. enterica* (all three serovars) after 20 seconds, ANOVA analysis revealed a significant difference between groups with a p-value of 1.84×10^{-6}. Subsequent posthoc analysis using the Tukey test demonstrated that the difference is primarily between *L. monocytogenes* and the other two bacteria. Specifically, there was no significant difference in reduction between *E. coli* O157:H7 and *S. enterica* after the 20-second treatment, with a p-value of 0.69.

Previous studies have demonstrated the capability of plasma in reducing pathogenic bacteria in water systems as well. For instance, Scholtz et al (2015) reported that pulsed plasma gas discharge reduced *E. coli*, *S. enterica*, and *L. monocytogenes* in wastewater to below the detection limit in just 30 seconds of exposure. Additionally, Rashmei et al. (2016) found that plasma spark treatment resulted in an impressive 8-log CFU/ml reduction in *E. coli* population in water during 900 s of treatment without producing any by-products unlike chlorination. Rashmei et al. (2016) reported that the bacterial inactivation was because of H_2O_2 and hydroxyl radicals generated by plasma, which can effectively attack various cell constituents and have a greater impact on Gram-positive bacteria compared to Gram-negative ones. Interestingly, this finding aligns with the results of our study, where *L. monocytogenes* demonstrated higher susceptibility to PAW-bubbles than *E. coli* O157:H7 and *S. enterica*. These findings suggest that the plasma could be a promising alternative to existing methods for efficiently inactivating pathogenic bacteria in water. However, further investigation is needed to explore the scalability of this setup for larger applications.

To investigate which of the RONS are responsible for bacteria inactivation, scavengers should be used. Scavengers are chemical compounds capable of quenching and neutralizing various RONS produced by plasma thereby preventing their effects, scavengers themselves do not possess any inherent antimicrobial activity (Rothwell et al., 2022). Several scavengers are available for this purpose, including 200 mM D-mannitol (which quenches hydroxyl radicals), 10 mM sodium pyruvate (for H_2O_2), 100 µM Uric acid (for O_3), 20 mM Tiron (for superoxide anion) (Kostya (ken) Ostrikov et al., 2020; Rothwell et al., 2022; T. Zhang et al., 2021). According to findings from a parallel project carried out in our lab under identical experimental conditions, it was noted that D-mannitol and Tiron acted as scavengers and effectively prevented plasma-induced inactivation by neutralizing superoxide and hydroxyl radicals. However, it is worth noting that said project involved *Bacillus subtilis* in DI water which is a spore-forming bacteria displaying relatively higher resistance compared to the bacterial strains used in this thesis. To validate these findings, scavenger test has been implemented with *S. enterica* 3139 and *L. monocytogenes* 3153. The same results were observed as in the other project with *Bacillus subtilis*, where the addition of

Tiron and D-mannitol noticeably inhibited the plasma's effect on inactivating *S. enterica* and *L. monocytogenes*. However, in our experiment addition of uric acid, which can quench O_3, also prevented the plasma inactivation effect. Notably, the only scavenger that did not affect the plasma's efficacy was sodium pyruvate, leading us to conclude that H_2O_2 does not contribute to the inactivation of bacterial cells. This outcome is illustrated in Figure 10. Based on the results, where all three scavengers inhibited inactivation to a certain content the selectivity of these compounds is questionable. Blemings et al. (2005) reported that while uric acid serves as a scavenger for ozone, it can also, to some extent, quench superoxide and hydroxyl radicals. Additionally, Bors et al. (1979) discovered that Tiron reacts very rapidly with superoxide; however, due to the highly reactive nature of hydroxyl radicals, they can also react with Tiron. On the other hand, based on some studies such as L. Lee & Wilson, (2016) who reported that D-mannitol is relatively selective for hydroxyl radical and do not quench ozone, we can conclude that ozone is not the only cause of antimicrobial effect of plasma and hydroxyl radicals seems to have greater effect. Therefore, we can state that hydroxyl radical, superoxide and O_3 are potential agents contributing to bacteria inactivation. To gain more precise insights further experiments are required to identify the concentration of each of these species produced during plasma treatment.

Scavengers were found to not have any antimicrobial effect since the survival after their addition did not significantly differ from the control where only bacteria were added to the DI water. After 30 seconds of plasma treatment, where no scavenger was added bacterial load was reduced below the detection limit while adding scavengers caused less than 1 log CFU/ml reduction for both strains (Figure 10, 11).

Xu et al. (2021) reported a comparable discovery, indicating that in their scavenger test, the primary agents responsible for plasma inactivation were hydroxyl radicals and singlet oxygen (L-histidine used as scavenger), where hydroxyl radicals were found to cause membrane leakage, while singlet oxygen primarily disrupted energy balance. Xia et al. (2023) also found that superoxide anion is the key species in inactivating *E. coli* biofilms in water tanks and instruments. Wu et al. (2012) reported that when utilizing a plasma jet with helium + 2% oxygen as the plasma-

producing gas, three ROS were identified: hydroxyl radicals, singlet oxygen, and superoxide anions. Among these three particles, it was found that hydroxyl radicals and singlet oxygen were the primary factors leading to the inactivation of *S. aureus* in water. Rothwell et al. (2023) also found that the antimicrobial effect of PAW generated using a DBD reactor, air and tap water is mainly due to the superoxide and other particles such RNS and H_2O_2 do not play a role in bacterial inactivation.

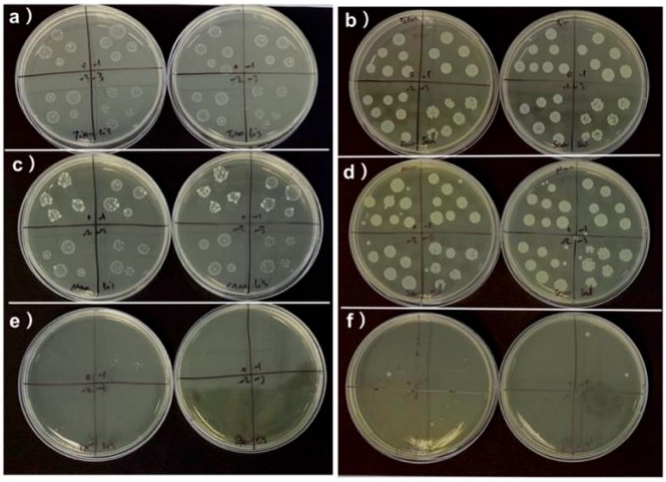

Figure 10. Photos of plates after 30 seconds of PAW-bubbles treatment, no scavenger added (e, f), with D-mannitol (c, d), with Tiron (a, b), L. monocytogenes 3153 (a, c, e) and S. enterica 3139 (b, d, f).

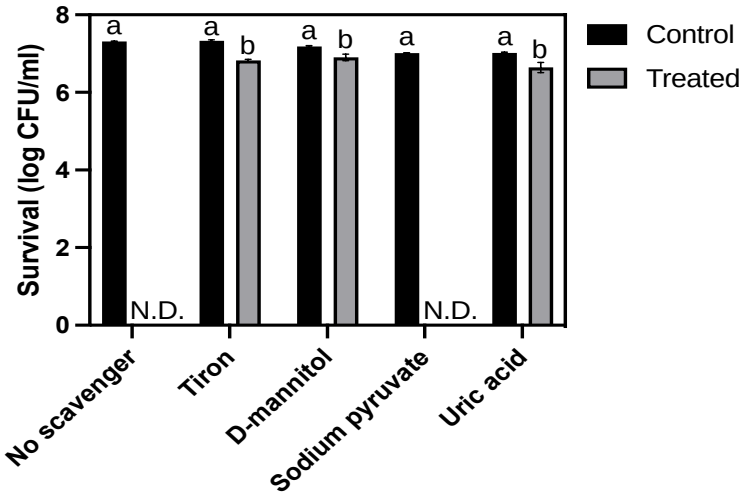

Figure 11. Survival of L. monocytogenes and S. enterica after 30-sec and before plasma treatment with no scavengers, with Tiron, D-mannitol, Sodium pyruvate, Uric acid (error bars represent standard deviation), (n=2), (N.D. means not detected)

