Efficacy of conventional and novel antimicrobial treatments on the inactivation of desiccated cells and biofilms of *Salmonella*

by

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Abstract

Repeated isolation of *Salmonella* from the dry and wet environments of the food industry poses a significant concern to human health. It is crucial to understand the influence of the various environmental conditions on *Salmonella's* survival. The overall objective of this research was to evaluate the efficacy of various conventional and novel disinfection approaches in the inactivation of *Salmonella* under diverse conditions of the food industry.

Salmonella is responsible for numerous foodborne outbreaks associated with low-water activity (a_w) foods. Thermal treatment is widely used to minimize the risk of *Salmonella* from such foods. An increase in the thermal resistance of *Salmonella* post-desiccation stress has been well documented. It is important to investigate the potential effect of food composition, a_w, and strain type on the desiccation and subsequent thermal survival of *Salmonella*. In the first study, three low-a_w foods (pet food, burger binder and skim milk powder) were wet inoculated with the different *Salmonella* spp. and desiccated to a_w of 0.33 and 0.75. The thermal inactivation kinetics of *Salmonella* spp. obtained following an isothermal treatment at 70 °C, demonstrated an interactive effect of the food composition, and a_w change at the treatment temperature, on its survival. Furthermore, the water sorption isotherms of the selected low-a_w foods were developed to relate the thermal survival of *Salmonella* to the a_w change at 70 °C. Sorption isotherms displayed a significant increase in the a_w of the pet foods as compared to burger binder and skim milk powder. However, the observed *Salmonella* thermal inactivation kinetics demonstrated the role of a complex interaction between the strain type, a_w, and food composition rather than being affected by a single parameter.

The low-a_w food contamination can also occur via contaminated food contact surfaces. Conventional disinfectants such as oxidizers (hydrogen peroxide, peracetic acid), dry heat and membrane-acting quaternary ammonium compounds are frequently used for the disinfection of food contact surfaces. However, *Salmonella* survival in response to the disinfection process can vary based on its cellular state. In the second study, *Salmonella* Enteritidis FUA1946 was inoculated on stainless steel under different desiccation conditions (air drying and air drying followed by equilibration to a relative humidity of 33%). Their inactivation was assessed using conventional and novel (plasma-activated water bubbles, plasma-activated hydrogen peroxide water bubbles) disinfectants. The results demonstrated a significant effect of the concentration, treatment temperature, and exposure time on the inactivation of the desiccated bacteria. The desiccation method significantly influenced the disinfection survival of *Salmonella*. Furthermore, the efficacy of plasma-activated water bubbles, recirculated under different hold and flow times was evaluated against the air-dried and equilibrated *Salmonella* on stainless steel surfaces. Increasing the plasma flow time significantly enhanced the inactivation of the surface-attached bacteria.

Salmonella uses biofilm formation as a mechanism for survival. Generally, true biofilms are formed inside close infrastructures like pipelines. Chemical disinfectants are used for the disinfection of the drinker lines. However, their persistent use can form hazardous by-products. In the third study, the disinfection effectiveness of plasma-activated water bubbles (PAWB) under different hydrodynamic variations against the mixed species biofilms was evaluated. A benchtop pipeline model simulating the industrial pipelines was built. *Salmonella* ATCC13311 and *Aeromonas australiensis* 03-09 were used to form mixed-species biofilms on the inner surfaces of the PVC pipes. The effectiveness of laminar (*Re* 1000), transitional (*Re* 2500) and turbulent (*Re*

4000) flow regimes in the inactivation of mixed-species biofilms was examined, followed by an investigation into the effect of treatment time on the biofilm inactivation. In the third set of experiments, the effectiveness of the constant volume flow rate was evaluated against the biofilm adhered to the pipe walls. PAWB recirculation under high Reynold's number resulted in a greater inactivation of the biofilms. The interactive effect of the PAWB reactive species, the volume of PAWB circulated, and the shear stress generated using the different flow regimes significantly improved the inactivation of the biofilms from the pipe walls. Moreover, bacteria in the bulk water was highly susceptible to the action of PAWB.

This PhD thesis research demonstrated the effectiveness of various conventional and novel disinfection technologies in the inactivation of *Salmonella* in low-a_w environments and in biofilms. The knowledge gained in this research would help in the appropriate selection of disinfection protocols for effective *Salmonella* control from the food industry.

Preface

This thesis is an original work done by Harleen Kaur Dhaliwal at the Food Safety and Sustainability Engineering Lab at the University of Alberta under the supervision of Dr. Roopesh Mohandas Syamaladevi.

Some sections of Chapter 2 of this thesis have been submitted as Dhaliwal, H. K., & Roopesh, M. S. "Process technologies for disinfection of food-contact surfaces in the dry food industry: A review" to Comprehensive Reviews in Food Science and Food Safety. H. K. Dhaliwal drafted the original manuscript and Dr. M. S. Roopesh reviewed and edited the manuscript. Some sections of the submitted manuscript are irrelevant to the scope of this thesis, hence, are not included here.

Chapter 3 of this thesis has been published as Dhaliwal, H. K., Gänzle, M., & Roopesh, M. S. (2021). Influence of drying conditions, food composition, and water activity on the thermal resistance of *Salmonella enterica*. Food Research International, 147, 110548. H. K. Dhaliwal conducted the experiments, did data analysis, and wrote the manuscript. Dr. M. S. Roopesh and Dr. M. Gänzle provided their timely suggestions, in experimental design, troubleshooting, and reviewed and edited the manuscript. Dr. F. Temelli provided suggestions regarding the experimental design. A virtual poster has been presented regarding the findings of this study as "Dhaliwal, H. K., Gänzle, M., & Roopesh, M. S. Influence of food composition and water activity on the thermal resistance of *Salmonella* spp. in selected low-a_w foods in 3rd Food Innovation and Engineering (FOODIE) Conference by AIChE. 2020 (Virtual)".

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manuscript. Three poster presentations related to the findings of this study have been presented as "Dhaliwal, H. K., Gänzle, M., & Roopesh, M. S. Efficacy of dry heat on the inactivation of *Salmonella enterica* on stainless steel. International Association of Food Protection Annual Meeting (IAFP), 2021 (Virtual)", "Dhaliwal, H. K., Gänzle, M., & Roopesh, M. S. Efficacy of wet and dry sanitizing techniques on desiccation resistance of *Salmonella enterica* on stainless steel. Canadian Institute of Food Science & Technology (CIFST), Guelph, Ontario, CA, June 1-3, 2022" and "Dhaliwal, H. K., & Roopesh, M. S. Plasma activated water bubbles: Characterization and assessment of desiccated *Salmonella* inactivation efficacy. 25th International Symposium on Plasma Chemistry, Kyoto, Japan, May 21-26, 2023".

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This thesis is dedicated to my parents, Satnam Singh Dhaliwal, and Sarabjit Kaur,

I am because they are! No amount of appreciation or words can ever be enough for my parents. They have seen me grow into what I am today. For all the sacrifices they have done for me, for their constant support and unconditional love that has sailed me through life's thick and thin, I am grateful to them!

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List of Abbreviations

$^{\bullet}NO$	Nitric Oxide
$^{\bullet}OH$	Hydroxyl Radical
$^{1}O_2$	Singlet Oxygen
ACC	Active Chlorine Concentration
AEW	Acidic Electrolyzed Water
AIEW	Alkali Electrolyzed Water
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
a_w	Water Activity
BAC	Benzalkonium Chloride
BET	Brunauer–Emmett–Teller
BSA	Bovine Serum Albumin
BSC	Biosafety Cabinet
CAP	Cold Atmospheric Plasma
CDC	Centre for Disease Control
CFU	Colony Forming Unit
CIP	Cleaning In Place
ClO_2	Chlorine Dioxide
ClO_2^-	Chlorite Ion
CO_2	Carbon Dioxide
COP	Cleaning Outside Place
CUT	Come-Up Time

CV	Crystal Violet
DBD	Dielectric Barrier Discharge
DE	Dey-Engley Neutralizing Broth
DNA	Deoxyribonucleic Acid
DPD	N,N-diethyl-p-phenylenediamine
DWS	Drinking Water System
EPS	Extracellular Polymeric Substances
EW	Electrolyzed Water
FCV	Feline Calicivirus
FDA	US Food and Drug Administration
FSPCA	Food Safety Preventive Controls Alliance
GAB	Guggenheim, Anderson and De Boer Model
GAD	Gliding Arc Discharge
GRAS	Generally Regarded as Safe
H_2O_2	Hydrogen Peroxide
HAV	Hepatitis A virus
HCoV-229E	Human Coronavirus 229E
HDPE	High-Density Polyethylene
hf	Head Loss
HOCl	Hypochlorous Acid
IPAQuat	Isopropyl Alcohol Quaternary Ammonium
LA-NS	Lennox Agar With No Salt
LB-NS	Lennox Broth with No Salt

LED	Light Emitting Diode
MNV-1	Murine norovirus 1
NaOCl	Sodium Hypochlorite
NEW	Neutral Electrolyzed Water
NO_2^-	Nitrite
NO_3^-	Nitrate
O	Atomic Oxygen
O_2^-	Superoxide
O_2NOO^-	Peroxynitrate
O_3	Ozone
OD	Optical Density
OES	Optical Emission Spectroscopy
OmpC, OmpF	Outer Membrane Porins
$ONOO^-$	Peroxynitrite
ONOOH	Peroxynitrous Acid
ORP	Oxidation Reduction Potential
OSHA	Occupational Chemical Database
PAA	Peracetic Acid
PAHP-WB	Plasma-Activated Hydrogen Peroxide Water Bubbles
PAW	Plasma-Activated Water
PAWB	Plasma-Activated Water Bubbles
PHAC	Public Health Agency of Canada
PVC	Polyvinyl Chloride

QAC/QUATs	Quaternary Ammonium Compounds
R_A	Arithmetic mean
Re	Reynold's Number
RH	Relative Humidity
R_K	Core Roughness Depth
RNA	Ribonucleic acid
RONS	Reactive Oxygen and Nitrogen Species
ROS	Reactive Oxygen Species
R_P	Levelling depth
R_{PK}	Reduced Peak Height
R_Q	Root Mean Square
R_{VK}	Reduced Valley Depth
SDBD	Surface Dielectric Barrier Discharge
SDS	Sodium Dodecyl Sulphate
SEM	Scanning Electron Microscopy
SHS	Superheated Steam
SLPM	Standard Liter Per Minute
SMD	Surface Micro-Discharge Plasma
SMP	Skim Milk Powder
SS	Stainless Steel
SSCs	Stainless Steel Coupons
TDT	Thermal Death-Time
TSA	Tryptic Soy Agar

TSB	Tryptic Soy Broth
UV	Ultraviolet
YE	Yeast Extract
σ^E, σ^S	Sigma Stress Factors

Chapter 1: General introduction and thesis objectives

1.1 Introduction

Salmonella is a ubiquitous pathogen causing salmonellosis (Finn, Condell, et al., 2013). *Salmonella* accounted for an average of 1459 foodborne outbreaks, resulting in 55 deaths and 36,559 illnesses, in the United States from 2011-2021 (CDC, 2023a). In Canada, yearly 87,500 illnesses, 925 hospitalizations and 17 deaths have been estimated due to *Salmonella* related food-borne infections (Public Health Agency of Canada, 2016).

Low-water activity (a_w) foods having $a_w < 0.85$ (Beuchat et al., 2013) are frequently associated with *Salmonella* outbreaks. The frequent implications of *Salmonella* in low-a_w foods including peanut butter (CDC, 2022), almonds (CDC, 2004), breakfast cereals (CDC, 2018b), flour (CDC, 2023c), pet food (CDC, 2012c), tahini paste (CDC, 2019b), and pistachio nuts (CDC, 2016b), increases the risk of infections. An increased occurrence of pathogens resulting from the use of contaminated raw ingredients, and food contact surfaces, raises significant public health concerns (Beuchat et al., 2013; Finn, Condell, et al., 2013).

Low-a_w food processing operations involve the use of desiccation and intervention technologies such as heat treatment to improve their shelf stability (Fong & Wang, 2016a). The bacterium under hyperosmotic conditions, induces the production of compatible solutes such as proline, trehalose, and glycine betaine to maintain the turgor pressure (Finn, Condell, et al., 2013; Greffe & Michiels, 2020). The resultant overcrowding of intracellular metabolites leads to a loss of cellular metabolic activity (Greffe & Michiels, 2020). Studies have reported an upregulation of sigma stress factors (σ^E and σ^S) (McMeechan et al., 2007) and membrane porins during the desiccation process (Finn,

Condell, et al., 2013). Moreover, the entry of cells to the viable but non-culturable state maintains their survival under harsh desiccated conditions (Oliver, 2010).

The fluctuations in the relative humidity of low-a_w food processing environments further make the bacteria challenging to eradicate (Alavi & Hansen, 2013). Studies have reported *Salmonella's* cross-tolerance to thermal (Frank & Koffi, 1990) and osmotic stress, post-exposure to desiccation stress (Gruzdev et al., 2011). Furthermore, several factors including strain type (Guillén et al., 2020), a_w, temperature (Bashir et al., 2022; Finn, Condell, et al., 2013) and food composition (Dhaliwal et al., 2021; Zhang et al., 2022) govern the survival of *Salmonella* in the desiccated state. Thus, it is crucial to comprehend the impact of inoculation methodology, a_w, and food composition, on the desiccation tolerance and thermal survival of *Salmonella* in various low-a_w foods.

Salmonella has an inherent ability to colonize the contact surfaces of the low-a_w conditions of food processing facilities (Bashir et al., 2022; Kusumaningrum et al., 2003; Møretrø et al., 2009; Veluz et al., 2012). Adaption of the bacterium to the environmental conditions including temperature, and relative humidity aids in its survival under stressful conditions of the dry food industry (Lee et al., 2020).

Adhesion of *Salmonella* on the food contact surfaces can potentially cross-contaminate low-a_w foods manufactured in the same facility (Frank, 2001). Studies have reported the use of cationic surfactants (Al-Qadiri et al., 2016), and conventional oxidizers including chlorine dioxide (Park & Kang, 2017), and hydrogen peroxide (Choi et al., 2012) for surface disinfection under low-a_w conditions. The increased usage of chemical-based disinfectants could result in the formation of resistant subpopulations (Fraise, 2002). Dry heat can also be used for surface decontamination (Mckelvey & Bodnaruk, 2013); however, an increase in the thermal resistance of *Salmonella* under

low-a_w conditions of the processing environment can further make the bacterium difficult to eradicate (Archer et al., 1998). This necessitates the need for sustainable approaches for *Salmonella* control in low-a_w food industry. A relatively new approach using plasma-activated water bubbles (PAWB) is gaining a lot of attention. PAWB is a sustainable alternative to chemical-based disinfectants (Hong et al., 2021). PAWB provides an increased mass transfer and better penetration of the reactive species in the form of bubbles (Mai-Prochnow et al., 2021). The dual air-phase and water-phase plasma discharge produces a complex environment consisting of ultraviolet (UV) photons, charged particles, ions, and reactive oxygen and nitrogen species (RONS) including ozone (O_3), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), superoxide (O_2^-), atomic oxygen (O), singlet oxygen ($^1O_2$), peroxynitrite $ONOO^-$), nitrite (NO_2^-), nitrate (NO_3^-), and nitric oxide ($^{\bullet}NO$) (Hong et al., 2021; Mai-Prochnow et al., 2021). Moreover, no study has evaluated the effectiveness of PAWB for the disinfection of low-a_w food contact surfaces.