3. Tomato decontamination results

The effect of PAW bubbles on the reduction of *S. enterica* and *L. monocytogenes* inoculated on cherry tomato surface was evaluated and the microbial reduction was compared for 1- and 3-minute exposure times. DI water was used as the wash water, and the number of bacteria entered the water was evaluated after plasma treatment and after using air bubbles to serve as a control for plasma efficiency. The decontamination level increased over time, and after 3 minutes of PAW bubbles treatment, there was an average reduction of 1.95 log for *Salmonella* serovars with serovar Thompson being the most susceptible which reduced 2.27 log. In comparison, as indicated in the results of water experiments, *L. monocytogenes* strains, particularly *L. monocytogenes* 15313 and *L. monocytogenes* BAA-3132 were found to be more sensitive and showed a reduction of 2.36- and 1.66- log, respectively, after just 1 minute of treatment. The other three isolates of *L. monocytogenes* (BAA-3132, BAA-3134, BAA-3153) were found to be more resistant and did not reduce more than 1 log after 1 minute. Consequently, they were exposed to 3 minutes of treatment. These findings highlight the variability in the susceptibility of different *L. monocytogenes* isolates to the PAW bubbles treatment. Understanding these differences in sensitivity among isolates is crucial for optimizing the decontamination process and ensuring effective microbial control in food safety applications.

3.1. L. monocytogenes

The survival rates and wash water quality of *L. monocytogenes* from different isolation sources after PAW bubbles treatment are presented in Figure 12 and 13. After 1 minute of treatment, *L. monocytogenes* 15313 was found to be significantly different from the other isolates in terms of reduction, with a p-value of 1.42×10^{-8}. On the other hand, *L. monocytogenes* 3132 showed a reduction of 1.66 log after 1-minute plasma exposure. As these two isolates showed >1.5 log reduction within 1 minute they were not further used in the experiments with 3 minutes exposure time. The effect of PAW bubbles on decontaminating these two strains from the tomato surface significantly differed from air bubbles, indicating the plasma efficacy in decontamination. After 3 minutes of treatment *L. monocytogenes* 3134 isolate was found to show a higher

reduction of 2.16 log than other isolates, *L. monocytogenes* 3153 and 3131 showed similar resistance to plasma on tomato surface where they were reduced with 1.17- and 1.20-log respectively. However, in contrast to the other two isolates, the decontamination levels caused by PAW and air bubbles did not differ significantly for these three strains, indicating that the detachment of bacteria is mostly due to the bubbling effect rather than the plasma. Regarding the presence of *L. monocytogenes* in the wash water, it was found that after both 1-minute and 3-minute plasma exposures, none of the *L. monocytogenes* isolates were detected in the water, highlighting the plasma's efficiency in effectively eliminating the bacteria from the water. In contrast, when using only air bubbles, bacteria were found to survive in the water, indicating that plasma treatment can play a critical role in preventing potential cross-contamination during the post-harvest washing step. The observed differences in reduction rates among the *L. monocytogenes* isolates may be attributed to their varying abilities to attach to the tomato surface, where the initial cell concentration will differ and lower levels might be easier to reduce (Ziuzina & Misra, 2016). Notably, *L. monocytogenes* 15313 and 3132 showed lower attachment levels compared to the other three isolates, which could explain their higher susceptibility to the plasma treatment. These findings align with similar research conducted by Han et al. (2023) which reported approximately 2 log CFU/g reduction in *L. monocytogenes* population after 3 minutes of plasma bubble-activated water treatment. However, Han et al. (2023) observed that *L. monocytogenes* can survive in wash water after 3 minutes of plasma treatment and can only be reduced to levels below the detection limit after 20 minutes of exposure which is contrary to our findings.

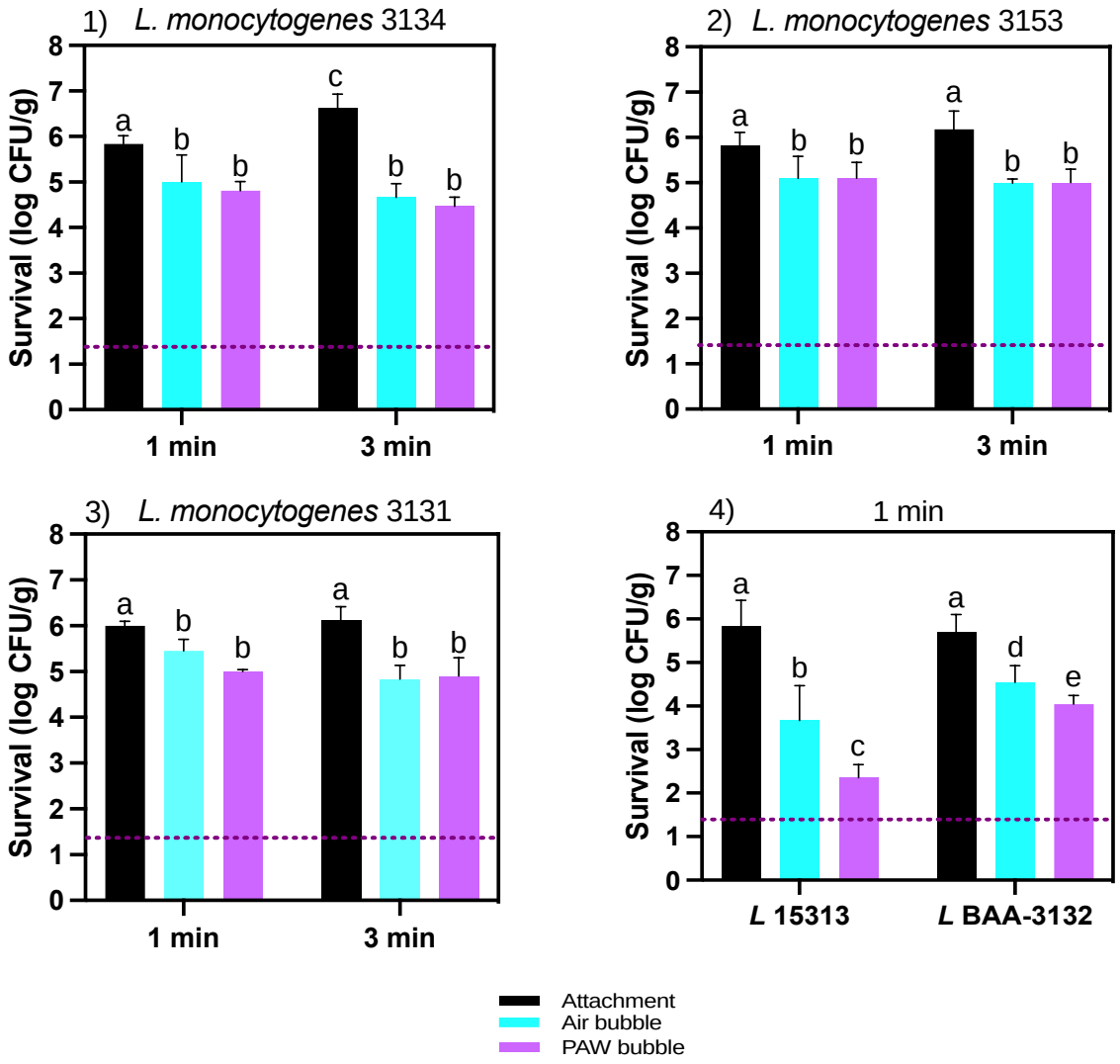

Figure 12. Mean ± SD attachment and survival of L. monocytogenes 3134 (1) L. monocytogenes 3153 (2) L. monocytogenes 3131 (3) after 1 and 3 min; L. monocytogenes 3132 and L. monocytogenes 15313 after one min (4) after Air and PAW bubble treatment inoculated on the tomato surface, dotted line is detection limit 1.4 log CFU/g(ml), (n=6)

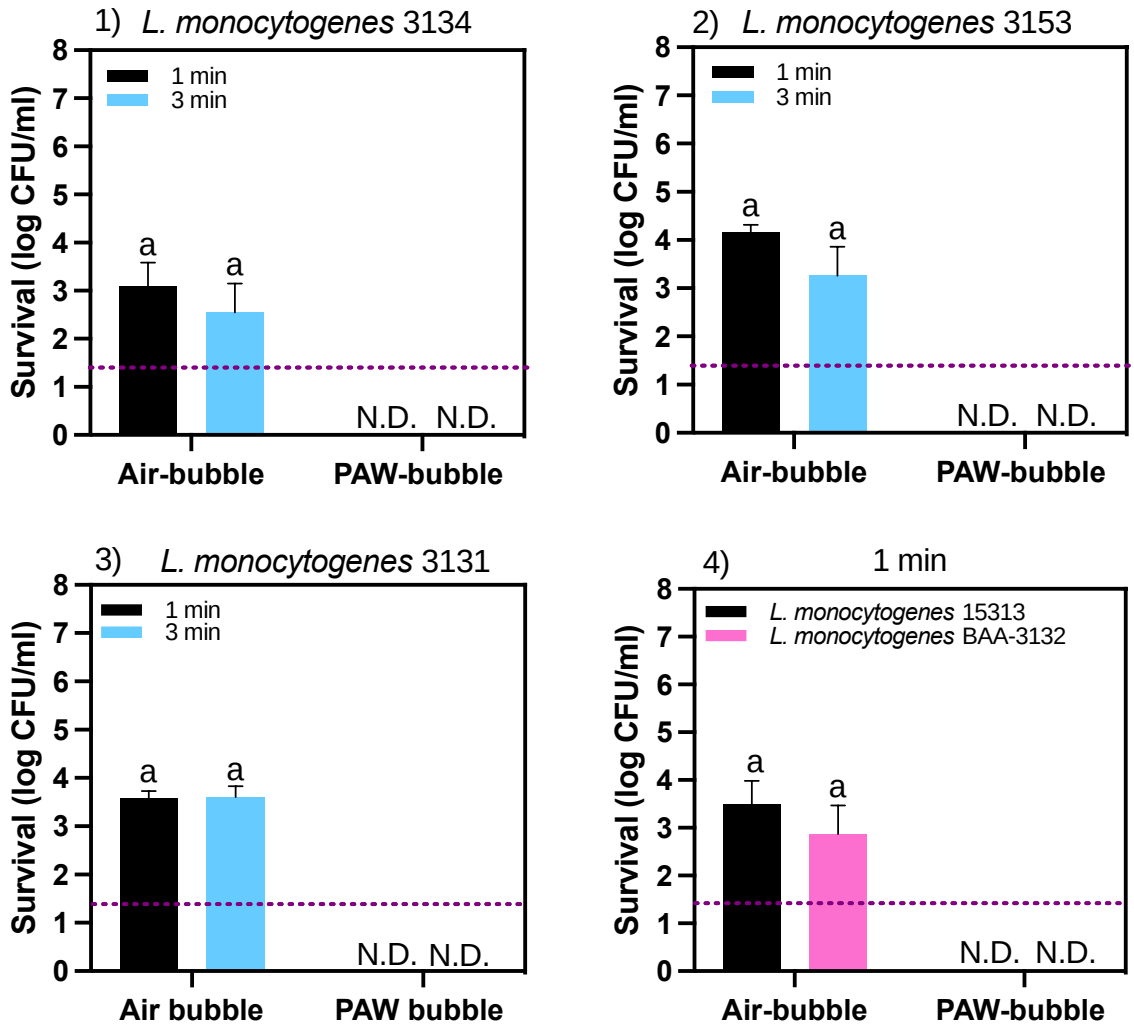

Figure 13. Mean ± SD survival of L. monocytogenes 3134 (1) L. monocytogenes 3153 (2) L. monocytogenes 3131 (3) after 1 and 3 min and L. monocytogenes 3132 and L. monocytogenes 15313 after 1 min (4) Air and PAW treatment in tomato wash water, dotted line is detection limit 1.4 log CFU/ml, N.D. means not detected, (n=6)

3.2. Salmonella

The survival rates and wash water quality of *Salmonella* from different serovars after PAW bubbles treatment are presented in Figure 14 and 15. After 1 minute of plasma exposure *S. enterica* 14028 (Typhimurium) showed 1.14 log reduction. Subsequently, longer contact time was used for all strains in further experiments. After 3 minutes of treatment, reductions of 1.82-, 2.27-, and 1.78-log CFU/g was observed for *S. enterica* 14028, 3141, and 3139 respectively. For *S. enterica* 14028 the reduction after 3 minutes was significantly different from 1 minute treatment with a p-value of 0.03. However, there was no significant difference in the reduction levels of different serovars after 3 minutes. Consistent with *L. monocytogenes*, none of the *Salmonella* serovars were detected in the water after 3 minutes of treatment, indicating the plasma's efficiency in eliminating these bacteria from the water. However, in contrast to *L. monocytogenes* isolates where all of them were reduced below the detection limit even after 1 minute of treatment, 1 minute of exposure was found to be insufficient for reducing *S.* Typhimurium in water below the detection limit. This is in accordance with the water disinfection results where *Salmonella* spp. were more resistant to plasma compared to *Listeria* spp. Previous studies investigating the use of plasma for reducing *Salmonella* on tomato surfaces have reported similar findings. M. Zhang et al. (2013), observed a reduction of 2.2 ± 1.1 log CFU/g in *S.* Typhimurium population inoculated on tomatoes after 10 minutes low-pressure oxygen plasma treatment. Additionally, Lee et al. (2023) reported 3.9 log CFU/tomato reduction in *Salmonella* inoculated on tomato surface after 3 minutes of atmospheric DBD plasma treatment. As discussed in plasma introduction part, different configuration and settings can induce different inactivation rates, and this could be the main reason in the different reduction values reported in various studies. Overall, these findings demonstrate the potential of plasma treatment in effectively reducing *Salmonella* on tomato surfaces and in water in short time periods.

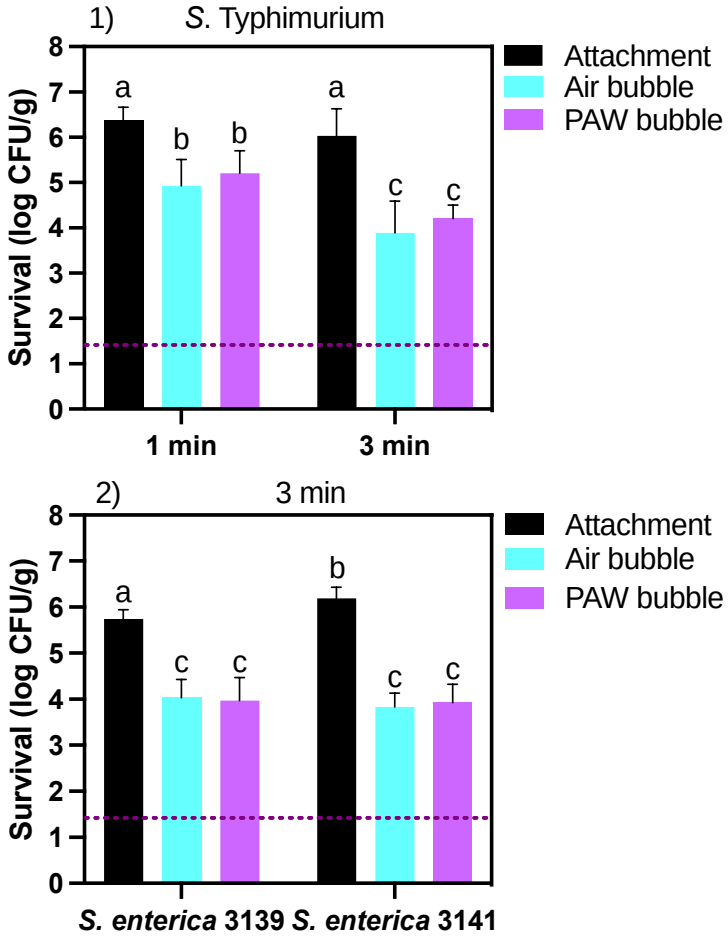

Figure 14. Mean ± SD survival of S. enterica 14028 after 1 and 3 min (1) S. enterica 3139 and 3141 after only 3 min (2) PAW and Air bubble treatment inoculated on the tomato surface, dotted line is detection limit 1.4 log CFU/g(ml), (n=6)