Salmonella's response to the disinfection agents is dependent on its cellular state, such as biofilms, planktonic, and surface-attached cells (Dhir & Dodd, 1995). The disinfection ability of several biocidal agents against the surface-attached *Salmonella* on various food contact surfaces is already studied (Djebbi-Simmons et al., 2019; Gruzdev et al., 2011). However, a comparative study on the efficacy of different conventional and novel oxidizers, dry heat and membrane-acting agents on inactivation based on the cellular state of bacteria i.e., planktonic, and surface-attached cells has not yet been conducted.

Biofilms are another survival strategy, in which bacterial cells colonize the contact surfaces and are protected against disinfectants (Maes et al., 2019). The solid-liquid interfaces of closed surfaces in the food industry provide a favourable environment for biofilm formation (Verran et al., 2008).

Moreover, the pipelines used to carry the wet ingredients in the food processing plants are generally associated with biofilms (Whitehead & Verran, 2015).

Salmonella infections related to broiler chickens, fresh poultry meat and eggs have been well documented (Chia et al., 2009; Cosby et al., 2015; Guard-Petter, 2001). Biofilm prevalence in the broiler drinking water systems facilitates its transmission in bulk water (Maes et al., 2019). Broiler chickens serve as a reservoir for the replication of *Salmonella* and can pose a risk for human infections (Cosby et al., 2015). Certain sugars, electrolytes, vitamins, and additives are dissolved into the drinking water to supplement the nutritional needs of the broiler (Maes et al., 2019). Biofilm presence indicates a decrease in the medicinal quantities, leading to broilers being administered a suboptimal dosage (Maes et al., 2020). The presence of bacterial biomass in the water systems decreases the pressure due to an obstruction of pipelines, leading to reduced water flow rates and eventually equipment failure (Maes et al., 2020).

Various oxidative and chlorine-based disinfectants are commonly employed for the disinfection of drinker lines (Watkins, 2006). However, their continued usage might produce hazardous disinfection by-products such as trihalomethanes, haloacetic acids, chlorite and chlorate (Simões & Simões, 2013). The chemical and microbiological contamination of drinker lines in broiler houses can pose a serious health hazard (Maes et al., 2019).

For effective *Salmonella* risk control, there is a need to evaluate novel sustainable approaches for disinfection in the food industry. Published literature has validated the effectiveness of PAW for biofilm inactivation on various food contact surfaces (Hozák et al., 2018; Mai-Prochnow et al., 2021; Xia et al., 2023). However, very few studies have explored the potential of plasma-activated water bubbles as a novel strategy for biofilm control (Xu & Tan, 2023). The bacterial contamination of the water facilitates the development of biofilm in the broiler drinking water

system (DWS) (Maharjan, et al., 2017a). Generally, a higher Reynold's number (*Re* $\geq$ 4000), representing the turbulent flow regimes is preferred for the disinfection of DWS. The synergistic action of the chemical disinfectants along with the high shear forces resulting from high *Re* can lead to a better inactivation of biofilms (Simões et al., 2005b). However, limited studies have evaluated the effectiveness of disinfectants under hydrodynamic fluctuations i.e. laminar, transitional and turbulent flow regimes representative of the DWS in biofilm inactivation (Douterelo et al., 2018). A comprehensive investigation on the utilization of PAWB under varying hydrodynamic conditions for the inactivation of *Salmonella* mixed species biofilms has not been conducted.

1.2 Hypothesis

1) Desiccation and thermal resistance of *Salmonella* are dependent on the food composition, a_w, inoculation methodology and the strain type selected.
2) The disinfection potential of the dry heat, oxidizing, and membrane-acting agents varies based on their mode of action and the cellular state of *Salmonella*.
3) Novel disinfectants such as PAWB can inactivate biofilms inside drinking water systems; however, the inactivation efficacy of PAWB against the mixed species biofilms is affected by the dynamic treatment conditions such as flow regimes, RONS concentration, the volume of PAWB circulated and exposure time.

1.3 Objectives

This PhD research aimed to understand the potential of conventional and novel technologies for the inactivation of the desiccated *Salmonella* in low-a_w food systems and on the contact surfaces, and *Salmonella* biofilms in pipelines of drinking water systems.

The specific objectives were :

1) To investigate the effect of food composition, a_w, and inoculation methodology on the thermal resistance of *Salmonella* in various low-a_w foods (Chapter 3).
2) (a) To evaluate the disinfection potential of different conventional and novel technologies for the inactivation of dry *Salmonella* on stainless steel (Chapter 4) and (b) To develop a continuous PAWB reactor with a coupon holder for the inactivation of dry *Salmonella* on stainless steel surfaces (Chapter 4).
3) To examine the inactivation effectiveness of PAWB under different flow regimes against the mixed species biofilms formed on the PVC surfaces (Chapter 5).

Salmonella is one of the leading causative agents of foodborne outbreaks (CDC, 2023b). *Salmonella* Enteritidis FUA1946 (dry heat-resistant wastewater isolate), *Salmonella* Senftenberg ATCC43845 and *Salmonella* Typhimurium ATCC13311 (wet heat-resistant strains) were selected to evaluate the desiccation and thermal resistance in various low-a_w foods (Chapter 3). Stainless steel, a material used for equipment fabrication in low-a_w food processing industry was used to assess the desiccation resistance of *Salmonella* (Chapter 4). Mixed-species biofilms of *Salmonella* ATCC13311 and *Aeromonas australiensis* 03-09 were developed on the inner surfaces of PVC pipe sections. In addition, the disinfection potential of PAWB in the inactivation of mixed-species biofilms was evaluated under flow conditions (Chapter 5).

Chapter 2: Literature Review

Salmonella is a gram-negative rod-shaped enteric bacterium causing intestinal infections (Foster & Spector, 1995). It can survive in various food products for few days to several years (Podolak et al., 2010). The frequent foodborne recalls and outbreaks associated with *Salmonella* causes a significant food safety concern (CDC, 2023a).

2.1 *Salmonella* survival in low-a_w foods

Foods naturally low in moisture or those that are dried to remove moisture are referred to as low-a_w foods (a_w < 0.85) (Beuchat et al., 2013). Generally, bacterial foodborne pathogens, including *Salmonella* spp., *E.coli* and *Clostridium perfringens* require a_w > 0.95 to maintain their cellular metabolic process (Fontana, 2020). Low-a_w foods were previously considered low-risk foods, due to the common misconception that low-a_w environments do not provide a favourable environment for bacterial growth. Most of low-a_w foods or ingredients undergo a thermal-kill step and were considered safe for consumption (Hiramatsu et al., 2005). However, their increased association with the foodborne outbreaks in common low-a_w foods such as wheat flour (CDC, 2023c), peanut butter (CDC, 2009a), pet food (CDC, 2008a), and chocolate (Werber et al., 2005), highlights the need to follow proper intervention steps to ensure food safety. Table 2.1 summarizes various recent *Salmonella*-related low-a_w food-borne outbreaks.

Table 2.1: Summary of the studies demonstrating *Salmonella* outbreaks linked with low-a_w foods.

Pathogen	Product	Year	Remarks	References
Salmonella spp.	All-purpose flour	2023	14 infected cases and 3 hospitalizations were reported from 13 states	CDC (2023c)
Salmonella Senftenberg	Peanut butter	2022	21 infected cases and 4 hospitalizations were reported from 17 states	CDC (2022)
Salmonella Concord	Tahini	2019	6 reported cases and 1 hospitalization were reported from 3 states	CDC (2019b)
Salmonella Mbandaka	Puffed wheat cereal	2018	135 cases and 34 hospitalizations were reported from 36 states	CDC (2018b)
Salmonella Typhimurium	Dried coconut	2018	14 infected cases were reported from 8 states	CDC (2018c)
Salmonella Montevideo and *Salmonella* Senftenberg	Pistachios	2016	11 infected cases and 2 hospitalizations were reported from 9 states	CDC (2016b)
Salmonella Paratyphi B	Nut butter spreads	2015	13 infected cases were reported from 10 states	CDC (2016c)
Salmonella Braenderup	Nut butter	2014	6 infected cases were reported from 5 states	CDC (2014c)
Salmonella Newport, *Salmonella* Hartford, *Salmonella* Oranienburg*, and* *Salmonella* Saintpaul	Sprouted chia seed powder	2014	63 infected cases were reported in Canada	PHAC (2014)
Salmonella Bredeney	Peanut butter	2012	42 infected cases were reported from 20 states	CDC (2012f)
Salmonella Infantis	Dry dog food	2012	49 infected cases were reported from 20 states	CDC (2012c)
Salmonella Montevideo, *Salmonella* Newport, and *Salmonella* Senftenberg	Pistachio nuts	2009	-	CDC (2009b)
Salmonella Agona	Puffed rice cereals	2008	28 infected cases were reported from 15 states	CDC (2008b)
Salmonella Schwarzengrund	Dry pet food	2007	62 infected cases and 10 hospitalizations were reported from 18 states	CDC (2007a)
Salmonella Tennessee	Peanut butter	2007	425 infected cases were reported from 44 states	CDC (2007b)
Salmonella Enteritidis	Almonds	2001	157 infected cases were reported in Canada	Isaacs et al., (2005)

2.1.1 Desiccation adaption of *Salmonella* in low-a_w foods

The long-term adaption of *Salmonella* under low-a_w conditions is a major food safety concern. Water is required for performing biological functions, and to maintain the structural integrity of macromolecules (Esbelin et al., 2018). *Salmonella* exposure to desiccation stress promotes the sudden loss of water and results in rapid cell shrinkage (Burgess et al., 2016). To maintain an appropriate turgor, certain stress factors (σ^S) upregulate the biosynthesis of osmoprotectants, to create a hypertonic environment and prevent the sudden cell death (Burgess et al., 2016). Increased production of low-molecular-weight solutes such as trehalose, maintains the structure and integrity of proteins and lipids (Spector & Kenyon, 2012). Other reported desiccation resistance mechanisms include reduced intracellular fluidity due to vitrification (Lee et al., 2020), production of cellulose and thin aggregative fimbriae (White et al., 2006), expression of outer membrane porins (OmpC, OmpF) (Spector & Kenyon, 2012), existence in viable but non-culturable state (Foster & Spector, 1995). Desiccation or matric stress also provides cross-resistance to oxidative and thermal stresses (Elbein et al., 2003).

2.1.2 Thermal resistance of *Salmonella* in low-a_w foods

Water is vital for the growth of bacteria; however, water levels below the threshold growth cause cell death due to starvation. Warth (1985) hypothesized that at higher water activity, water molecules are in close contact with the proteins. When bacterial cells are exposed to heat stress, water molecules vibrates and induces the protein unfolding, thereby resulting their subsequent denaturation. Whereas in the desiccated conditions, lesser protein-water interactions are observed, hence substantial thermal energy would be required to break the disulphide and hydrogen bonds in the proteins. These heat-stressed proteins play a significant role in increasing the heat resistance of the bacteria markedly. Observations made by researchers suggested that at lower a_w, dipoles of

the protein molecules interact, and form stabilized complexes, which further contributes to the survival mechanism of bacteria in hostile conditions (Warth, 1985).

Low-moisture food safety can be achieved using various food decontamination methods including; thermal processes involving dry heat (Beuchat & Mann, 2011), steam (Lee et al., 2006), radiofrequency (Villa-Rojas, Zhu, et al., 2017) and infrared heating (Brandl et al., 2008) as well as non-thermal processes including high hydrostatic pressure processing (D'Souza et al., 2012), irradiation (Ban & Kang, 2014), cold plasma (Chaplot et al., 2019), light emitting diode (LED) (Du et al., 2020), and propylene oxide treatment (Danyluk et al., 2005).

2.2 *Salmonella* survival in low-a_w food processing environments

The desiccation of microorganisms in a low-a_w environment in the dry food processing industry renders them resistant to various decontamination processes and causes food safety issues (Gruzdev et al., 2011). Cross-contamination and poor sanitation are identified as key contributors linked to foodborne outbreaks (Podolak et al., 2010). Surface-adhered microorganisms may detach and colonize nearby surfaces and subsequently cross-contaminate the foods (Frank, 2001; Norwood & Gilmour, 1999). Furthermore, the existence of persistent strains in food processing environments plays a significant role on the subsequent contamination (Miettinen et al., 1999). Table 2.2 outlines the outbreak investigations linked to the contaminated dry food production environments.

Microorganisms can enter food manufacturing facilities and industrial environments via airflow, water, personnel, or raw materials (Figure 2.1) (Gupta & Adhikari, 2022). Open and closed systems make up a food processing plant. Open systems comprise of cutting boards, knives, slicers,

conveyor belts etc. The food utilized in open systems may be solid or liquid and is exposed to air. It creates favourable conditions for bacterial adhesion and growth.

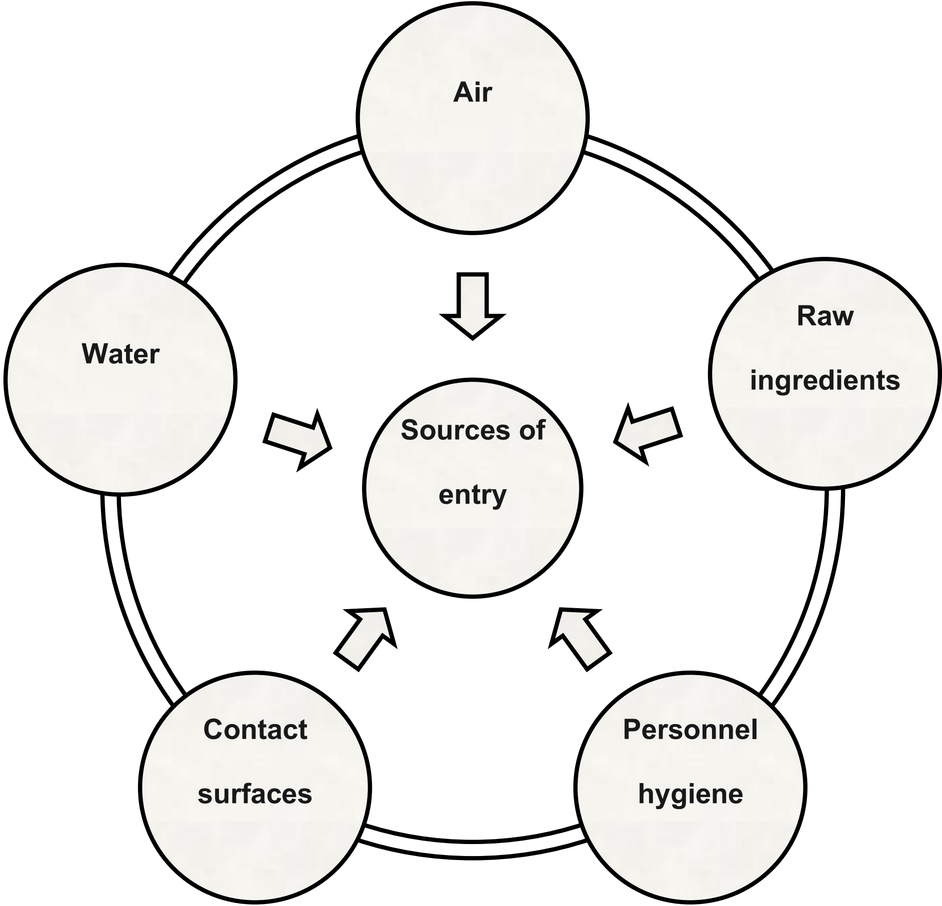


Figure 2.1: Possible contamination routes of microorganisms into the dry food processing facility.