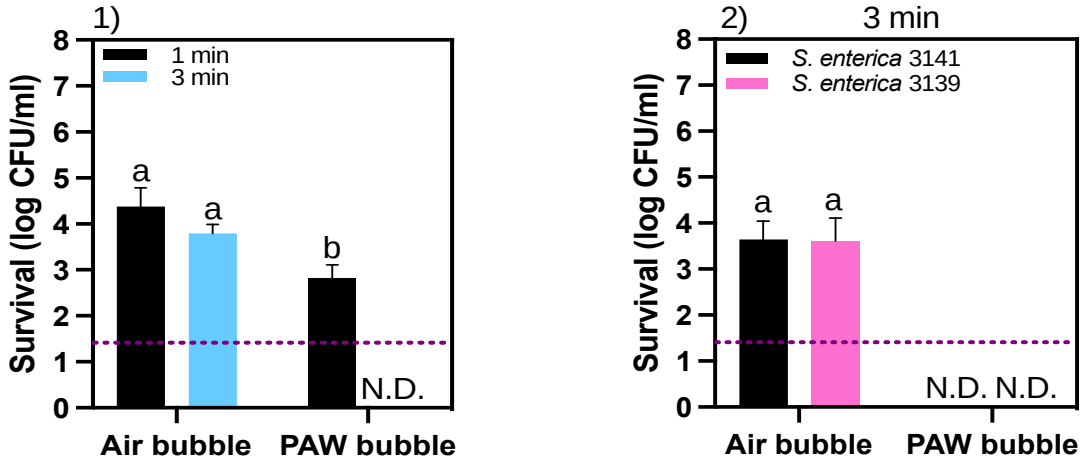

Figure 15. Mean ± SD survival of S. enterica 14028 after 1 and 3 min (1) S. enterica 3139 and 3141 after only 3 min (2) PAW and Air bubble treatment in tomato wash water, dotted line is detection limit 1.4 log CFU/ml, N.D. means not detected, (n=6)

Contrary to the water disinfection, the results after 3 minutes of PAW bubbles treatment showed no significant difference in the reduction of *Salmonella* and *L. monocytogenes* from the tomato surface, with a p-value of 0.107. However, checking the data for 1 minute treatment, revealed a significant difference with p-value of 1.27×10^{-8} between *S.* Typhimurium and *L. monocytogenes* 15313 where *L. monocytogenes* indicated more reduction. Bhagat et al. (2010) also reported similar findings, where they treated tomatoes with 0.5 mg/l of ClO_2 gas and observed no difference in the reduction of *S. enterica* and *L. monocytogenes* within an 8-minute contact time. This treatment resulted in an approximate reduction of 4 log CFU/cm^2 of both pathogens on tomato surface. Timmons et al. (2018) also observed no significance difference (p-value <0.05) between *Salmonella* and *L. monocytogenes* reduction after 4- and 10-minute treatment with SDBD plasma, an average reduction of 1 log CFU/ml from cherry tomatoes surface was reported after 4-minute exposure.

A voltage of 150 V was also employed in the tomato decontamination experiment to evaluate the impact of increased voltage on the decontamination level (Figure 16). The results for *L. monocytogenes* 15313 showed a significant enhancement in the decontamination rate, reaching a reduction of 3.05 log within just 1 minute of treatment. A similar finding was reported by Kim et al. (2011), where the higher power input of atmospheric pressure plasma has led to more reduction of *L. monocytogenes* inoculated on the bacon.

In contrast, the reduction observed for *S. enterica* 14028 did not exhibit significant difference following the increase in voltage and the reduction value only changed from 1.14- to 1.28-log. The variability in tomatoes and the complex attachment of bacteria to the surface can explain why changes in plasma setting did not affect the detachment of *Salmonella*. However, in contrary with 120 V the increased voltage reduced the *Salmonella* entered the water below the detection limit within 1-minute. Other two serovars reduction were similar to *S.* Typhimurium after plasma exposure with the values of 1.26- for *S. enterica* 3141 and 1.52- log for *S. enterica* 3139.

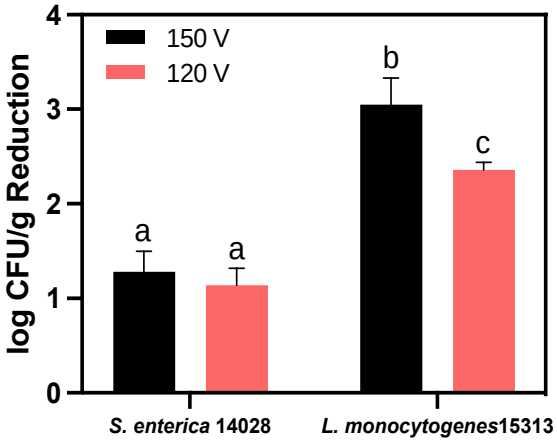

Figure 16. Mean ± SD reduction from tomato surface after 1-minute PAW-bubble treatment with 120 and 150 V

To assess the effect of bubbling on detaching bacteria from the tomato surface, air bubbles were used, and the corresponding data are presented in Table 14. No significant difference was observed in the mean initial attachment of different bacteria to tomato surface with a p-value of 0.44. However, after air bubble exposure for 3 minutes, a significant difference was observed in the number of attached bacteria on the tomato surface for *Salmonella* and *L. monocytogenes*. Specifically, *Salmonella* exhibited significantly higher detachment compared to *L. monocytogenes* with a p-value of 4.01×10^{-4} after air bubble treatment. Furthermore, it can be concluded that the bubbles aid in removing *Salmonella* more effectively than *L. monocytogenes*, resulting in a higher number of *Listeria* cells remaining on the tomato surface.

These results demonstrate that in the most cases the reduction after air and PAW bubbles do not show any significance difference. Alternately, air bubble was done for 1 minute only for *S.* Typhimurium and *L. monocytogenes* 15313 to assess how much detachment occurred. After air bubble exposure, the number of bacteria on the tomato surface was 4.02 ± 0.34 and 5.29 ± 0.26 for *L. monocytogenes* and *S.* Typhimurium respectively, which was significantly higher than the PAW bubble for *L. monocytogenes*. Therefore, PAW bubble seems effective for decontaminating *L. monocytogenes* 15313 and 3132, but not for other strains. Further experiments are required for enhancing the PAW bubble efficacy in decontamination.

Table 14. Mean SEM values of initial tomato surface attachment, reduction after Air bubble and 1- and 3-min PAW bubble, and the number of bacteria entered water after Air bubble treatment (n=6)

Strain	Attachment (log CFU/g)	Air bubble reduction	water after Air bubble (log CFU/ml)	1 min PAW bubble reduction	3 min PAW bubble reduction
S. enterica Typhimurium	6.17 ± 0.10	1.88 ± 0.18	3.79 ± 0.20	1.14 ± 0.18	1.82 ± 0.28
S. enterica Poona	5.74 ± 0.10	1.71 ± 0.16	3.61 ± 0.22		1.78 ± 0.20
S. enterica Thompson	6.19 ± 0.11	2.36 ± 0.13	3.64 ± 0.17		2.27 ± 0.13
L. monocytogenes 3153	6.08 ± 0.17	1.03 ± 0.14	3.72 ± 0.29	0.73 ± 0.09	1.17 ± 0.20
L. monocytogenes 3134	6.23 ± 0.20	1.13 ± 0.30	2.81 ± 0.26	1.03 ± 0.19	2.16 ± 0.32
L. monocytogenes 3132	5.70 ± 0.16	1.16 ± 0.16	2.87 ± 0.27	1.66 ± 0.21	
L. monocytogenes 3131	6.06 ± 0.11	0.91 ± 0.17	3.59 ± 0.08	0.99 ± 0.04	1.20 ± 0.04

4. Cross-contamination results

In this experiment, two tomatoes were used: one was a non-inoculated tomato, and the other was inoculated with *L. monocytogenes* or *Salmonella* strains. Each tomato was enclosed within individual mesh and submerged in a beaker containing approximately 500 ml of DI water. The distance between the two tomatoes was roughly 3 cm. Both PAW bubble and air bubble treatments were conducted, air bubbles to verify whether cross-contamination occurred and served as blanks, PAW bubbles to assess if plasma was efficient in preventing it.