Table 2.2: Summary of the studies demonstrating the outbreaks linked with low-a_w food processing environments.

Pathogens	**Year**	**Remarks**	**References**
Salmonella Newbrunswick	1965 – 1966	Inadequate hygiene standards in the spray dryer resulted in the isolation of *Salmonella* from the air filter	Collins et al. (1968)
Salmonella Eastbourne	1975	Dust-induced airborne contamination of chocolate	Craven et al. (1975)
Salmonella Agona	1998 & 2008	Long-term persistence of *Salmonella* in the dry environments of the cereal manufacturing plant	CDC (2008b)
Salmonella Wandsworth & *Salmonella* Typhimurium	2007	The initial examination revealed that the puffed rice snack was contaminated with *Salmonella*. The recalls was expanded to include other items containing the same components or processed with the same equipment	CDC (2007c)
Salmonella Schwarzengrund	2007	Two prominent brands of dry dog food related to *Salmonella* contamination manufactured in the same facility	CDC (2007a)
Salmonella Tennessee	2007	Outbreak related to the peanut butter of 2 different brands (Peter Pan & Great Value brand) manufactured in the same facility	CDC (2007b)
Salmonella serotypes Montevideo, Newport, and Senftenberg	2009	Contamination of pistachio nuts and pistachio nut containing products produced in the same facility	CDC (2009b)
Salmonella	2011	Presence of *Salmonella* in the air, broom, floor, and processing equipment of the feed mills	Binter et al. (2011)
E. coli O157:H7	2011	*E. coli* contamination of hazelnuts and hazelnut-containing products, sourced from the same distributor	CDC (2011a)
Salmonella Bredeney	2012	Outbreak related to the peanut butter of 2 different brands manufactured in the same facility.	CDC (2012f)

Table 2.2. (Continued)

Pathogens	Year	Remarks	References
Salmonella Montevideo and *Salmonella* Mbandaka	2013	*Salmonella* infection of tahini sesame paste. To avoid the potential risk of *Salmonella*, subsequent batches produced on the same production line were also recalled.	CDC (2013c)
Salmonella Braenderup	2014	Contamination of almond and peanut butter manufactured in the same plant.	CDC (2014c)
Salmonella Montevideo and *Salmonella* Senftenberg	2016	Pistachios contaminated by the same farms' production.	CDC (2016b)
Salmonella Paratyphi B	2016	Nut butter, sprouted nut butter, and all other items made on the manufacturing line were recalled owing to potential contamination.	CDC (2016c)
E. coli	2016	Recalled various varieties of the flour manufactured at the same plant	CDC (2016d)
Salmonella Typhimurium	2018	Multiple products of dried coconut contamination	CDC (2018c)
Salmonella Newport	2018	The outbreak was linked to two distinct brands of dry shredded coconut manufactured in the same plant.	CDC (2018a)

Closed systems are those used for carrying liquid and solid materials, such as pipelines. It creates a solid-liquid interface for bacterial colonization and is incapable of being cleaned effectively, posing a risk of cross-contamination. Biofilms pose a big concern in closed systems. Open systems, on the other hand, represent solid-air and solid-liquid-air interfaces, facilitating bacterial desiccation in a low-a_w food processing environment (Verran et al., 2008). Microorganisms adhering to food-contact surfaces can survive in the starvation phase without the presence of nutrients. This can raise their pathogenicity and subsequently increase their resistance to additional stresses (Frank, 2001; Lisle et al., 1998). Since production settings have a significant role in cross-contamination, it is essential to follow hygienic zoning within the facilities. It aids in the identification of niches and harborage areas, where prevalent pathogens are found. The zones are determined by possible risk sites (Gupta & Adhikari, 2022).

Cleaning, sanitation, disinfection, and sterilization are some of the different approaches that can be taken to lower the level of microbial contamination (Figure 2.2). Cleaning refers to the removal of surface soils and involves scrubbing, washing, and rinsing food-contact surfaces to get rid of visible contaminants like dust or food particles (Carpentier & Cerf, 2011). Soil tends to accumulate in cracks, crevices, and irregular contact surfaces; however, it can be easily removed from smooth and nonporous surfaces. Soil deposits left on food processing equipment after use are often contaminated with microorganisms that thrive off the soil's nutrients Usually, soap or another detergent and water are used to remove the soil deposits from the contact surfaces. After cleaning, sanitation and or disinfection is done to remove the microorganisms from the contact surfaces (Moerman & Mager, 2016). Sanitation is the reduction of microorganisms to a level deemed safe by the public health guidelines. Disinfection results in the destruction of all the pathogenic microorganisms except spores. Sterilization refers to complete destruction of all life forms and is

the most lethal of all the processes (Block, 2001). There is currently no known antimicrobial agent that is both broadly effective and capable of combining all the necessary properties required for efficient microbial eradication (FSPCA, 2016).

Dry food processing facility generally prefers the use of dry methods for cleaning and disinfection. The addition of moisture could potentially cause product putrefaction and lump formation. The dry food processing environment, in addition to the processing equipment, must also be kept clean. For instance, the production of dust is a common occurrence in operations that involve the handling of dry materials. Dust clouds can be created, when dry ingredients are handled in the normal course of weighing, milling, etc. Pathogens and allergens may be entrapped in process dust, causing cross-contamination (Jones & Richardson, 2004; Moerman & Mager, 2016). This highlights the significance of preventing and clearing away the dust in dry food-handling areas. Foods manufactured on contaminated equipment, compromise the product quality thereby endangering customer health. Moreover, the abrasive nature of the dust particles could cause equipment deterioration. Periodically, wet cleaning is performed with oxidizing antimicrobials, such as chlorine-based agents, peracetic acid, hydrogen peroxide, and quaternary ammonium compounds. Generally, wet cleaning occurs after a production run and before the beginning of another production cycle. It can be accomplished with cleaning in place (CIP) or cleaning outside place (COP). Before beginning the production of a new product, it is essential to thoroughly dry all food-contact surfaces. To control pathogenic microorganisms in food facilities, strict adherence to appropriate hygiene and production control measures are essential (Moerman & Mager, 2016).

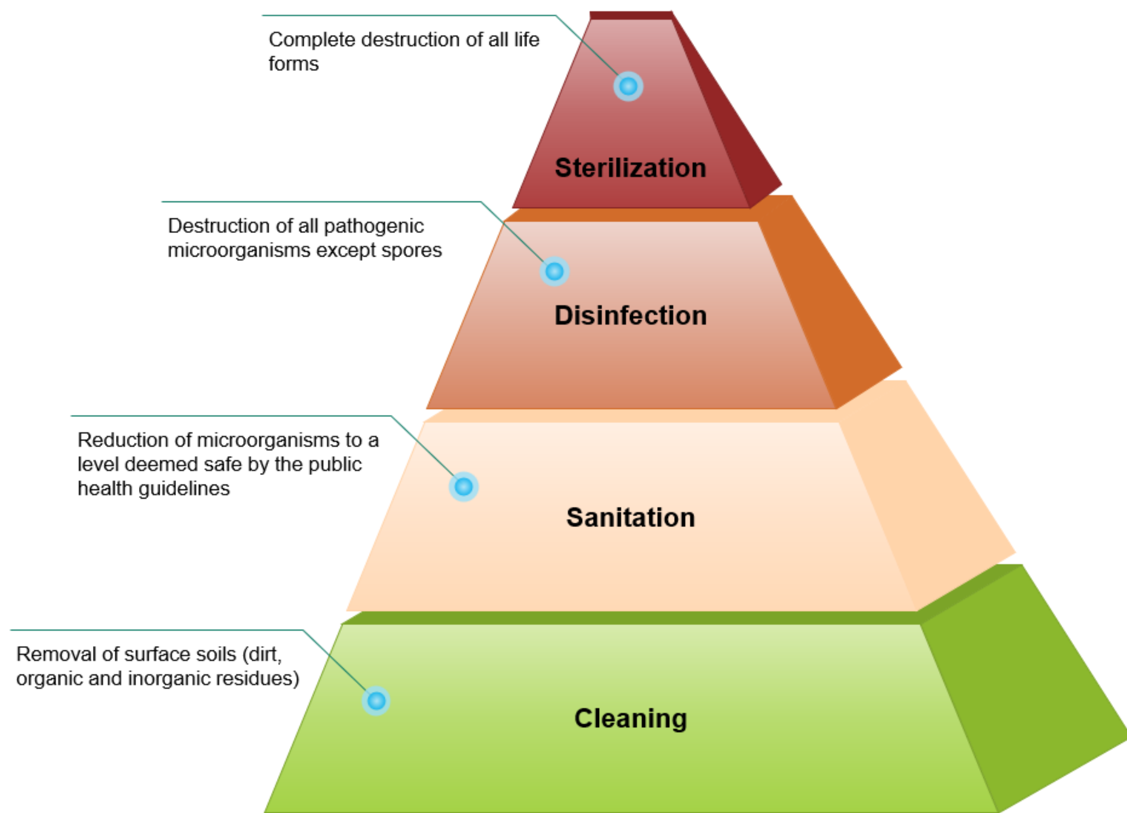


Figure 2.2: Techniques used to reduce the level of microbial contamination in a food processing facility.

2.2.1 Factors influencing the disinfection process in low-a_w food processing environments

Disinfection practices are the cornerstone of any food safety management system. Inadequate disinfection practices may result in foodborne outbreaks (Marriott et al., 2005). The heterogeneous nature of the resident microorganisms on the surfaces of food processing facilities may have an effect on the survival of surface-attached microorganisms (Carpentier & Chassaing, 2004). If microorganisms persist after cleaning, they can replicate and spread, leading to contamination. Several factors have been proven to affect bacterial retention on contact surfaces, including disinfection procedure, bacterial type, presence of organic residues, surface chemistry, type of attachment, and surface free energy of the bacteria and contact surface, thereby reducing the efficacy of the disinfection process (Verran et al., 2008). The presence of viable cells on biotic and abiotic surfaces contributes to their biotransfer potential (Hansen & Vogel, 2011). Moreover, studies have demonstrated that surface-adherent cells are more resistant to disinfectants than planktonic cells (Kastbjerg & Gram, 2009).

2.2.1.1 Biotransfer potential

Bacterial contamination in the dry food industry can occur either through the air or via food-contact surfaces. Airborne dust and aerosols released during the processing of powdered dry ingredients may contaminate subsequent batches of foods manufactured in the same facility (Pérez-Rodríguez et al., 2008). The second type of biotransfer occurs when food-borne pathogens are transmitted from contaminated surfaces to the food itself. The latter form typically occurs when food is contaminated due to contact with unsanitary surfaces or improper handling. Poor manufacturing practices and improper handling of raw materials can introduce pathogenic bacteria into finished products (Pérez-Rodríguez et al., 2008). Zhao et al. (1998) investigated the transfer of *Enterobacter aerogenes* B199A from chickens to cutting boards. The research showed that there

was a total of 5 logs attached to the cutting board, with an additional 3-4 logs contaminating the vegetables. Furthermore, Chen et al. (2001) reported that *Enterobacter aerogenes* B199A had a high biotransfer capability from hands to common kitchen surfaces including cutting boards, lettuce, etc.

There is a risk of biotransfer if exposed surfaces host cells that are alive but not necessarily dividing (Verran et al., 2008). The transferability of various microorganisms in both static and dynamic systems has been thoroughly investigated. The transfer of the surface-attached microorganisms to food varies by strain and is proportional to the viable counts present on the surfaces (Zhao et al., 1998). Kusumaningrum et al. (2003) observed that *S. aureus* (10^3-10^5 CFU/cm^2) dried at 22 °C at 40 - 45% relative humidity survived for at least four days on the stainless steel, but when the inoculum load was lowered to 10 CFU/cm^2, the bacteria reached below the detection limit within two days. The study also reported *S. aureus* dried on stainless steel was more resistant to air-drying than *C. jejuni* and *S. enteritidis*. In addition, the presence of the organic matter, food composition, type of contact surface, temperature, and relative humidity (RH) will impact its transferability (Zhao et al., 1998). Furthermore, Miranda & Schaffner (2016) demonstrated that the food's moisture content significantly influenced the rate at which bacteria transfer from surfaces to the food. For instance, *E. aerogenes* inoculum in tryptic soy broth with nalidixic acid and buffer was dried for 5 h on stainless steel, tile, wood, and carpet. The study reported a greater transfer rate from all the indicated surfaces to watermelon (~0.2 – 97%) regardless of the contact period (1, 5, 30, and 300 s), and that the transfer rate was lowest for the gummy candy (~0.1 – 62%)

Mishandling and improper treatment of food are one of the leading causes of foodborne outbreaks. Hansen & Vogel (2011) observed a higher efficiency of transfer of desiccated non-biofilm cells than desiccated biofilm cells of *Listeria monocytogenes* on stainless steel. Transferability occurs

as a result of the capillary effect and the possible formation of the liquid bridges between the contact bacterium and the food surface. Furthermore, the study found that rehydrating the desiccated biofilm bacterial cells after contact with the salmon led to greater overall water contact, resulting in less transfer than desiccated non-biofilm cells.

2.2.1.2 Presence of persistent and non-persistent strains

Certain strains are capable of surviving in food processing conditions and are repeatedly isolated from similar environments (Carpentier & Cerf, 2011; Larsen et al., 2014). Several outbreaks traced back to the same processing plant have been related to the persistent strains. In 2008, *S.* Give outbreak in infant formula was associated with the contaminated production facility (Jourdan et al., 2008). *S.* Poona has been related to another infant formula outbreak that occurred in 2018-2019 (Jones et al., 2019). Genomic study demonstrated genetic similarity between *S.* Poona isolates collected in 2018–2019 and in 2010–2011, proving the persistence of the pathogen through time (Jones et al., 2019).

Cross-contamination is mostly caused by the occurrence of strains with comparable molecular subtypes, which are associated with persistence (Carpentier & Cerf, 2011; Wulff et al., 2006). Therefore, it is of the utmost importance to comprehend the genetic and physiological behaviour of the persistent strains. A multitude of variables, including the temperature and relative humidity of the manufacturing site play a significant role in microbial persistence. Hiramatsu et al. (2005) observed that *Salmonella* serovars dried on paper disks survived for 24 months when stored at 4 °C. However, the bacteria only survived for 70 days when stored at 35 °C. In another study, Chaitiemwong et al. (2010) observed a greater reduction in *L. monocytogenes* cells on the conveyor belt at 37 °C and 20% RH with or without antimicrobials than at 10 and 25 °C at 60-75% RH. The results suggested that rapid drying at 37 °C led to a greater decrease, whereas delayed drying at 10

and 25 °C led to a greater resistance to inactivation. This implies that the environmental conditions of the processing facility must be properly managed to prevent the eventual persistence of microorganisms. Bashir et al. (2022) assessed the viability of factory, veterinary, and clinical isolates of *Salmonella* on stainless steel after drying. At 37 °C and 20% relative humidity, all the isolates were viable for 48 h; however, at 25 °C and 15% relative humidity, they were viable for 22 days. At 10 °C, only *S.* Senftenberg, *S.* Schwarzengrund and *S.* Typhimurium retained viability for 22 days. It has been reported that higher temperatures cause cellular damage, resulting in a decrease in bacterial survival.