4.1. 120 V

For *L. monocytogenes* 15313 and *S. enterica* 14028, a 1-minute treatment was applied, while all the *Salmonella* strains and other *L. monocytogenes* isolates underwent a 3-minute treatment. Throughout the experiment, a total of 23 trials were conducted with the three *Salmonella* strains. Among them, cross-contamination was only observed in 9 trials with blanks out of 23 (air bubble treated). Notably, PAW bubbles treatment prevented cross-contamination in 18 out of these 23 trials, and 5 still showed contamination even after plasma treatment. The problem was the fact that even without plasma treatment cross-contamination did not occur in most trials (in 14 out of 23). However, only looking to the data from *S. enterica* 14028 after 1-minute where among 6 trials, all the blanks showed positive results for cross-contamination and PAW bubbles were only able to prevent it in 2 trials out of 6. Nevertheless, we conclude that this plasma setting seems

to be not strong enough to prevent cross-contamination and an increase in voltage may improve the plasma effect. Upon examining the bacterial load in the water in the cases where cross-contamination occurred after using air bubbles, it was observed that in those events mostly the number of bacteria entering the water exceeded 4 log CFU/ml, the non-inoculated tomato was more susceptible to cross-contamination. As part of further experimentation, attempts were made to increase the initial attachment of bacteria to the tomatoes by using a more concentrated main inoculum. For *L. monocytogenes* 15313, a total of 7 trials were conducted, and cross-contamination was observed in 6 of these trials. Remarkably, PAW bubble treatment successfully prevented cross-contamination in 5 out of these 7 trials. For the other *L. monocytogenes* isolates, a total of 24 trials were performed, and cross-contamination occurred in 18 of these trials. However, PAW bubble treatment was only able to prevent cross-contamination in 9 out of these 24 trials. During some batches of the experiment, when a few tomatoes were picked randomly, enriched in BHI broth, and incubated, initial contamination with *Listeria* was detected. A challenge we encountered during this process was the difficulty in differentiating between *L. innocua* and *L. monocytogenes* on the selective PALCAM media used in the experiments. Both strains can grow with the same colony appearance, leading to the possibility of false results for cross-contamination. Furthermore, when it appeared that the non-inoculated tomatoes were contaminated, it can be as a result of initial microbial load of the tomatoes and not the result of cross-contamination. To address this issue, three solutions were proposed. First, the use of another media, such as Brilliance *Listeria* agar, which can differentiate between *L. monocytogenes* and other *Listeria* strains, could be considered. Second, an alternative approach involved washing the tomatoes in 70% ethanol for one minute and then rinsing them with sterile DI water prior to their use in experiments. Second approach was not used further because it might change surface properties and become a variation cause in the experiments. Lastly, make the studied bacteria antibiotic resistant and then use antibiotics in media to prevent background flora grow. However, this could interfere with the bacteria's ability to attach to the tomato surface. Moreover, it creates experimental conditions that may deviate from reality, and we cannot be certain that resistant bacteria will exhibit the same responses as wild-type strains. Due to the time limit all *Listeria* strains were not further used for new setting, only *L. monocytogenes*

15313 isolate was used as a reference only when the tomatoes were checked for initial contamination, and they did not show any contamination.

4.2. 150 V

Overall, our experimental design with 120 V, 60 kHz resonance frequency encountered challenges for both *Salmonella* and *L. monocytogenes* strains. Specifically, we observed that our setting was not able to reduce the *Salmonella* population in tomato wash water below the detection limit within 1 minute, which may indicate the possibility of cross-contamination during this exposure time. As a result, our current approach appears to be inefficient in preventing *Salmonella* cross-contamination. Based on these results, we conclude that a higher voltage is necessary to effectively prevent cross-contamination. The rationale behind this conclusion is that with a higher voltage, the bacteria will be killed more rapidly in the water, and thus, they will have less time to attach to the good tomatoes. As a result, we selected a voltage of 150 V for further experiments. Initially, this voltage was implemented in water disinfection to assess how much faster the bacterial cells would be inactivated compared to 120 V. With the previous voltage setting of 120 V, the reduction for the *Salmonella* strains after a 10-second exposure was as follows: 1.78-log CFU/ml for *S. enterica* 14028, 0.66-log CFU/ml for *S. enterica* 3139, and 0.58-log CFU/ml for *S. enterica* 3141. However, upon increasing the voltage to 150 V, the reduction at a 10-second exposure significantly increased, with values of 4.73-log CFU/ml for *S. enterica* 14028, 4.73-log CFU/ml for *S. enterica* 3139, and 4.59-log CFU/ml for *S. enterica* 3141. With a longer treatment time, specifically 20 seconds, a reduction of >5 log was achieved. No significant difference was observed between different serovars with a p-value of 0.654. As a result, we anticipated to observe less cross-contamination using this voltage, as it has the capability to rapidly inactivate bacterial cells in water. The inactivation levels of *S. enterica* from different serovars and different *L. monocytogenes* isolates after PAW bubbles treatment are presented in Figure 17.

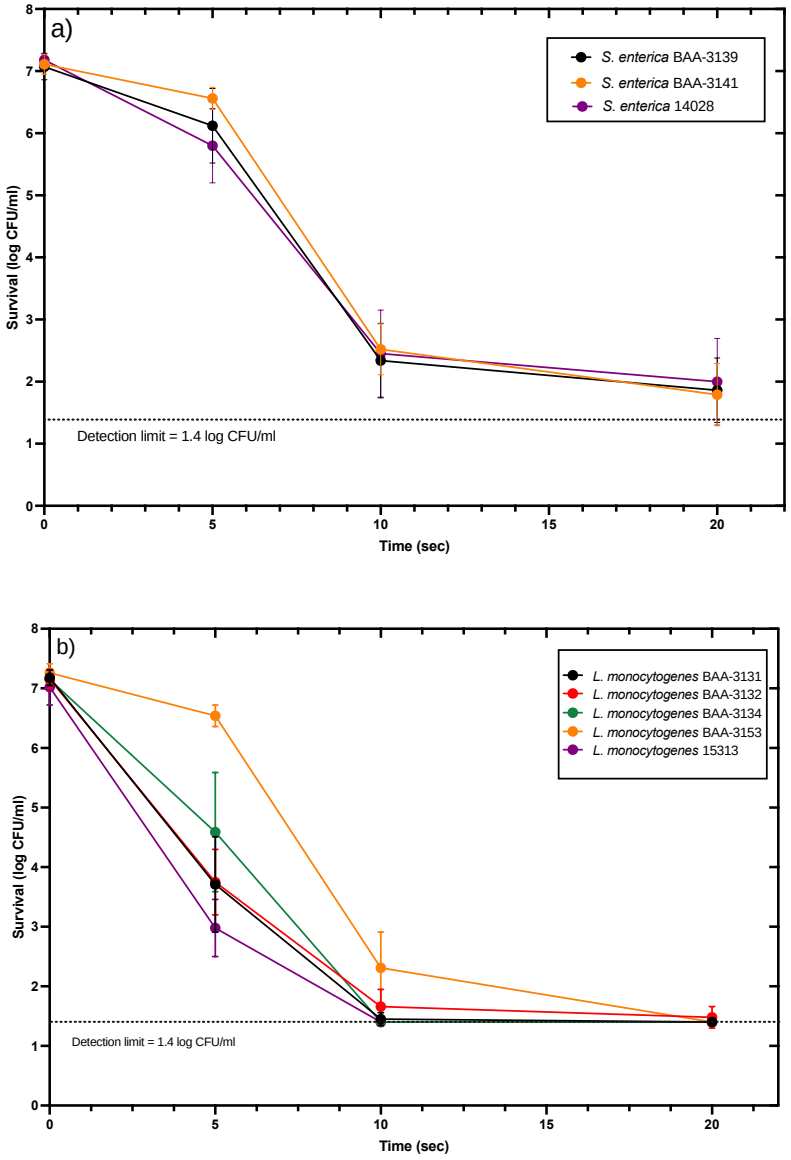

Figure 17. Mean ± SD survival of a) Salmonella enterica from different serovars and b) L. monocytogenes from different isolation sources in DI water subjected to PAW bubbles with 150 V (n=6)

Same as *Salmonella*, *L. monocytogenes* isolates also demonstrated significantly higher reduction levels after increasing the voltage to 150 V (Figure 15). With a 10-second PAW bubble exposure, all the isolates showed a reduction of >4.5 log, and with a 20-second treatment, their number were reduced below the detection limit. *L. monocytogenes 3153* appeared to be significantly more resistant than other *L. monocytogenes* strains after 5 and 10 seconds of treatment; however, when a longer contact time was used, no significant difference was observed in their

reduction rates. Consistent with the previous setting where *L. monocytogenes* was significantly more susceptible to plasma exposure, the use of 150 V was found to be stronger treatment for *L. monocytogenes* after 20 seconds, where it was significantly weaker than *S. enterica* with a p-value of 0.008.

Table 15. The mean ± SEM reduction of all strains after 5-, 10-, and 20-second PAW bubbles treatment (red shades indicates higher reduction values starting from 5 and blue shades demonstrates lower reduction below 5) (n=6)

Strain	**Reduction**		
	Time (seconds)		
	5	**10**	**20**
S. enterica Typhimurium	1.38 ± 0.27	4.73 ± 0.35	5.12 ± 0.31
S. enterica Poona	0.95 ± 0.30	4.73 ± 0.28	5.21 ± 0.18
S. enterica Thompson	0.55 ± 0.12	4.59 ± 0.20	5.31 ± 0.31
L. monocytogenes 15313	4.04 ± 0.29	5.62 ± 0.12	5.62 ± 0.12
L. monocytogenes 3153	0.73 ± 0.01	4.96 ± 0.39	5.86 ± 0.02
L. monocytogenes 3134	2.56 ± 0.89	5.75 ± 0.01	5.75 ± 0.01
L. monocytogenes 3132	3.41 ± 0.23	5.50 ± 0.08	5.68 ± 0.08
L. monocytogenes 3131	3.48 ± 0.32	5.74 ± 0.07	5.79 ± 0.05

Afterward, this setting (150 V) was utilized for cross-contamination experiments to assess whether the efficiency improved compared to the previous setting (120 V). In this set of experiments, in addition to drop plating, streaking was performed after enrichment to validate the results. A total of 18 trials were conducted for three *Salmonella* strains. Distinct outcomes were observed between the dropped and streaked plates (Table 17). Streaking appears to be a more reliable method than drop plates.

Following a 1-minute treatment with PAW bubbles, the drop plate data indicated that cross-contamination was observed in 4 out of the 18 trials, representing a 22.23% possibility of cross-contamination occurring. In contrast, air bubble treatment resulted in contamination in 14 out of the 18 trials, corresponding to a 77.78% possibility of cross-contamination. On the other hand, when streaking was employed, the probability of cross-contamination following PAW bubble treatment reduced to 55.58% from the 94.44% after air bubble treatment. It is noteworthy that although plasma did not completely prevent cross-contamination in all trials, it severely reduced the contamination load on the good tomatoes after implementation, as shown in Figure 18.

To figure out why cross-contamination is still occurring even though in the water samples, bacteria is not detected, a larger water sample of 25 ml was collected and was filtered with a vacuum pump, then the filter was put on the selective media and incubated overnight. Results demonstrate that bacterial cells are still present in the water in very low concentration less than 1 log CFU/ml that could move and contaminate the non-inoculated tomato (Table 16).

Table 16. Number ± SEM of survived bacteria identified on XLD plates through filtering 25 ml sample in water after 1-minute PAW bubble treatment with 150 V

Bacteria strain	Number present in water (log CFU/ml)
S. enterica 14028	0.32 ± 0.01
S. enterica 3139	0.19 ± 0.16
S. enterica 3141	0.55 ± 0.09

Table 17. Number of positive plates in 18 trials after PAW bubbles and Air bubbles treatment for 3 Salmonella strains, and total of 6 trial for L. monocytogenes

strain	Plating method	Number of contaminated plates		Number of total trials
		Air-bubbles	PAW-bubbles	
S. enterica	drop	14	4	18
	streak	17	10	
L. monocytogenes	drop	5	0	6
	streak	6	0	

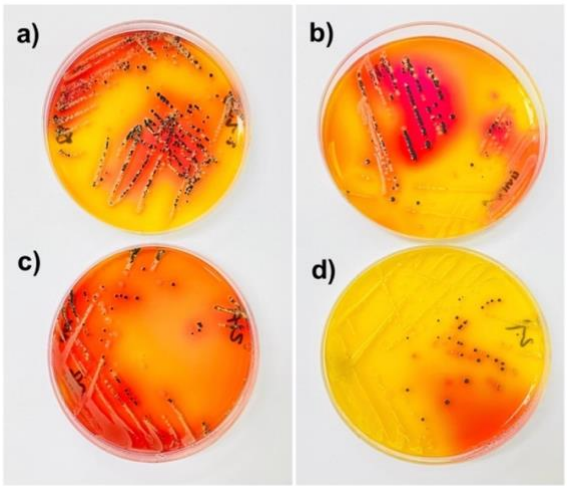

Figure 18. Photo of cross-contamination XLD plates after 1-minute PAW bubble (c, d) and 1-minute air bubble (a, b) treatment

Given that the cross-contamination aspect primarily served as a proof of concept to assess the plasma's capability to prevent such contamination and to determine the optimal settings for maximum prevention, few limitations were identified during the experiments. For instance, in our procedure, we placed the non-inoculated tomato first, followed by the inoculated tomato once all the steps were done. There was, however, a short lag time of approximately 10 seconds while closing the biosafety cabinet, during which both tomatoes were in the water, and the plasma was turned off. This lag time increased the possibility of cross-contamination, which, in turn, affects the reliability of our data. Furthermore, our data cannot be relied on totally as it overestimates the likelihood of cross-contamination occurrence. It is crucial to implement an optimized experimental design that eliminates any lag time between placing the tomatoes and turning on the plasma. It is recommended that the plasma be turned on for a few seconds with only the non-inoculated tomato present before introducing the inoculated tomato into the beaker. This modification would help mitigate the risk of cross-contamination and ensure more accurate data collection. However, few trials were conducted with the optimized design, and cross-contamination persisted after plasma treatment. Furthermore, we contemplate that the initial pathogen load of tomatoes is very high (>6 log CFU/g), which does not reflect the actual situation. As mentioned before, the initial microbial load is a crucial factor in establishing the effectiveness of plasma treatment.

Suggestions for future work:

- It would also be valuable to quantify the bacterial load on good tomatoes with and without plasma treatment, aiming to assess the plasma's efficiency for this purpose.
- Additionally, as previously mentioned, it is recommended to utilize specific media capable of distinguishing the studied bacteria from other bacteria within the same family.
- Using antibiotics in the media, after making the study strains resistant to them, is another suggestion to prevent background microflora from growing and therefore having more accurate data.
- In future studies, it is of significance to examine the effect of decreasing the initial inoculum to a realistic level in fresh produce and subjecting them to plasma treatment.