In addition, persistence is also dependent upon the growth phase. Gruzdev et al. (2012) observed that stationary *S.* Typhimurium SL 1344 cells dried at 40% RH and 25 °C for 22 h and subsequently stored at 40-45% RH at 4 °C for 8 weeks on polystyrene had a greater desiccation tolerance than those in the early and mid-log phases. This could be due to the potential formation of the Rpos-stress operon (Navarro Llorens, J. M., Tormo, A., & Martínez-García, 2010). In the same study, the dried *S.* Typhimurium SL 1344 cells grown in Luria-Bertani broth supplemented with 0.5-5% salt conditions showed higher desiccation tolerance and long-term survival at 4 °C, as compared to the cells grown in the absence of salt. It has been reported that salt induces cross-tolerance to a variety of stressors (Gibson et al., 1988).

Certain studies have related the persistence of the strains with their ability to adhere to the surfaces (Norwood & Gilmour, 1999). Lunden et al. (2000) reported that the adherence of persistent poultry plant strains was from 9.1 x 10^2 cells/cm^2 - 1.43 x 10^3 cells/cm^2 within 1 and 2 h. The adhesion of persistent strains was 2.7 - 4.6 times greater than that of non-persistent or commonly termed as sporadic strains. Adherence numbers are crucial from a disinfection standpoint. As reported by Lunden et al. (2000), shorter contact time led to a greater attachment of the strains, indicating that

extending the contact time could pose a food safety risk. The persistence of the organism after sanitation or disinfection suggests that the microorganisms may have created microenvironments and developed resistance to the sub-inhibitory concentration of the disinfectant (Martínez-Suárez et al., 2016). It could also have persisted in harborage places such as nooks or crevices (Carpentier & Cerf, 2011). To minimize the persistence and subsequent cross-contamination of pathogens and to limit the associated risks to food safety (Beuchat et al., 2013), it is essential that the dry food industry adheres to proper disinfection measures.

2.2.1.3 Presence of organic matter

Microbial adhesion to the food processing equipment offers a major risk of pathogen transmission and contributes to foodborne outbreaks (Giaouris & Nychas, 2006). A variety of disinfectants including quaternary ammonium compounds, amphoteric surfactants, and hypochlorous and alcoholic solutions are used for sanitation and disinfection. The presence of organic matter can reduce the efficacy of the disinfection procedure (Kuda et al., 2008; Li et al., 2014). It is essential to comprehend the nature of the food soil or organic matter on food-contact surfaces before and after disinfection. Surface attachment of the bacteria (Kuda et al., 2012; Kuda et al., 2015) and spores (Park et al., 2022) in the presence of organic residues provides a protective effect against the desiccation (Kuda et al., 2008), disinfection, and heat (Leslie et al., 1995). The studies on food residues and desiccation resistance are summarized in Tables 2.3 and 2.4. The degree of microbial adhesion will depend on the presence of organic residues on the contact surfaces. Hingston et al. (2013) reported a significantly ($p < 0.05$) lesser reduction of *L. monocytogenes* on stainless steel, when exposed to desiccation stress for 20 days at 15 °C at 43% RH in the presence of 20% animal lard (3.13 ± 0.12 log CFU/cm^2) than in the control 0% animal lard (3.76 ± 0.17 log CFU/cm^2). Abban et al. (2012) observed that *E. coli* attached to residue-free stainless steel at a higher rate

(5.18 log cell counts) than it did in the presence of chicken residues (1.5 - 2.0 log cell counts). However, the cell counts of dried bacteria without organic residues were reduced by 50.8% following rinsing and spraying, whereas they were reduced by only 5.8-16.2% in the presence of chicken residues. Organic residues provide microbial cells with a barrier under desiccated conditions and decrease the disinfectant's efficacy due to its reduced penetrability (Kuda et al., 2015; Li et al., 2014).

2.2.1.4 Types of the food-contact surfaces

The survival population of microorganisms will vary based on the various food-contact surfaces (Verran et al., 2008). Djebbi-Simmons et al. (2019) observed greater survival of *S.* Typhimurium on the plastic cutting board and laminate than on the stainless steel cutting board (SS). As a hydrophilic material, stainless steel provides a larger contact area, resulting in the rapid evaporation of the inoculum. Cutting board and laminate, on the other hand, are hydrophobic and offer a smaller droplet surface area, resulting in a slower evaporation rate of the bacterial inoculum. Numerous studies attempted to relate the surface characteristics to bacterial adhesion (Boulangé-Petermann et al., 2009). The surface energy parameters and the contact angle of the various food contact surfaces are summarized in the Table 2.5. Many factors, such as increased surface area, greater convection mass movement, and decreased shear stress after cleaning, have been associated with higher bacterial adherence on hydrophobic surfaces (Characklis et al., 1981).

The relationship between roughness values and antibacterial efficacy has been the subject of debate, with some research indicating that lower the roughness characteristics of the abiotic materials, the greater the microbial inactivation and, consequently, increasing the hygienic status (Bower et al., 1996; Park & Kang, 2017). Kim & Kang (2020) investigated the relationship between roughness values (mean arithmetic (R_a) and root mean square (R_q)) and microbial

inactivation utilizing UV-C LEDs. The study reported that dried *S.* Typhimurium, *E. coli* O157:H7, and *L. monocytogenes* were inactivated more effectively on glass, which had lowest R_A (0.0204 ± 0.0005 µm), R_Q (0.0492 ± 0.0099 µm), and surface contact angle (53.04 ± 1.01°), indicating that it is hydrophilic in nature. However, regardless of bacterial species, silicon with the highest R_A (0.96 ± 0.02 µm), R_Q (2.76 ± 0.11 µm) and surface contact angle (118.85 ± 0.03°) exhibited lowest inactivation. However, the relationship between the roughness parameters and microbial inactivation remains controversial. Faille et al. (2000) explored the relationship between the surface topographies and free energies of various stainless-steel finishes and bacterial adhesion. *Bacillus thuringiensis* was dried with an organic food soil (composed of carbohydrates, proteins, fats, and minerals) on stainless steel surfaces with various finishes. They compared various roughness profile characteristics, including levelling depth (R_P), arithmetic mean (R_A), reduced peak height (R_{PK}), reduced valley depth (R_{VK}), and core roughness depth (R_K) amongst others. Generally, the roughness factor of 0.8 µm for stainless steel is regarded as applicable for maintaining equipment hygiene in the food industry (Abban et al., 2012). The study indicated that a single roughness parameter is inadequate for determining the hygienic status of a surface, as R_A (0.15 - 1.56 µm) and R_{PK} (0.21 - 3.49 µm) roughness values differed substantially across the different finished stainless steel surfaces (Faille et al., 2000). Thus, the type of disinfection technique chosen should rely on the type and roughness of the food-contact surface materials.

Table 2.3: Summary of studies demonstrating the desiccation survival of microorganisms with or without the presence of food residues.

Pathogens	Contact surface	Drying conditions	Food sediment	Log reduction	Reference
L. monocytogenes N53-1	Stainless steel	Storage at 15 °C at 43% RH for 91 days	Smoked salmon juice with 5% salt	~4.5	Vogel et al. (2010)
S. enterica	Stainless steel	Storage at 6.5 °C at 60-70% RH for 168 h	Chard (66.8 g/100 mL)	6.26	Posada-Izquierdo et al. (2013)
			Romaine lettuce (66.8 g/100 mL)	7.68	
L. monocytogenes	Stainless steel	Air dried, storage for 30 days at 25 °C	Minced tuna (100 g/100 mL)	~5	Takahashi, Kuramoto, et al. (2011)
			No food residue	> 7	
L. monocytogenes	Stainless steel	Biosafety drying for 120 h	Soy milk (50%)	0.48	Lim et al. (2020)
			No food residue	3.08	
S. Enteritidis	Stainless steel	Biosafety drying for 120 h	Soy milk (50%)	1.83	
			No food residue	4.4	
S. aureus	Stainless steel	Biosafety drying for 120 min	Carrot juice (50%)	< 1	Kuda et al. (2015)
			Distilled water (no residue)	~2	
Murine norovirus-1 (MNV-1)	Stainless steel	Storage for 30 days	Cabbage (100 g/100 mL)	1.4	Takahashi, Ohuchi, et al. (2011)
			No food residues	6.2	
Enterobacter sakazakii	Stainless steel	Biosafety drying for 2 h, and storage at 43% RH at 4°C for 60 days	Infant formula	1.07 - 1.21	Kim et al. (2008)
			No food residues	1.73 - 2.02	
L. monocytogenes	Stainless steel	Drying at 43% RH at 15 °C for 23 days	0.5% NaCl	2.46	Hansen & Vogel (2011)
			5% NaCl	0.88	
Salmonella spp.	Paper discs	Drying for 25 h at 35 °C	No food residues	2.43 – 3.51	Hiramatsu et al. (2005)
		Storage of the dried cells at 4 °C for 22-24 months		< 1	

Table 2.4: Summary of studies demonstrating the survival of microorganisms in the presence of food residues and when treated with disinfectants.

Pathogens	Contact surface	Drying conditions	Disinfection technique	Treatment conditions	Food sediment	Log reduction	Reference
E. coli O26	Stainless steel	Biosafety drying for 90 min	Benzalkonium chloride	2 mg/L, 10 min	Milk	0.39	Kuda et al. (2008)
S. Typhimurium	Stainless steel	Biosafety drying for 120 min	Sodium hypochlorous acid	0.01% w/v, 10 min	Carrot	< 1	Kuda et al. (2016)
S. Typhimurium	Glass	Biosafety drying for 180 min	Benzalkonium chloride	2 mg/mL, 10 min	Whole egg solutions	< 1	Kuda et al. (2011)
S. aureus	Polystyrene	Biosafety drying for 90 min	Benzalkonium chloride	0.5 mg/mL, 10 min	Bovine serum albumin (BSA)	N. D.	Li, Kuda, et al. (2014)
S. aureus	Polystyrene	Biosafety drying for 90 min	Benzalkonium chloride	2.0 mg/mL, 10 min	Milk	1.85	Li, Kuda, et al. (2014)
E. coli	Stainless steel	Biosafety drying for 120 h	Benzalkonium chloride	500 mg/L, 10 min	Soy milk (25%)	1.5	Lim et al. (2020)
S. Typhimurium	Glass	Biosafety drying for 180 min	UV-C (254 nm)	1 min	Egg yolk (15%)	~3	Kuda et al. (2012)

Table 2.5: Surface characteristics of various materials

Materials	Contact angle	Surface energy parameters			References
	θ_w (°)	γ^{LW} (mJ/m^2)	γ^{+} (mJ/m^2)	γ^{-} (mJ/m^2)	
Stainless steel (type 304, P80 finish)	51.8 ± 9.8	ND	ND	ND	Medilanski et al. (2002)
Stainless steel (type 304, diamond-polished)	76.1 ± 10.6	ND	ND	ND	Medilanski et al. (2002)
Stainless steel (type 304, electropolished)	58.9 ± 4.4	ND	ND	ND	Medilanski et al. (2002)
Stainless steel (type 304, #4 finish)	32.0 ± 3.6	37.9	0.5	1.8	Nguyen et al. (2011)
Stainless steel 304	65.8	39.62	0.0	18.43	Zhao et al. (2007)
Stainless steel 316L	48.8	39.0	0.02	36.39	Zhao et al. (2007)
Stainless steel (type 304)	86 ± 2	35.5	0.0	3.8	Meylheuc et al. (2006)
Titanium	42.0	41.32	0.04	41.14	Zhao et al. (2007)
Glass	73.5 ± 3.1	29.6	0.0	20	Simões et al. (2007)
Glass with metal oxide finish (TiO_2)	59 ± 2	ND	ND	ND	Li & Logan, (2004)
Glass with metal oxide finish (Fe_2O_3)	68 ± 5	ND	ND	ND	Li & Logan, (2004)
Glass	12 ± 3	39.9	1.5	51.8	Bayoudh et al. (2006)
Silicone	122 ± 1.8	12.4	0.0	0.9	Simões et al. (2007)
Polyethylene	102 ± 2.4	36.4	0.0	0.6	Simões et al. (2007)
Polypropylene	107 ± 3	28.4	0.0	1.7	Simões et al. (2007)
Polyurethane	80.4	36.34	0.00	7.85	Zhao et al. (2007)
Polyvinyl chloride	95.4 ± 2.9	33.9	0.0	5.8	Simões et al. (2007)

θ_w (°) indicates the contact angle measurement with water.

γ^{LW} , γ^{+}, and γ^{-} indicates the Lifshitz–van der Waals, electron accepting, and electron donating surface energy parameters, respectively.

ND indicates not determined.

2.2.2 Conventional dry disinfection methods for microbial inactivation in low-a_w processing facilities

The effectiveness of the disinfection process is reduced in the presence of organic and inorganic soils. Consequently, a prior cleaning step is required to remove the soiled residues from the food-contact surfaces. Dry or wet procedures may be utilized for cleaning in food processing facilities (Stanga, 2010). Introduction of moisture in the dry food industries can be a source of microbial harborage. Thereby, wet cleaning processes are typically unacceptable in facilities that process dry components or low-a_w foods, as the addition of water can promote bacterial adhesion (Kane et al., 2016). Dry cleaning methods have gained popularity among food manufacturers in recent years due to an increased risk of food-borne contamination. Conventional dry cleaning methods such as brushing, sweeping, scraping, vacuum cleaning, and blowing with compressed air are effective in removing the unwanted dust, dirt, and food deposits from the equipment surfaces, walls, ceilings, and floors of the processing plants. However, these techniques are only advantageous for dislodging the 'caked on' residues from the contact surfaces and are not effective in removing the bacteria or allergens (Moerman & Mager, 2016). Moreover, they are beneficial for cleaning easily accessible locations but may not be able to reach inaccessible areas such as corners and nooks. Inappropriate cleaning methods may also result in equipment deterioration and damage. In addition, they are associated with known constraints, such as the possible generation of dust clouds and the subsequent contamination of the air (Moerman & Mager, 2016). Though dry cleaning cannot guarantee a completely sanitary environment, it can help reduce product quality loss and food safety hazards. A microbiological assessment of the raw materials used, the process characteristics, and the types of food residues determine the frequency of cleaning. It is crucial to follow appropriate cleaning and disinfection procedures to prevent the spread of food-borne

diseases to ensure food safety (Wirtanen & Salo, 2016). This section provides a brief overview of selected conventional and novel dry disinfection methods and their effectiveness in eliminating surface-attached microorganisms in the dry food industry.

2.2.2.1 Alcohol-based disinfectants

Traditional dry cleaning techniques are not appropriate for all low-a_w foods, such as nut products with a high-fat content (Grasso et al., 2015) and insufficient sanitation practices may result in microbiological cross-contamination.

Alcohols are generally used for surface disinfection due to their antimicrobial properties; they permeate the cell membrane and denature proteins resulting in the lysis of the cells. Quaternary ammonium compounds (QUATs) possess bactericidal and fungicidal properties. They are positively charged cations that are attracted to negatively charged cellular components. They function by causing damage to the cell membrane and result in lipid and protein denaturation and cell death (Pereira & Tagkopoulos, 2019). It is possible to improve the microbial inactivation efficacy by combining QUATs and alcohols (Kane, 2012). IPAQuat (isopropyl alcohol quaternary ammonium) formulations have a relatively modest concentration of quaternary ammonium compounds along with a varying concentration of isopropyl alcohol. Alcohol-quaternary formulations are non-corrosive and suitable for disinfecting non-porous surfaces (Grasso et al., 2015; Kane et al., 2016). Moreover, carbon dioxide (CO_2) can be used as a propellant for IPAQuat spray application. CO_2 displaces oxygen and renders it non-flammable, hence eliminating the potential of a fire hazard (Kane, 2012). Kane et al. (2016) studied the effectiveness of IPAQuat-CO_2 (200 ppm, 58.6% isopropyl alcohol) spray for eliminating *Salmonella* cells dried (16-18 h) on stainless steel. An IPAQuat-CO_2 exposure for 30 s resulted in a reduction of the dried *Salmonella* cells by 6.18 log CFU/25 cm^2. However, *Salmonella* dried in the presence of

breadcrumbs were reduced to below the detection limit (0.70 log CFU/25 cm^2) after 30 s of the IPAQuat treatment.

IPAQuat formulation does not require water and has a high evaporation rate, making it appropriate for disinfecting dry food processing plants. The IPAQuat's active quaternary ammonium component generates an antimicrobial film for surface sanitation. The concentration of the QUATs can be controlled to avoid an excessive buildup of the residual film on the contact surfaces (Kane et al., 2016). Prior to the application of IPAQuats, food soil must be eliminated through cleaning (Moerman & Mager, 2016). Grasso et al. (2015) followed a 2-step cleaning and sanitation procedure to decontaminate a *Salmonella* infected (7.4 ± 0.4 log CFU/g) peanut butter stainless steel processing equipment. A hot oil cleaning alone at 90 °C for 2 h resulted in 0.25 ± 1.12 log CFU/cm^2 of *Salmonella* survival counts. However, a 2-step combination of cleaning using hot oil (90° C for 2 h), followed by disinfecting using isopropanol with quaternary ammonium compounds (60% for 1 h) resulted in a 5-log reduction of *Salmonella* from the various sampling sites of the contaminated equipment. Moreover, the disinfection efficacy of IPAQuats will vary based on the type of surface. Du et al. (2007) studied the effectiveness of different cleaning and disinfection treatments in reducing the aerobic load from the typical surfaces found in the almond huller-sheller facilities. A combination treatment of blowing air for 30 s followed by an IPAQuat (200 ppm, 58.6% isopropyl alcohol) spraying for 60 s resulted in a higher reduction of the aerobic counts by 2.1 and 3.6 log CFU/cm^2 from the conveyor belt and galvanized steel respectively. Limited research has been conducted using IPAQuats for the eradication of microorganisms from the dry food industry.

2.2.2.2 Chlorine dioxide gas fumigation

Chlorine dioxide (ClO_2) gas is a non-thermal technology used for equipment sanitation (Park & Kang, 2018). It is a synthetic reddish-yellow gas (Morino et al., 2011; Sun et al., 2019), that functions as a potent biocidal disinfectant (Yeap et al., 2016), by removing an electron from electron-rich sites on biological molecules and subsequently being reduced to chlorite ion (ClO_2^-). It penetrates the bacterial cell membrane and causes lipid peroxidation, protein denaturation, and DNA damage. The oxidation capacity of ClO_2 is 2.5 times more than that of liquid chlorine (Bang et al., 2014). Extensive research has been undertaken on the use of ClO_2 gas to disinfect food products and remove biofilms from contact surfaces (Trinetta et al., 2012). It works over a broad pH range (3.0 - 8.0) (Sun et al., 2019) and prevents the formation of chloramines and other halogenated organic compounds (Li et al., 2012). Moreover, its fast action, and on-site production (Thorn et al., 2013) make it appropriate for use in the dry food industry disinfection. The investigations on the usage of ClO_2 gas for the dry microorganisms on the food-contact surfaces are summarized in Table 2.6.

It is generally recommended that the equipment used in dry procedures should not be wet cleaned, except under specific conditions. ClO_2 in gaseous form penetrates uneven surfaces more effectively than aqueous chlorine dioxide (Montazeri et al., 2017). The application of aqueous chlorine in the dry food industry is not preferred as it can introduce unwanted moisture if it is not drained properly, resulting in microbial growth. Gaseous ClO_2 has greater diffusivity and produces lesser toxic residues, leading to a greater reduction of pathogenic microorganisms (Nam et al., 2014). Amino acids are oxidized by ClO_2 according to pseudo-first-order kinetics, which impedes protein synthesis. Furthermore, the oxidative stress coupled with respiration inhibition causes cell destabilization (Sun et al., 2019).

Table 2.6: Summary of studies demonstrating the use of ClO_2 in inactivating microorganisms dried on food contact surfaces.

Pathogens	Contact surface	Drying conditions	ClO_2 gas parameters	Log reduction	References
S. Typhimurium	Stainless steel	Biosafety drying for 1 h	20 ppmv, at 15 °C for 30 min	< 1	Park & Kang (2018)
			20 ppmv, at 25 °C for 30 min	1.5 - 2.0	
L. monocytogenes	Stainless steel	Biosafety drying for 2 h	2 mg/L for 10 min	3.8	Trinetta et al. (2012)
E. coli O157:H7	Polyvinyl chloride	Biosafety drying for 1 h	20 ppmv for 15 min	3.0	Park & Kang (2017)
Bacillus subtilis	Glass	Biosafety drying for 12 h	0.080% for 3 h	> 6.5	Li et al. (2012)
	Stainless steel			< 5	
Bacillus thuringiensis	Wood	Biosafety drying for 3 h	5 mg/L under 85-92% RH for 12 h	3.6	Han et al. (2003)

The effectiveness of ClO_2 gas depends on intrinsic parameters such as roughness (Park & Kang, 2017) and hydrophobicity, as well as extrinsic parameters such as temperature (Park & Kang, 2018), contact time, and gas concentration (Byun et al., 2021). Moreover, pre-humidification and the state (wet and dry) of the microbial culture are crucial to the inactivation efficacy of ClO_2 gas (Morino et al., 2011). In a study conducted by Yeap et al. (2016), the stainless steel coupons were contaminated with murine norovirus 1 (MNV-1). Prior to ClO_2 treatment, coupons were preconditioned at 85% RH at 25 °C for 10 min. ClO_2 treatment at 2.5 mg/L for 2 min resulted in a 3 log reduction of the virus. Moreover, no infectious virus was recovered when the dosage was increased to 4 mg/L. Morino et al. (2009) investigated the efficacy of a low concentration ClO_2 (0.08 ppm) at 45-55% against feline calicivirus (FCV) in the dry and wet states on glass. The wet condition resulted in a greater reduction of > 6 log after 6 h, while the dry state resulted in a smaller reduction of < 2 log after 48 h. In another study conducted by Morino et al. (2011), ClO_2 at 0.05 ppmv for 4 h was used to treat suspension cultures of *E. coli* and *S. aureus* on glass surfaces. The reduction of *E. coli* (> 5.0 log reduction) was greater than that of *S. aureus* (> 2 log reduction).

The surface characteristics influence the effectiveness of ClO_2 gas against microbial inactivation (Park & Kang, 2017). In general, hydrophilic surfaces allow for a more uniform attachment and distribution of microorganisms than hydrophobic surfaces, resulting in a higher level of inactivation. Park & Kang (2017) evaluated the efficacy of a 15 min ClO_2 treatment at 20 ppmv against *Salmonella* Typhimurium on a variety of food-contact surfaces. The treatment of the dehydrated cells was conducted under humidified conditions (90% RH). Compared to hydrophobic materials such as silicon (0.96 ± 0.45) and rubber (1.32 ± 0.32), the high hydrophilicity of glass resulted in a greater reduction of > 6.76 log. The research indicated that the dosage or duration of ClO_2 treatment should be increased under dry conditions in order to get a greater microbial

reduction. ClO_2 gas has several drawbacks, including its instability at higher concentrations, its difficulties in handling and transportation, and its high cost for on-site generation and mixing (Sun et al., 2019).

2.2.2.3 Ozone gas fumigation

Ozone is a colourless, highly reactive gas with strong antioxidant and biocidal properties (Moore et al., 2000). It is composed of three singlet oxygen atoms covalently linked together (Torres-Mata et al., 2022). Ozone can be produced photochemically by bombarding an oxygen-containing gas with ultraviolet light, or by passing air through a corona discharge, utilizing a high-energy electric field to trigger the creation of free radicals (Oliveira et al., 2020). Ozone is an unstable oxygen allotrope with a half-life of nanoseconds (Canut & Pascual, 2008). It auto decomposes, produces oxygen, and equilibrates with air, so it neither leaves behind residual by-products (Sujayasree et al., 2022) nor increases the water salinity. In addition, it minimizes the cost of sewage disposal by oxidizing organic material and facilitating its biodegradation, hence minimizing surface deterioration and environmental impact (Canut & Pascual, 2008). Ozone can be administered in either its gaseous or aqueous (ozonated water) forms (Sujayasree et al., 2022). Gaseous ozone provides an alternative sanitation technology to chemical sanitizers. With an oxidation-reduction potential (ORP) of +2.07 V, ozone is a suitable alternative to chlorine's lower ORP of +1.49 V (Cullen & Norton, 2012). Numerous studies have highlighted the advantages of gaseous ozone over conventional disinfection procedures, as it does not include water and hence eliminates the need for rinsing (Canut & Pascual, 2008). It can be effectively used to inactivate the airborne microorganisms generated during the handling of the dust clouds. It is spreadable, and accessible and it can easily penetrate the cracks and crevices of the contact surface (Volkoff et al., 2021). Moreover, ozone can expand and take up the volume of the whole room, making it advantageous

over other surface treatments such as UV light (Torres-Mata et al., 2022). Nevertheless, the efficiency of ozone as a disinfectant is much enhanced at higher humidity levels (Cullen & Norton, 2012) and the magnitude of the bactericidal activity will be reduced at lower humidity levels.

The surface disinfection efficiency of the ozone is dependent on pH, temperature, ozone concentration, treatment time, surface type and the presence of organic residues (Torres-Mata et al., 2022). It can be generated on-site and requires no additional handling and storage (Canut & Pascual, 2008). Generally, ozone at a higher concentration is effective for antimicrobial action (Candia et al., 2015). However, the major disadvantage of ozone is its potential toxicity, which can have detrimental impacts on contact surfaces and human health (Tseng & Li, 2008). In its gaseous form, ozone is more harmful than when it is present in water. Inhalation of ozone might cause peripheral vasoconstriction (Mascarenhas et al., 2021). The recommended threshold for ozone exposure is 0.1 ppm (OSHA). Degassing is necessary before entry or human exposure, and adequate implementation or application procedures are required (Volkoff et al., 2021). Therefore, sufficient time must be allowed for ozone breakdown prior to entry.

Ozone has been the subject of numerous investigations due to its broad spectrum of antimicrobial potential against bacteria (Candia et al., 2015), viruses (Volkoff et al., 2021; Tseng & Li, 2008), and mycotoxin degradation (Sujayasree et al., 2022). Ozone has strong penetrating power and destroys cells by removing hydrogen atoms from carbon-carbon bonds, hence causing metabolic interferences and cell lysis. Furthermore, the generation of reactive species upon ozone decomposition affects cellular metabolism by targeting membrane glycolipids and glycoproteins and further oxidizes protein and lipid components, resulting in cellular leakage (Candia et al., 2015). Kim & Yousef (2000) investigated the efficacy of ozone against airborne and surface-

attached *Pseudomonas aeruginosa*. Ozone (2 ppm) exposure for 2 h resulted in 2 log reduction in the number of cells of both types.

Gas phase application of the ozone can be used for the surface disinfection of the dry food industry. The dried cells of *Escherichia coli*, *Shigella liquefaciens*, *Listeria innocua*, *Rhodotorula rubra*, and *Staphylococcus aureus* on stainless steel had their microbiological viability reduced from 7.56 to 2.41 log values after being treated with ozone at 2 ppm for 4 h at 77% RH (Moore et al., 2000). Bailey et al. (2007) reported that *Micrococcus luteus* on stainless steel was reduced by 2-3 logs after being treated with 2 ppm ozone for 1 h at 50% RH. While increasing the humidity levels has been shown to boost antibacterial efficacy, however, it would introduce unwanted moisture into the dry food manufacturing sector. Candia et al. (2015) demonstrated the efficacy of low-concentration gaseous ozone treatment (1.07 mg m^{-3}) in inactivating the dried cells of *Listeria monocytogenes*, *E. coli*, *S. aureus* on the stainless steel, glass, polystyrene and polypropylene, and observed no viable cells after 6 days of the treatment at 4 °C. Dubuis et al. (2020) discovered that gaseous ozone treatment at 0.05 ppmv for 30 min led to the inactivation of the SARS-CoV-2 virus by causing damage to the viral capsid protein (Tseng & Li, 2008).

Ozone is approved as an antimicrobial addition by the US Food and Drug Administration (FDA) and has the GRAS (generally regarded as safe) affirmation for use in food-contact applications (O’Donell et al., 2012). Moreover, European Union has authorized the use of ozone for the treatment of wastewater (Moore et al., 2000).

2.2.2.4 Superheated steam

The use of superheated steam (SHS) for surface disinfection has only been the subject of a small number of studies, despite its widespread investigation as a dehydration method (Park et al., 2022).

SHS is a promising technology that can be implemented as a dry disinfection tool in low-a_w food processing industry (Kim, Park, et al., 2019). SHS is produced by raising the temperature of the saturated steam by supplying sensible heat at a constant pressure (Park et al., 2022). SHS can inactivate vegetative cells, spores, and biofilms (Kim et al., 2020).

SHS is non-polluting and does not require the use of chemicals. It is advantageous for surface disinfection, as it offers a higher heat transfer to the contact surface, and upon condensation, raises the temperature of the contact surfaces thereby providing a better latent heat transfer (Kim et al., 2020). In addition, it is safe and results in a lower quality loss. The distinctive advantage of SHS over saturated steam is that a slight decrease in temperature does not result in condensation. Moreover, SHS has higher efficiency and better penetration into cracks and crevices and does not produce toxic by-products. High temperature denatures the cytoplasmic protein and induces membrane fluidization resulting in cell death (Moerman & Mager, 2016). Kim et al. (2020) reported that SHS treatment (250 °C for 1 min), reduced *B. cereus* inoculated on the stainless steel by 0.68 ± 0.27 log CFU. At temperatures > 250 °C, spores were eliminated within 1 min. The combined efficacy of UV-C (15 min) followed by SHS (250 °C, 1 min) offered a synergistic effect and reduced spores by 2.30 ± 0.50 log CFU.

The organic residues on the contact surfaces must be removed by a thorough cleaning. Inadequate cleaning prior to the SHS exposure might result in baked-on residues. Kim et al. (2020) investigated the effect of food composition on the antimicrobial effectiveness of SHS against *B. cereus* spores. SHS (161 ± 1° C) resulted in lower *D*-values (46.53 ± 4.48 s) for a low-fat peanut butter (6% fat and 55% moisture) and a higher *D*-value (79.21 ± 14.87 s) for a high-fat peanut butter (43% fat, 10% moisture). On the contrary, non-fat dry milk and whole milk powder had lower *D*-values of 24.73 ± 6.78 s and 34.38 ± 20.08 s, respectively. Kim et al. (2020) further

investigated the efficacy of SHS treatment for removing food residues from aluminum foil. The study showed that the ease of removing food residues depended on the food composition and the duration of SHS exposure. SHS (161 ± 1 °C, 30 s) resulted in the residual weight removal of 99.07 ± 0.15% of peanut butter (50% fat, 18% carbohydrate) as compared to the 36.22 ± 2.88% residual weight removal of the non-fat dry milk (0% fat, 52% carbohydrate). Due to the impact of food components on surface adherence, SHS treatments must be preceded by adequate cleaning. Park et al. (2022) studied the efficacy of the SHS treatment in inactivating *Geobacillus stearothermophilus* inoculated along with wheat flour on various food surfaces (stainless steel, rubber, and concrete). The study reported a higher heat transfer and temperature increase in the concrete, which resulted in rapid drying of the wheat flour, resulting in a lesser reduction of *G. stearothermophilus*. On the contrary, irrespective of the treatment temperature, a higher reduction of *G. stearothermophilus* was observed on the stainless steel. There is a lack of research into the potential of supersaturated steam for disinfection application in the dry food industry.