D. Conclusion

In this study, it was determined that a contact time of 20 seconds suffices to achieve a >3.5 log reduction of pathogens, utilizing a plasma setting with a voltage of 120 V, a duty cycle of 100 µsec, and a resonance frequency of 60 kHz (refer to Table 13). By elevating the plasma voltage to 150 V, this contact time can be further reduced to 10 seconds. Notably, the efficacy of plasma treatment was found to be a function of treatment time and the technological settings of the plasma generator. In other words, different configuration and settings can induce different inactivation levels, and this could be the main reason in the different reduction values reported in various studies.

Additionally, this study revealed that air bubble itself had the potential to provide microbial decontamination of tomato achieving reductions to >1.5 log levels after 3 min with 120 V for all pathogens. PAW bubbles treatment was only significantly different from air bubble in decontaminating *L. monocytogenes 3132 and 15313* on tomato surfaces. The number of pathogens in wash water after plasma treatment was below detection limit proving its efficacy to inactivate pathogens in wash water, while after air bubble there was still high survival of them in water. The inactivation mechanism was found to be as a result of hydroxyl radicals, superoxide, ozone, and not due to the hydrogen peroxide.

While PAW bubbles did not achieve complete prevention of cross-contamination, it did lead to a reduction in the contamination load of non-inoculated tomatoes in the cases where complete prevention was not achieved.

In summary, PAW bubble treatment was effective for water disinfection, while additional optimization of experiments and further research is warranted to comprehensively evaluate at which initial inoculum concentration plasma can prevent cross-contamination and to figure out why plasma was not very effective in decontaminating most species from tomato surfaces (possibly change tomato inoculation method or concentration). The potential impact of plasma treatment on the quality of treated samples, as well as the scalability of the setup when maintaining the same level of efficacy need to be evaluated as well.

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Appendix

- Spread plating vs. Spot plating

First three trials of water disinfection experiments with 120 V for *E. coli* O157:H7 were performed using both methods in duplicates to assess if they differ or not. Results demonstrated that there is no significant difference between spread and spot plating (p-value > 0.05). Furthermore, to save time and media, spot plating was done later for all experiments in duplicates.