2.2.3. Novel dry disinfection methods for microbial inactivation in low-a_w processing facilities

2.2.3.1 UV light disinfection

UV has a broad electromagnetic spectrum ranging from 100 to 400 nm. It can be divided into UV-A, UV-B, UV-C and UV-vacuum categories. UV-C has the maximum germicidal potential, it causes the alteration of DNA/RNA at 254 nm (Haughton et al., 2011). The U.S. Food and Drug Administration has approved ultraviolet 254 nm as a non-thermal decontamination technology for disinfecting food-contact surfaces (Lim & Harrison, 2016; Sommers et al., 2010), and for treating surface microorganisms on food products (Park et al., 2015). It is also commonly utilized for its ability to disinfect the air and water.

UV triggers the transition from the ground state to the excited state of a molecule. Its mode of action has been attributed to numerous mechanisms including (Haughton et al., 2011);

(a) Fluorescence, wherein the molecule returns to its ground state by emitting a photon.

(b) Phosphorescence, which indicates that the molecule will maintain its excited state.

(c) Internal conversion, in which heat is lost as the medium returns to its initial state.

(d) Chemical conversion, by altering the chemical structure of the molecules such as DNA/RNA.

UV has been primarily linked to the photochemical modification of pyrimidine bases, which leads to the formation of dimers between successive pyrimidines in a DNA strand (Haughton et al., 2011) and results in the formation of a DNA bend, which in turn inhibits the action of DNA polymerase, thereby blocking transcription and replication and causing cell death (Calle, et al., 2021; Lim & Harrison, 2016). It inactivates the pathogen, rendering it incapable of reproduction. UV application has many benefits, including the absence of odours and harmful residues, low costs, ease of use, and lack of regulatory constraints and limits (Yoon et al., 2018). It is chemical- and heat-free and relatively inexpensive (Haughton et al., 2011). Table 2.7 provides a summary of the studies demonstrating UV light's effectiveness in inactivating the dry microorganisms from the food-contact surfaces.

Table 2.7: Summary of studies demonstrating the use of UV light in inactivating the microorganisms dried on food contact surfaces.

Pathogens	Contact surface	Drying conditions	UV exposure conditions	Log reduction	References
S. enterica	Stainless steel	Biosafety drying for 90 min	UV-C light (254 nm) at 656 μW/cm^2 for 5 s (3.3 mJ/cm^2)	2.75	Lim & Harrison (2016)
	High-density polyethylene			2.93	
	Waxed cardboard			1.39	
	Polyvinyl chloride			1.91	
S. enterica	Stainless steel 304 hairline	Biosafety drying for 4 h	UV-C (254 nm) at 15 W for 0-180 s	>4	Gabriel et al. (2018)
S. Typhimurium	Stainless steel	Air drying for 30 min	UV-C (254 nm) at 250 μW/cm^2 for 3 min	4.35	Kim et al. (2002)
E. coli O157:H7				5.2	
Salmonella spp.	Electroplated stainless steel	Biosafety drying for 30 min	UV-C (254 nm) at a dose of 0.20 J/cm^2	3.34	Sommers et al. (2010)
L. monocytogenes				2.89	
S. aureus				2.58	
L. monocytogenes	Polyurethane	Biosafety drying for 30 min	UV light (254 nm) at 5.53 mW/cm^2 for 3 s	4.97	Morey et al. (2010)
S. Typhimurium DT104	Stainless steel	Biosafety drying for 30 min	UV (253.7 nm) at 0.236 ± 0.013 mW/cm^2 for 30 min	0.82	Bae & Lee (2012)
	Polypropylene			1.62	

UV disinfection has several disadvantages, including low penetrability and a shadowing effect (Lim & Harrison, 2016). Conventional UV lights utilize UV mercury lamps, but they operate at high voltages and are frequently linked to the formation of ozone, which is hazardous to human health. Ultraviolet light emitting diodes (UV-LEDs) are therefore becoming increasingly popular. Calle, et al., 2021 examined the effectiveness of UV-C LED (250-280 nm) at 2 mW/cm^2 (50%) and 4 mW/cm^2 (100%) for 60 s against *Salmonella* attached to stainless steel and high-density polyethylene (HDPE). The results from the study showed that treatment with 50% irradiance resulted in 1.97 log CFU/cm^2 and 1.25 log CFU/cm^2 reduction of *Salmonella* on stainless steel and HDPE, respectively. Increasing the irradiance to 100% caused a higher reduction of *Salmonella* by 3.48 log CFU/cm^2 and 1.77 log CFU/cm^2 on stainless steel and HDPE respectively. The antibacterial efficacy of UV light against foodborne microorganisms adhered with the food residues on contact surfaces has been widely investigated. Kuda et al. (2012) studied the antibacterial effectiveness of UV-C (254 nm) on dried suspensions of *Salmonella* Typhimurium and *Staphylococcus aureus* soiled with 1.5-15% w/v egg albumen, 1.5-15% yolk, or 3.0-30% whole egg solutions on glass surfaces. It was observed that the food sediments had a protective effect on the bacteria against drying and UV-C treatment.

Certain microorganisms, including *E. coli*, *Salmonella* and *Shigella dysenteriae*, can repair UV-induced DNA damage by photoreactivation. The photolyase enzyme reverses the thymine dimerization mutation in the presence of sunlight. Additionally, certain species are capable of independent repair without light; endonucleases can cleave the altered DNA, and polymerase can then synthesize a new DNA fragment. Finally, ligases can join these fragments together (Lim & Harrison, 2016).

2.2.3.2 Cold plasma

Cold plasma also referred to as the fourth state of matter (Katsigiannis et al., 2021) is an emerging non-thermal technology used for the disinfection of bacteria (Gabriel et al., 2018), virus (Park & Ha, 2018) and spores (Butscher et al., 2016). Gas, when subjected to a source of energy, dissipates and creates free radicals, reactive oxygen (O, $^{1}O_2$, $^{\bullet}OH$, O_3, H_2O_2) and nitrogen species (N, NO_3^-, NO_2^-, $^{\bullet}NO$, $ONOO^-$, HNO_3), electrons, photons, neutral and charged particles, They are primarily produced through the collision of high-energy electrons with heavy particles such as atoms, molecules, and ions (Dasan et al., 2017; Katsigiannis et al., 2021).

The antimicrobial mechanism of plasma involves multiple mechanisms, including thymine dimerization, inhibition of replication in response to UV-induced DNA damage, and surface etching of the cell membrane due to the accumulation of excessive charge (Katsigiannis et al., 2022; Sen & Mutlu, 2013). It leads to the abstraction of the H-atoms resulting in cleavage of C–O, C–C, and C–N bonds and production of C=O bonds (Leipold et al., 2010). In addition, ROS can disrupt the peptidoglycan linkages of the outer membrane leading to lipid peroxidation, protein denaturation and DNA damage thereby contributing to cell death (Dasan et al., 2017). ROS-generated oxidative stress additionally leads to the inactivation of microorganisms (Sen & Mutlu, 2013).

Microbial inactivation will differ depending on the plasma source, such as dielectric barrier discharge (DBD), plasma jet, gliding arc, microwave-based discharge, corona discharge, or glow discharge (Gonzalez-gonzalez et al., 2021; Katsigiannis et al., 2021). DBD is appropriate for surface disinfection due to the fact that it is a direct treatment that can disinfect larger surfaces, is inexpensive and scalable, and can be operated under ambient conditions (Katsigiannis et al., 2021).

The treatment with DBD plasma for 340 s reduced *L. innocua* inoculated on spinning knives by more than > 5 log (Leipold et al., 2010).

Several other parameters, including voltage, frequency, flow rate, structural properties of the contact surface, and treatment time, influence the effectiveness of cold plasma in inactivating foodborne microorganisms (Timmons et al., 2018). The inactivation efficacy of cold plasma reduces as the distance between the plasma source and the sample increases. *E. coli* surface dried for 1 h on polyvinyl chloride followed by 5 min of air DBD plasma treatment at spacings of three, four, and five centimetres correspondingly led to reductions in the germicidal efficacy of 99.99%, 99.1%, and 98.9%, respectively (Miao & Yun, 2011). The efficacy of this technique also depends on the type of soil accumulated on the contact surfaces (Lis et al., 2018). Gonzalez-Gonzalez et al. (2021) examined the inactivation efficacy of the piezoelectric plasma applied at 10 and 20 mm distances, for inactivating *Salmonella* and *Listeria* inoculated on stainless steel surfaces with or without protein residues. The results demonstrated better protection of both bacteria in the presence of the protein soils. This could be due to the protection against the oxidative stress provided by the protein water complex, as well as the probable quenching of the plasma species in the presence of food soils, resulting in a reduction of bactericidal effectiveness (Lis et al., 2018). The plasma inactivation efficacy will also depend on the type of the microorganisms (Katsigiannis et al., 2022). Laroussi et al. (2003) observed a higher disruption of the *E. coli* cell membrane, as compared to *Bacillus subtilis* cell membrane, which remained unaffected. Several investigations have shown that gram-negative cells are more susceptible to plasma inactivation than gram-positive cells because their thin cell wall is easily ruptured, resulting in a leakage of the intracellular components (Katsigiannis et al., 2022).

Cold plasma can be used as a dry disinfection treatment. Several studies have documented the decontamination potential of cold plasma on industrially relevant contact surfaces (Table 2.8). Aboubakr et al. (2020) investigated the effectiveness of dry and moist exposure of DBD plasma against *Salmonella enterica* inoculated on stainless steel. The addition of water resulted in a reduction of ~ 6.5 logs after 3 min of treatment. However, the dry conditions caused a lower reduction of 2.5 log after 10 min of plasma exposure. It was observed that bacteria treated under dry conditions were more resistant to inactivation as compared to wet bacteria. Sen & Mutlu (2013) assessed the efficacy of radio frequency plasma treatment using air, O_2, N_2, and water vapour (H_2O, RH 95%) against the inactivation of *E. coli* K12 dried on stainless steel. *D*-values observed were the least when water vapour (12.9 min) was used as the working gas, followed by air (22.8 min), nitrogen (37.1 min) and oxygen (62.8 min). Ionization of water molecules into H^- and $^\bullet OH$ radicals (Eq. 2.1) in the plasma chamber usually leads to lower *D*-values and, thus, greater inactivation.

$$H_2O + e^- \rightarrow {}^\bullet OH + H^- \quad (2.1)$$

Cold plasma offers a broad spectrum of antibacterial activity and is environmentally safe (Katsigiannis et al., 2021). As a non-thermal technique involving low-to-moderate temperatures, it does not degrade the product characteristics, making it superior to conventional processes. It provides surface treatment and can be effectively applied for the treatment of temperature-sensitive products. Moreover, as opposed to chemical procedures, there is no production of hazardous residues (Butscher et al., 2016; Leipold et al., 2010). However, the transition from lab scale to industrial scale is challenging due to the equipment size, working gas, relative humidity conditions, and microbial resistance (Katsigiannis et al., 2022).

Table 2.8: Summary of studies demonstrating the use of cold plasma in inactivating microorganisms dried on food contact surfaces.

Pathogens	Contact surface	Drying conditions	Plasma type	Plasma exposure conditions	Log reduction	References
E. coli	Stainless steel	Biosafety drying for 30 min	Surface micro-discharge (SMD) plasma	Air (90% RH, 5 SLPM), for 20 min	4.13	Lis et al. (2018)
S. aureus					3.38	
S. enterica	Stainless steel	Biosafety drying for 4 h	Atmospheric pressure plasma jet system	Air (5 SLPM), for 14 s	≥ 6	Gabriel et al. (2018)
E. coli	Stainless steel	-	Atmospheric pressure plasma jet system	Air (12 SLPM) for 90 s	3.40	Cahill et al. (2014)
	Polypropylene				3.40	
S. Typhimurium	Stainless steel	Biosafety drying for 1 h	Piezoelectric cold atmospheric plasma (CAP)	15 V, 50 kHz, Air for 300 s at 10 mm distance	3.5	Gonzalez-gonzalez et al. (2021)
S. enterica	Glass	Biosafety drying for 1 h	Surface dielectric barrier discharge (SDBD)	7 kV, 13.5 V, Air, 1 cm distance, 4 min	3.0	Timmons et al. (2018)
S. epidermidis	Stainless steel	1–2 h drying at 35 °C	Gliding arc discharge (GAD)	Nitrogen (0.5 m^3/h) for 5 min	3.94	Dasan et al. (2017)
E. coli					3.65	
E. coli	Wood chopping board	Biosafety drying for 30 min	Atmospheric dielectric barrier discharge (DBD) plasma	Nitrogen (1.5 SLPM) for 60 min	1.6	Kim et al. (2021)

2.2.4. Conventional wet disinfection methods for microbial inactivation in low-a_w processing facilities.

Adequate sanitation and or disinfection practices are needed to control the entry of microorganism in the dry food industry. However, the dry food facilities prefer dry disinfection methods over wet disinfection methods. The greatest difficulty associated with dry disinfection techniques is that they may not eliminate all food soils and surface-attached microorganisms (Margas et al., 2014). This necessitates the periodic application of wet disinfection techniques. Cleaning and disinfection operations require the use of toxic-free processes. Common chemical agents used for equipment disinfection, include peracetic acid, hydrogen peroxide, ozone, chlorine, chlorine dioxide, sodium hypochlorite and quaternary ammonium compounds. However, large amounts of water are needed for surface cleaning in these operations to get rid of any leftover disinfectants. Moreover, bacteria may develop resistance to the overuse of disinfectant due to chromosomal gene mutation (Skåra & Rosnes, 2016). Following is some of the selected conventional and novel wet disinfection methods, which can be used in the dry food industry.

2.2.4.1 Quaternary ammonium compounds

Quaternary ammonium compounds (QACs) are the slow-acting (Al-Qadiri et al., 2016) cationic surfactants, extensively used for cleaning and disinfection. They are composed of a positively charged nitrogen centre and are surrounded by the aryl or alkyl groups. Hydrophobic cations in QACs bond with acidic phospholipids in microbial cell membranes, thereby preventing the uptake of nutrients and the efflux of waste products (Loyawattananan, 2020). A maximum concentration of 200 mg/L of QAC-based sanitizers is permitted in food processing facilities (U.S. Food and Drug Administration, 2016). QACs have foaming properties, are colourless, odourless, and effective at a broader pH range (Al-Qadiri et al., 2016). They are neither corrosive nor irritant to

the skin, and possess superior germicidal and wetting properties (Hui, 2014; Marriott & Robertson, 1997).

The antimicrobial efficacy of QACs depends on the microorganism. Al-Qadiri et al. (2016) observed that 200 mg/L QUAT reduced *Listeria monocytogenes* and *Staphylococcus aureus* by > 5 log, whereas *Salmonella* Typhimurium and *E. coli* inoculated on wooden cutting boards were reduced by < 5 log. Gram-positive bacteria due to the absence of an outer membrane are vulnerable to QAC-based disinfectants. In another study, Mustpha & Liewen (1989), QAC (50 ppm for 1 min) effectively reduced the number of *Listeria monocytogenes* cells dried for 1 h and 24 h on stainless steel by > 4 log.

The disinfection efficacy of QACs will be affected in the presence of organic residues (Kuda et al., 2008). Li et al. (2014) evaluated that benzalkonium chloride treatment at 0.5 mg/L for 10 min reduced *S. aureus* dried on the stainless steel to below the detection limit; however, a lesser reduction of 0.28 logs was observed in the presence of milk sediments. It was reported that organic layers can block chemicals from entering the cytoplasm of cells.

For surface disinfection, the QACs should be employed at the appropriate concentration (Moerman & Mager, 2016). However, increasing the disinfectant concentration may not necessarily have a linear relationship with bacterial inactivation (Ríos-Castillo et al., 2018) and can promote antibiotic resistance. Benzalkonium chloride (1% for 30 min) applied at 1:1, 1:2, and 1:4 against *E. hirae* inoculated on the stainless steel, resulted in a reduction of 6.30 ± 0.11, 6.31 ± 0.08, and 5.82 ± 0.10 log, respectively. The chemical properties of the QACs permit their adhesion to the contact surfaces and give a lasting biocidal effect. Ríos-Castillo et al. (2018) dried a mixture of benzalkonium chloride 1.0% + NaOH (0.20%) + NaClO (1%) on stainless steel for 1 h and 24 h and evaluated its immediate and long-term antibacterial efficacy against *S. aureus*. Disinfectants

dried for 1 h and 24 h resulted in a greater reduction of 6.74 ± 0.19 and 6.89 ± 0.13 log, respectively.

Moreover, the germicidal efficacy of QACs will vary depending on the formulations' pH and concentration of the active ingredient. Kim et al. (2007) observed that regardless of the strain type, QAC (pH 12.04) having alkyl dimethyl benzyl ammonium chloride and n-alkyl dimethyl ethylbenzyl ammonium chloride as a microbicide reduced dry *Enterobacter sakazakii* cells on the stainless steel to < 1.48 log CFU/coupon after 1 min of treatment. The study postulated the significance of highly alkaline conditions in rupturing the cell membrane and causing cell death. The bactericidal effect of QACs is due to the generation of electrostatic bonds with the cell membrane, resulting in protein denaturation and membrane disruption (Block, 2001). They disrupt the peptide bonds of protein moieties, which leads to the instability of the cell membrane and the cytolytic leaking of intracellular components (Crismaru et al., 2011). The primary drawback of QACs is the formation of bacteriostatic film on food processing equipment, which can lead to residual accumulation. Moreover, they are incompatible with anionic synthetic detergents and have a higher dilution requirement for their germicidal effect (Hui, 2014; Marriott & Robertson, 1997).

2.2.4.2 Peracetic acid

In an aqueous solution, PAA essentially exists in the chemical equilibrium mixture of acetic acid and hydrogen peroxide (André et al., 2012). PAA is a potent disinfectant having a broad spectrum of activity against vegetative bacteria, viruses (Choi et al., 2022), and spores (André et al., 2012; Kreske et al., 2006). It functions as a non-rinse eco-friendly disinfectant because its decomposition produces acetic acid, water, and oxygen (Block, 2001). The biocidal effect of PAA is due to the oxidation of the cellular macromolecules, resulting in cytoplasmic disruption. The bactericidal action is also linked to the formation of H_2O_2 as an intermediate degradation product (Horn &

Niemeyer, 2022). PAA is a weak acid that is very efficient at low concentrations but can also be utilized effectively at alkaline pH when used in higher concentrations. It is thermodynamically unstable, especially in its diluted form, and must be stored at cold temperatures in its original containers (Block, 2001).

Most of the research on PAA disinfection focussed on food and biofilms. The FDA has approved the usage of PAA in the wash water for fruits and vegetables at a maximum concentration of 80 ppm (Sapers, 2014). Limited studies have evaluated the effectiveness of PAA against the dry microorganisms adhered to the food-contact surfaces (Table 2.9).

Dried cells have a greater resistance to disinfectants than their planktonic counterparts. The effectiveness of PAA (pH 5.24), both on dry *Enterobacter sakazakii* 3231 on stainless steel and in a planktonic solution was studied by Kim et al. (2007). Planktonic cells were reduced to below the detection limit of 0.30 log CFU/mL after being treated with PAA for 1 min, while dry cells observed a lesser reduction of 1.58 log CFU/coupon. Desiccation triggers the starvation phase in cells, which increases their resilience to various stresses. Moreover, PAA's ability to disinfect is reduced in the presence of organic residues. In the same experiment, a 1 min PAA treatment reduced the number of planktonic and surface-dried cells in the presence of infant formula by 0.94 log CFU/mL and 0.58 log CFU/coupon, respectively.

Table 2.9: Summary of studies demonstrating the use of PAA in inactivating the microorganisms dried on food contact surfaces.

Pathogens	Contact surface	Drying conditions	PAA concentration	Log reduction	References
A. brasiliensis	Aluminium	Biosafety drying for 1 h	1000 mg/L at 40 °C	~6	Scaramuzza et al. (2020)
Geobacillus stearothermophilus spores	Stainless steel	Biosafety drying	200 ppm for 5 min	< 1.5	Magulski et al. (2009)
Murine norovirus (MNV-1)	Stainless steel	Drying for 18-24 h, soiled with bovine serum albumin	200 ppm for 3 min	N. C. (no cytopathic effect)	Moon et al. (2021)
Feline calicivirus (FCV)	Stainless steel	Biosafety drying for 30 min	15% PAA and 11% H_2O_2 at 1:500 dilution	3.00	Gulati et al. (2001)
Hepatitis A virus (HAV)	Stainless steel	Biosafety drying for 1 h	200 ppm for 10 min	4.43	Song et al. (2022)

Moreover, the PAA disinfection efficacy will vary based on the type of surface, concentration, and treatment time. Choi et al. (2022) reported that Human coronavirus 229E (HCoV-229E) dried on stainless steel was reduced by 4.73 ± 0.09 to 1.06 ± 0.10 $\log_{10}$ $TCID_{50}$/coupon when treated with PAA at concentrations between 50 and 200 ppm for 5 min. However, HCoV-229E dried on polypropylene exhibited a reduction in viral titers from 4.70 ± 0.04 to below the detection limit (1.0 $\log_{10}$ $TCID_{50}$/coupon) following treatment with 50-200 ppm PAA for 5 min.

Spores have a very high resistance to peroxyacetic acid as compared to vegetative bacteria and fungi. Kreske et al. (2006) compared the effectiveness of peroxyacetic-based sanitizer (40 and 80 µg/L), chlorine (10 to 100 µg/mL), and chlorine dioxide (10 to 200 µg/mL) on the inactivation of *Bacillus cereus* spores on the stainless steel. The efficacy of the sanitizers was assessed by drying

the spores in the presence of water and 5% horse serum. Under all the treatment conditions, a higher susceptibility of the *Bacillus cereus* spores in the order of chlorine > chlorine dioxide > peroxyacetic acid was observed.

2.2.4.3 Hydrogen peroxide

Hydrogen peroxide is a fast-acting, versatile disinfectant with extensive biocidal activity against a wide range of bacteria (Møretrø et al., 2019), viruses (Goyal et al., 2014) and spores (Johnston et al., 2005). It is thermodynamically unstable, and the decomposition of H_2O_2-based disinfectants generates water and oxygen, making them environmentally safe (Eq. 2.2) (Mikutta et al., 2005).

$$H_2O_2 + H_2O_2 \rightarrow 2H_2O + O_2 \quad (2.2)$$

The bactericidal effects of H_2O_2 are a direct result of its oxidizing properties, which include the oxidation of biomolecules (proteins, nucleic acids, and lipids), peroxidation of lipid membranes, inhibition of enzymes, resulting in the genomic cell damage (Møretrø et al., 2012). The principal reactive oxygen species can be produced via two distinct pathways: the reaction of superoxide with the H_2O_2 yields a highly reactive hydroxyl radical ($^{\bullet}OH$) (Linley et al., 2012) (Eq. 2.3).

$$O_2^{-\bullet} + H_2O_2 \rightarrow O_2 + OH^- + {}^{\bullet}OH \quad (2.3)$$

In addition, in Fenton's reaction, transition metal ions decompose H_2O_2 to generate a highly reactive hydroxyl radical (Linley et al., 2012) (Eq. 2.4).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH \quad (2.4)$$

It possesses better material compatibility and does not produce any toxic by-products when in contact with organic wastes. Conventional procedures such as spraying, and immersing involves a direct administration of antimicrobial chemicals to the contact surfaces. For the disinfection of

food processing plants, fumigation-based strategies are routinely employed. They offer the distinctive advantages of greater dispersion and improved penetrability (Kure et al., 2021).

H_2O_2 decomposes at higher temperatures and is highly corrosive. It can also be used in combination with other agents (Møretrø et al., 2012). Hydrogen peroxide in combination with peracetic acid, is a potent oxidant and environmentally safe (Briñez et al., 2006). Kim et al. (2007) tested the effectiveness of a disinfectant based on peroxyacetic acid (5.1%) and hydrogen peroxide (21.7%) against *E. sakazakaii* that had been dried on stainless steel in water and infant formula. The *E. sakazakaii* cell counts were reduced to 1.48 log CFU/coupon after 10 min of treatment with the disinfectant under both drying conditions. In another study by Gulati et al. (2001), peroxyacetic acid (15%) + hydrogen peroxide (11%) based sanitizer at 1:2000, 1:1000, and 1:500 dilution reduced Feline calicivirus (FCV) dried on the stainless steel by 0.4 ± 0.1, 0.6 ± 0.05 and 3.00 ± 0.0 $\log_{10}$ FCV, respectively.

The fundamental issue with foam or gel-based disinfectants is their limited accessibility in the niche areas of the food processing equipment and machines. Therefore, the distribution of the disinfectant in the form of aerosol and mist is a possible alternative for surface disinfection (Kure et al., 2021). H_2O_2 based mist system (5-10%) is commercially used in the healthcare setting (Doll et al., 2015). Choi et al. (2012) examined the survival rates of *E. coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* Typhimurium after being air dried for 2 h on stainless steel and then equilibrated for 7 days at 70% RH. All three pathogens were below the detection limit (1 log CFU/mL) following a 60 min treatment with 0.25 percent H_2O_2 aerosol.

Møretrø et al. (2019) investigated the efficacy of 5% hydrogen peroxide mist containing 0.005% silver, for whole-room disinfection under conditions relevant to the food industry. A suspension of *Listeria monocytogenes* was inoculated onto the stainless steel without drying, and another set

of coupons was dried for one hour in the biosafety cabinet. Hydrogen peroxide was applied to both sets of coupons. Under all tested conditions of H_2O_2 concentration (40-80 ppm), contact time (53-126 min), and temperature (20 °C), suspension cells exhibited a decrease of at least 5 logs. A greater reduction may be attributable to the dissolution of hydrogen peroxide in liquid-phase suspension cells. However, the dried *Listeria monocytogenes* cells were more resistant and showed an overall reduction of 1.5 log. The effectiveness of H_2O_2 against surface-dried microorganisms has only been studied to a little extent (Table 2.10). Decontamination efficacy for H_2O_2 in mist/aerosol has been established against several moulds dried on food-contact surfaces. Kure et al. (2021) discovered that exposure to H_2O_2 mist (40 – 80 ppm) for 2 h resulted in a > 3 log reduction of *Alternaria alternata* adhered to stainless steel. The primary drawback is that H_2O_2 is highly sensitive to the presence of heavy metals. In addition, for a better antimicrobial effect, a concentration ≥ 3% is necessary (Stanga, 2010).

Table 2.10: Summary of studies demonstrating the use of H_2O_2 in inactivating the microorganisms dried on food contact surfaces.

Pathogens	Contact surface	Drying conditions	H_2O_2 concentration	Log reduction	References
Feline calicivirus (FCV)	Stainless steel	Air drying for 45 min in the biosafety cabinet	H_2O_2 (7.5%) for 5 min	4.3	Montazeri et al. (2017)
E. coli	Glass	Biosafety drying for 30 min	H_2O_2 (5%) micro aerosol mist for 30 min	100% inactivation	Neighbor et al. (1994)
S. Typhimurium	Glass	Biosafety drying for 22 h at 25 °C and 40% RH	H_2O_2 (2%) for 5 min	4.3	Gruzdev et al. (2011)
S. Typhimurium	Stainless steel	0, 2 and 24 h of biosafety drying of the stationary phase cells	H_2O_2 (0.88%) for 10 min	100% inactivation	Djebbi-Simmons et al. (2019)

2.2.4.4 Sodium hypochlorite

Sodium hypochlorite (NaOCl), generally known as household bleach, is a major disinfectant used in the food industry (Fukuzaki, 2006). NaOCl is relatively inexpensive and easy to use. It has biocidal potential against bacteria (Djebbi-Simmons et al., 2019), viruses (Gulati et al., 2001) and spores (Dye & Mead, 1972). NaOCl's active form, ^{-}OCl (Eq. 2.5), has germicidal properties and can potentially damage DNA and limit protein synthesis (Fukuzaki, 2006). Sodium hypochlorite in aqueous solution is in equilibrium with ^{-}OCl, HOCl and Cl_2 (Fukuzaki, 2006) (Eqs. 2.5, and 2.6).

$$HOCl \rightleftharpoons {}^{-}OCl + H^{+} \quad (2.5)$$

$$HOCl + H^{+}\, Cl^{-} \rightleftharpoons Cl_{2\,(aq.)} + H_2O \quad (2.6)$$

Hypochlorite solutions with an acidic pH between 4 and 6 have a greater biocidal action due to the presence of a greater amount of hypochlorous acid (HOCl) (Sapers, 2014). As the pH of the solution drops below 4, HOCl decomposes into Cl_2 (Eq. 2.6). The appropriate NaOCl concentration is determined by the total available chlorine, which includes both free and combined chlorine (Sapers, 2014). As a no-rinse food-contact surface sanitizer, the maximum concentration of sodium hypochlorite permitted by the FDA is 200 ppm of available chlorine (FDA, 1999). Table 2.11 provides a summary of the studies demonstrating the effectiveness of NaOCl in inactivating the dry microorganisms from the food-contact surfaces.

Table 2.11: Summary of studies demonstrating the use of NaOCl in inactivating microorganisms dried on food contact surfaces.