Figure A. Plasma power generator

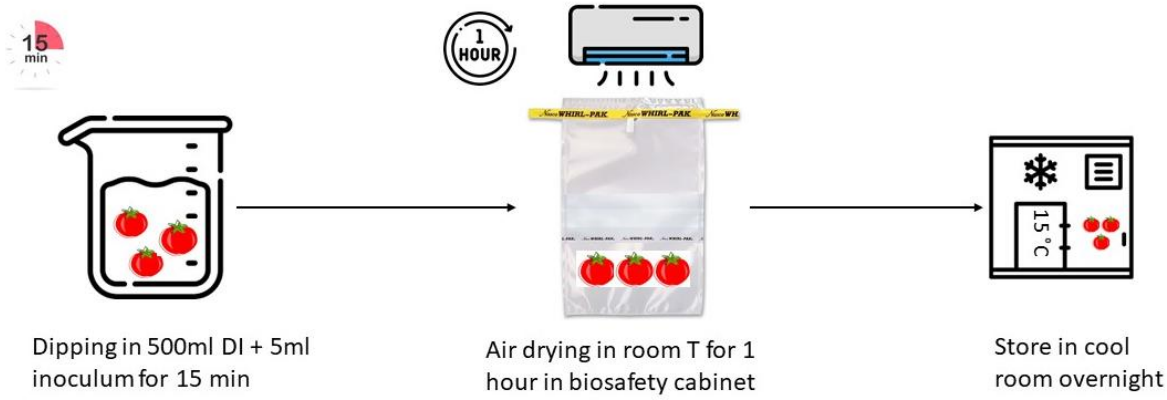

Figure B. Tomato inoculation

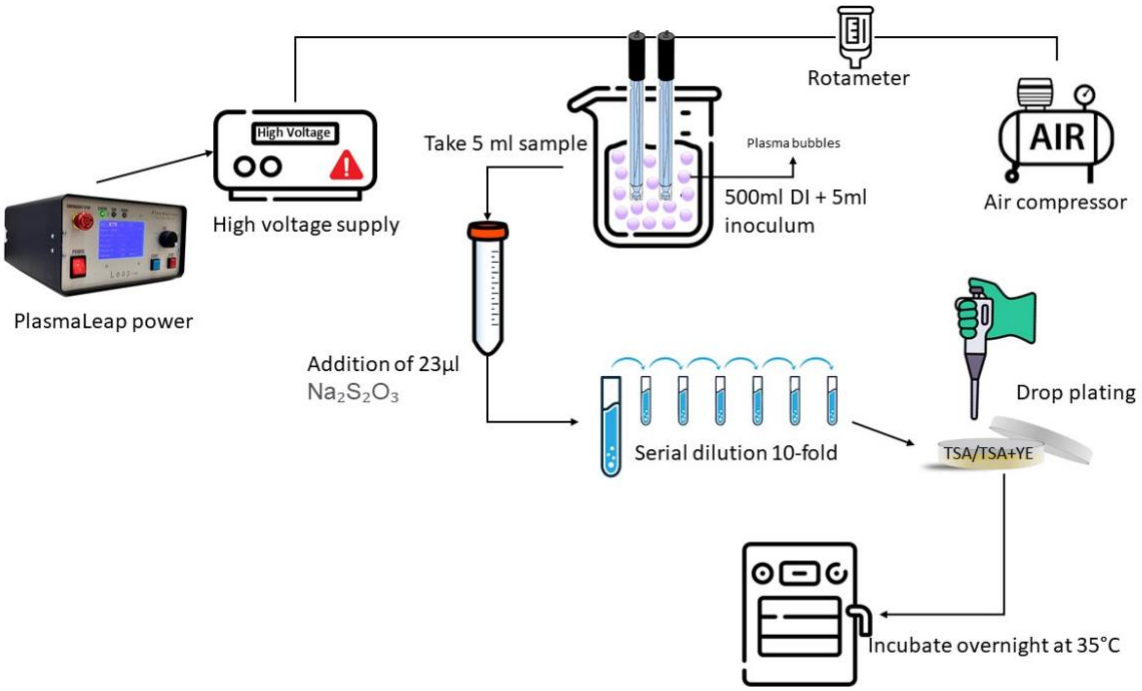

Figure C. Water disinfection steps

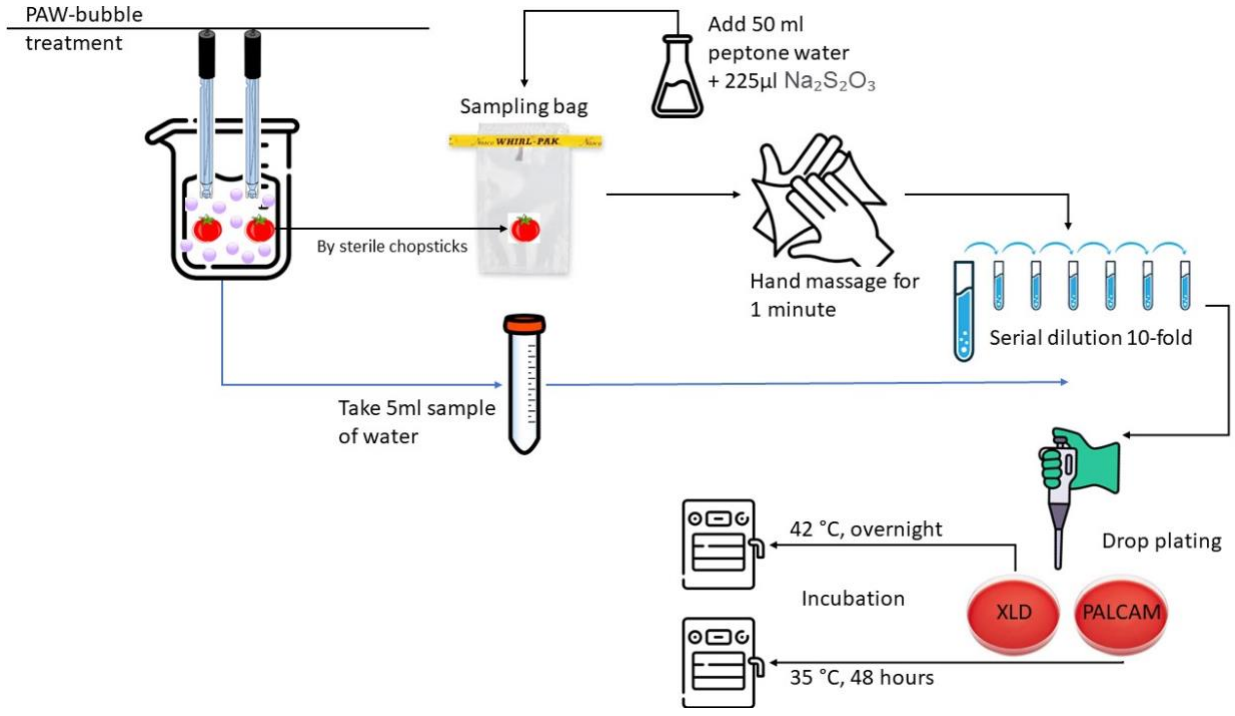

Figure D. Tomato decontamination experiment steps

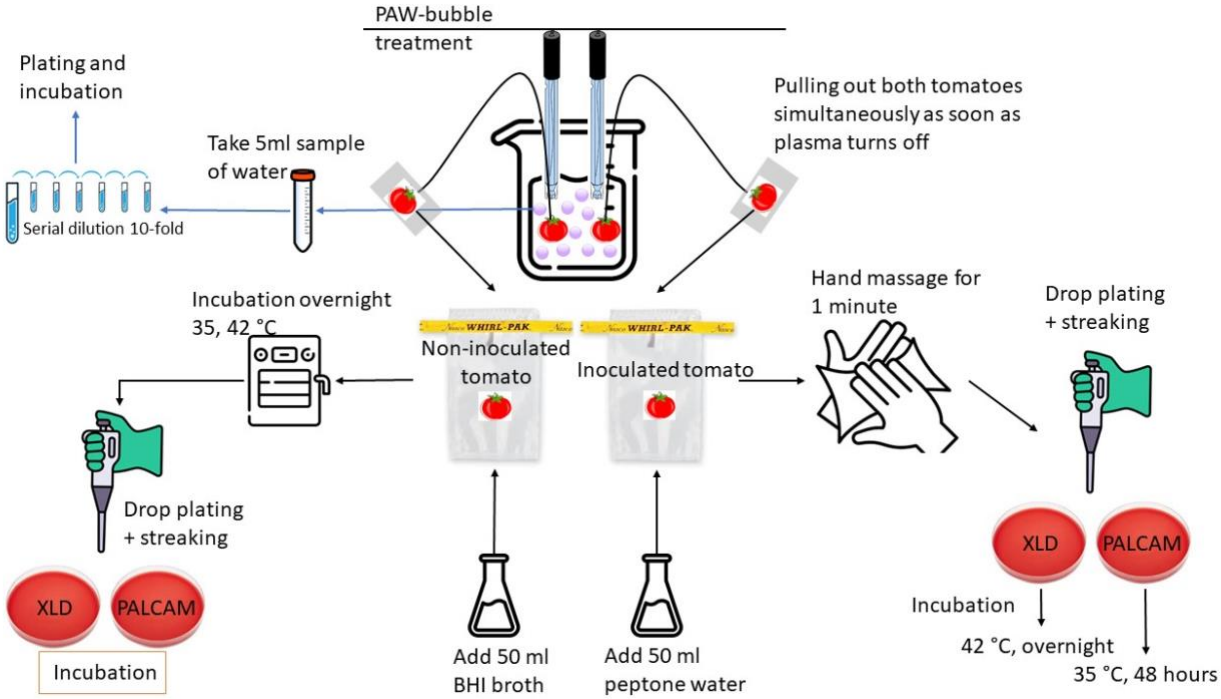

Figure E. Cross-contamination experiment steps

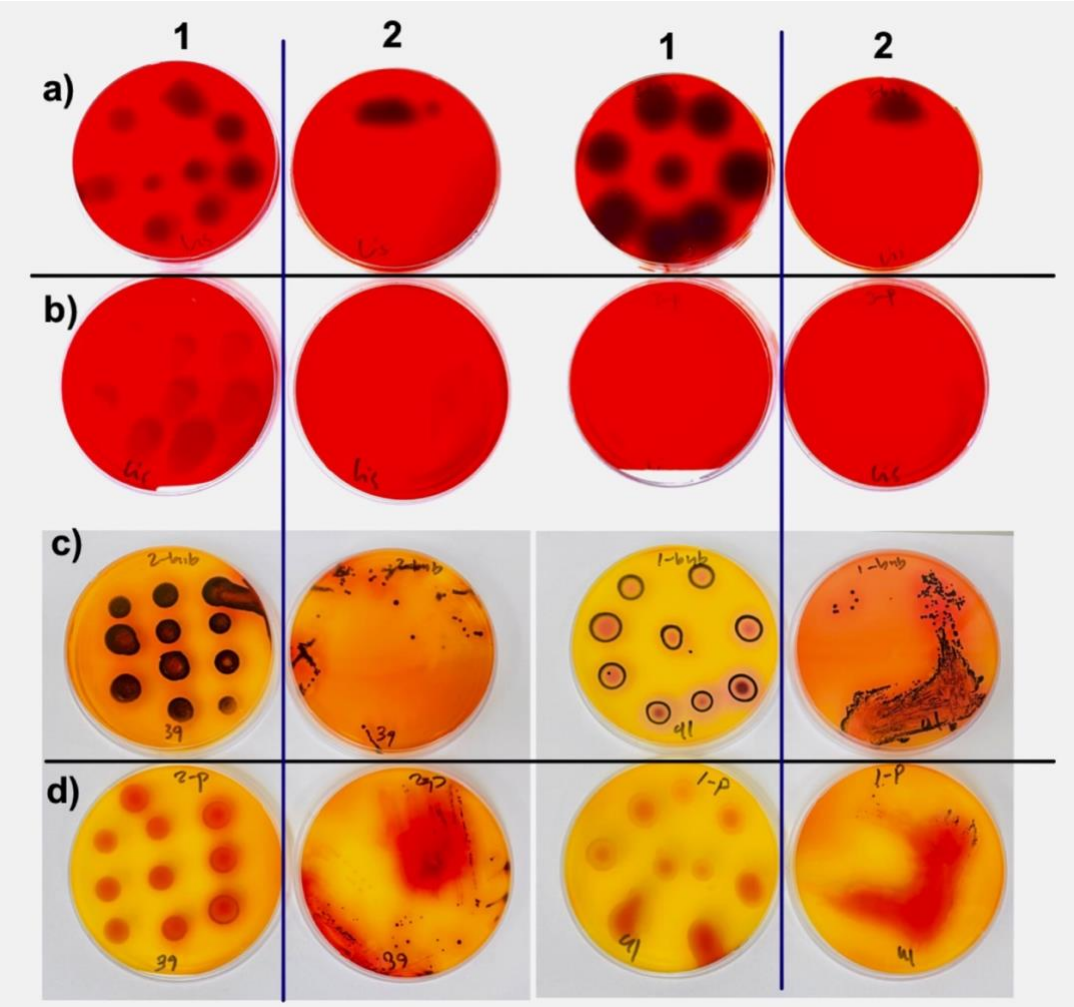

Figure F. Cross-contamination plates picture after 1-minute air bubble treatment (a, c) and after PAW bubble treatment 150 V (b, d), 1 represents dropped plates and 2 represent streaked plates