Pathogens	Contact surface	Drying conditions	NaOCl concentration	Log reduction	References
S. Typhimurium	Plastic cutting board	Biosafety drying for 24 h (using high microbial load)	0.0095% for 2 min	1.75	Djebbi-Simmons et al. (2019)
S. Enteritidis	Glass	Biosafety drying for 22 h at 25 °C and 40% RH	100 ppm for 5 min	5.8	Gruzdev et al. (2011)
L. monocytogenes	Stainless steel	Biosafety drying for 24 h	200 ppm chlorine for 5 min	>3	Mustpha & Liewen (1989)
S. aureus	Wood	Biosafety drying for 20 min	62.3 mg/L chlorine for 1 min	5.54	Deza et al. (2007)
	Polypropylene			6	
Human norovirus (NoV)	Stainless steel	Biosafety drying for 40 min	3% for 5 min	< 2	Maryline et al. (2010)
Feline calicivirus (FCV)	Stainless steel	Biosafety drying for 30 min	5.25% for 10 min	1.1	Gulati et al. (2001)
Feline calicivirus (FCV)	Stainless steel	Drying for 1 h	12% (5000 ppm) for 5 min	5.20	Kim et al. (2012)
Feline calicivirus (FCV)	Polystyrene	Drying for 30 to 60 min	5.7% (100 ppm available chlorine) for 1 min	< 2.27	Whitehead & McCue (2010)
Hepatitis A virus (HAV)	Stainless steel	Biosafety drying for 1 h, in the presence of 5% soil	PAA 500 ppm for 10 min,	3.76	Song et al. (2022)
Murine norovirus (MNV-1)	Stainless steel	Drying for 30 min	500 ppm for 5 min	< 4	Takahashi, Ohuchi, et al. (2011)
Murine norovirus (MNV-1)	Stainless steel	Biosafety drying for 60 to 90 min	1350 ppm for 5 min	5.5	Chiu et al. (2015)
Alicyclobacillus spp. spores	Stainless steel	-	2000 ppm for 30 min	1.0	Friedrich et al. (2009)

The efficacy of the NaOCl treatment varies depending on concentration, sanitizer exposure time (Mustpha & Liewen, 1989) and the presence of organic residues. Takahashi, Ohuchi, et al. (2011) observed complete inactivation of the murine norovirus after 30 min of drying on stainless steel after treatment with NaOCl at 1000 ppm for 5 min. However, when MNV-1 was dried on stainless steel in the presence of organic residues (cabbage, ground pork, and lettuce), its survival and resistance to the disinfectant increased. The efficiency of NaOCl is also dependent on the drying time of microorganisms on the contact surface (Chiu et al., 2015). Mustpha & Liewen (1989) reported that *Listeria monocytogenes* was more susceptible to NaOCl after being dried on stainless steel for 24 h compared to 1 h. This could be attributed to the variations in drying times and humidity.

A NaOCl treatment (2,700 ppm for 1 min) of murine norovirus (MNV-1) on stainless steel resulted in a greater reduction under wet conditions (6.8 log_{10} reduction) than under dry conditions (5.9 log reduction) (Chiu et al., 2015). Furthermore, the resistance of the foodborne pathogens to disinfectants will vary based on the growth phase such as log, stationary and long-term stationary phases. Djebbi-Simmons et al. (2019) compared the resistance of low, medium, and high microbial load cells dried for 24 h on the stainless steel towards disinfection by sodium chlorite (0.0095%). Under the tested condition, cells in the long-term stationary phase were more resistant to disinfection than those in the log and stationary phases. Bacteria upon entry to the stationary phase initiate the expression of the alternative sigma factor to prevent desiccation tolerance. The requirement for handling precautions is one of sodium hypochlorite's primary drawbacks. In addition, antimicrobial resistance can be avoided through the rotation of disinfectants (Meyer, 2006).

2.2.5 Novel wet disinfection methods for microbial inactivation in low-a_w processing facilities

2.2.5.1 Electrolyzed water (EW)

Electrolyzed water (EW) or electro-chemically activated water has a broad spectrum of antimicrobial activity against bacteria (Yan et al., 2021), fungi, viruses, and spores (Phuvasate & Su, 2010). It is produced by electrolyzing a diluted NaCl solution (Yan et al., 2021) and produces active chlorine concentration (ACC) compounds (Cl_2, OCl^-, and HOCl) as well as reactive oxygen species (H_2O_2 and O_3). The presence of the oxidizing compounds increases the ORP of the EW. Liao et al. (2007) examined the role of ORP in inactivating the *E.coli* suspensions and observed that as ORP increased, the outer and inner membranes of *E.coli* were more severely damaged. According to several studies, sulfhydryl groups on cell surfaces are oxidized in the presence of a greater ORP (Rahman et al., 2016). Multiple factors, including pH (Possas et al., 2021), ACC, ORP, surface type (Deza et al., 2007), and the presence of organic matter, govern EW's microbial efficacy.

Hypochlorous acid (HOCl) with a pH of 5.0 – 6.5 is the active chlorine form present in the EW (Possas et al., 2021). The germicidal action of HOCl results from its passive diffusion into the cell wall and membrane, and production of the $^{\bullet}OH$ radicals and cause oxidation of the cellular components (Yan et al., 2021). HOCl is a relatively weak acid with an approximate pKa of 7.46 (Yan et al., 2021), At a pH < 4.0, it dissociates to generate Cl_2, while at a higher pH, the reversible reaction causes HOCl to decompose into H^+ and OCl^-. Ionized OCl^- possesses low bactericidal action. It is unable to penetrate the hydrophobic lipid bilayer and thus only oxidizes the outer cell wall components. Moreover, the reactive oxygen species (H_2O_2 and ozone) produced by EW contribute to its antimicrobial activity (Rahman et al., 2016).

EW can be divided into acidic electrolyzed water (AEW), neutral electrolyzed water (NEW), and alkali electrolyzed water (AIEW), based on the various electrolytes, equipment, and electrolysis conditions (Yan et al., 2021). AEW (Deza et al., 2007) with a pH 2.3 – 3.7; ORP >1000 mV; and ACC 10 -100 ppm, is a natural sanitizer used for food-contact surface disinfection (Jee & Ha, 2021). Ni et al. (2016) reported exposure to AEW decreased the dehydrogenase activities of *E. coli* and *S. aureus*, resulting in the rapid loss of intracellular DNA, potassium, and proteins and an increase in the permeability of the bacterial membrane. However, the application of AEW is limited by various factors such as equipment corrosion and skin irritation. In addition, AEW can lose its bactericidal activity during storage, and a low pH can favour the loss of Cl_2 gas.

NEW (pH 7.0 -8.0; ORP 750-900 mV) is more effective in disinfecting than AEW (Deza et al., 2007; Yan et al., 2021). It has been reported that NEW is non-corrosive and does not lose its antibacterial properties upon storage. It can be substituted for sodium hypochlorite (NaOCl), a chemical sanitizer widely used for surface disinfection (Jee & Ha, 2021). Deza et al. (2007) compared the efficacy of NEW (pH: 7.76 ± 0.35; ACC: 64.11 ± 6.29 mg/L) and NaOCl (8.11 ± 0.41; ACC: 62.3 mg/L) in disinfecting plastic cutting boards inoculated with *Listeria monocytogenes*. No significant ($p \geq 0.05$) difference was observed in reduction efficacy of NEW and NaOCl after treatment for 1 min. A similar study by Deza et al. (2005) observed no significant difference in the effectiveness of NEW (pH 8.0 ± 0.5; ACC: 60 mg/L) and NaOCl (ACC: 60 mg/L) in reducing *E.coli* and *Listeria monocytogenes* inoculated on the stainless steel and glass surfaces after 1 min of treatment.

EW posseses numerous advantages, tt does not involve the use of harmful chemicals and has lesser environmental impact than conventional chemical sanitizers. It is cost-effective and it does not affect the sensory properties of the foods. EW is more convenient for on-site manufacturing (Deza

et al., 2005; Rahman et al., 2016) as the primary expenses are the initial outlay for the EW generator, chemical salts, and water.

Loss of EW's antibacterial characteristics due to a lack of Cl_2, H^+, and HOCl via electrolysis, and the high initial cost of the necessary equipment, are the biggest drawbacks. Furthermore, the powerful chlorine gas released by different EW generators when the pH is below 5 may cause operator discomfort. Antimicrobial action in EW can also be diminished due to improper storage. Moreover, the presence of the organic matter neutralizes the disinfection efficacy of EW and reduces its shelf life, thereby an additional cleaning step is necessary before its application (Rahman et al., 2016). Diverse studies have advocated combining EW with other disinfection technologies to overcome these shortcomings.

2.2.5.2 Plasma activated water

Plasma treatment of water results in the generation of plasma-activated water (PAW) (Katsigiannis et al., 2022). It is a non-thermal technique with broad antimicrobial activity against bacteria (Smet et al., 2019), virus (Guo et al., 2018), and spores (Bai et al., 2020) and can also be effectively used for surface decontamination (Kamgang-Youbi et al., 2018; Mai-Prochnow et al., 2021). PAW can be generated either at the interface between gas and liquid or within the liquid phase (Mai-Prochnow et al., 2021). RONS production will vary depending on whether the discharge is submerged, on the water surface, or in bubbles. It is an easy to use technology and produces no hazardous by-products, unlike chemical disinfectants (Ölmez & Kretzschmar, 2009). The distinctive advantage of PAW compared to cold plasma is that it can be produced and stored and later utilized for surface disinfection, or it can be produced continuously and circulated (Kamgang-Youbi et al., 2018). However, certain ROS have a shorter shelf life, thereby necessitating

improvement of the procedure. Low-temperature storage provides plasma radicals with higher stability (Katsigiannis et al., 2022).

The antimicrobial activity of PAW results from the production of reactive oxygen species, reactive nitrogen species, charged particles, ions, and electrons. Moreover, low pH and the high ORP also contributes to the antimicrobial effect of PAW (Zhao et al., 2020). The effectiveness of PAW can be increased by optimizing the process parameters, such as applied voltage, gas type, flow rate, reactive species composition, presence of organic matter and the exposure period. Moreover, the microbial properties, such as species type and state (planktonic, dry, biofilm), will also influence the efficacy (Zhou et al., 2020; Zhang et al., 2017).

The antimicrobial effect of PAW is attributed to the electroporation-induced surface etching and membrane permeability caused by the production of charged particles. In addition, the generated electric field increases membrane permeability, and the interaction between biological molecules causes DNA damage, protein disruption, and lipid peroxidation, which ultimately results in cell death. ROS production across the membrane induces oxidative stress and the oxidation of polyunsaturated fatty acids, leading to lipid peroxidation. In addition, as membrane permeability increases, water can enter the cell and cause swelling (Herianto et al., 2021). Baek et al. (2021) dried *S. aureus* ATCC 27213 for 60 min on stainless steel and treated with PAW generated using atmospheric-pressure plasma dielectric generator (2.2 kHz and 4.2 kV for 10 min) and observed a 1.07 log reduction in the cell counts. Most of the antimicrobial research has focused on the use of different generations of plasma-activated water against the treatment of biofilms. However, limited research has examined its effectiveness against surface-dried bacteria on the contact surfaces.

2.3 *Salmonella* survival in the biofilms

Biofilms are polymeric matrix enclosed complex bacterial communities firmly attached to biotic or abiotic surfaces (Lamas et al., 2016). The extracellular polymeric matrix (EPS) is composed of polysaccharides, DNA, and proteins. Biofilms EPS forms a stable microenvironment conducive of bacterial growth. The thickness of the biofilms varies from few micrometers to milimeters (Whitehead & Verran, 2015). Biofilm bacteria generally occurs as a mixed species consortium and is protected against the action of various stressors including disinfectants, temperature, and shear forces (Khu et al., 2023; Whitehead & Verran, 2015).

2.3.1 Factors affecting bacterial attachment

Bacterial adhesion is the first step in the biofilm formation. Biofilms can be formed gradually over time (Whitehead & Verran, 2015). The physicochemical properties of the substratum including contact angle and surface irregularities, and various cell surface properties including hydrophobicity, presence of flagella, and fimbriae aids in the bacterial attachment (Chia et al., 2009). PVC is the most commonly used material for the fabrication of the drinker lines (Maharjan et al., 2017b). The initial bacterial *attachment* also depends on the presence of organic matter or mineral supplements retained on the pipe walls. Insufficient removal of the organic deposits increases the surface roughness and helps in promoting the bacterial adhesion (Whitehead & Verran, 2015). The microbial load in the DWS must be effectively controlled as the EPS plays a crucial role in providing the mechanical stability to the biofilms resulting in a blockage of the pipeline (Khu et al., 2023).

2.3.2 Commonly found bacteria in the broiler DWS

Campylobacter jejuni (Zimmer et al., 2003), *Aeromonas* (Mohammed & Attia, 2022), and *Salmonella* spp. (Jafari et al., 2006) are commonly prevalent in the broiler DWS. Maes et al. (2019) analyzed the microbiological quality of the drinking water obtained from five broiler farms. The study reported the presence of *Shewanella* spp., *Pseudomonas* spp., *Acinetobacter* spp., *Comamonas* spp., *Stenotrophomonas* spp. The high microbial load in the broiler drinking water post disinfection using oxidizing agents, necessitates the need for adoption of a better protocol for the disinfection of the drinker lines (Maharjan et al., 2017b).

2.3.3 *Salmonella* outbreaks associated with poultry

Fecal and water contamination represents the potential transmission routes of *Salmonella* in the broiler farms (Cosby et al., 2015). The daily basis water consumption of the broilers is doubletheir feed intake. Thereby, it is crucial to maintain the microbiological quality of water within acceptable range (Maharjan et al., 2017b). Public health agency of Canada (PHAC) reported a total of 18 outbreaks and 584 cases due to *Salmonella* contamination of the frozen chicken products from 2015-2019 (Morton et al., 2019). Table 2.12 lists the recent *Salmonella* outbreaks associated with the live hatcheries and raw poultry meat. The high susceptibility of *Salmonella* colonization in the broilers is attributable to their less mature gut microbiota (Cosby et al., 2015).

2.3.4 Biofilm formation in the broiler DWS

DWS biofilms are challenging to eradicate as no visual inspection is possible due to their enclosed structure (Maharjan et al., 2017b). Moreover, the samples collected for microbial evaluation are either from the end of the pipeline, or from the nipple drinkers or drinking cups (Maes et al., 2019). The flow rate of the water in the pipelines affects the biofilm architecture (Liu et al., 2016; Perni

et al., 2006). Studies have documented the role of flow regimes in biofilm formation in the DWS (Chen et al., 2023; Khu et al., 2023). Turbulent flow regimes generally result in the formation of strong biofilms with tightly packed bacterial cells. In contrary, laminar regime is a smooth flow that result in the formation of weak biofilms with loosely packed bacterial colonies (Araújo et al., 2016).

Water circulation in the DWS experiences fluctuations in the flow rate and can consequently alter the biofilm architecture (Oder et al., 2018). An understanding of the flow rates of water in the DWS is important for effective design of the disinfection protocol. Disinfectant action under the turbulent regimes having high flow rates is used for the disinfection of the drinker lines (Simões et al., 2005b). Inadequacy in cleaning implies that the biofilm residing on the pipe walls can disperse and attach on the clean surfaces (Khu et al., 2023) and can subsequently increase the microbial load in water. This results in an increase in the turbidity of the drinking water due to an increase in the cell density resulting from the sedimentation and biomass accumulation (Chaves Simões & Simões, 2013; Lehtola et al., 2004).

Table 2.12: Salmonellosis outbreaks resulting from contaminated live poultry and raw poultry meat.

Pathogen	Year	Source	Reference
Salmonella Enteritidis	2021	Raw frozen chicken products	CDC (2021)
Salmonella Reading	2020	Raw chicken	PHAC (2020)
Salmonella Enteritidis	2019	Frozen raw breaded chicken products	PHAC (2019)
Salmonella Infantis	2019	Raw chicken products	CDC (2019a)
Salmonella I 4,[5],12:i:-	2018	Kosher chicken products	CDC (2018e)
Salmonella Typhimurium	2018	Chicken Salad	CDC (2018d)
Salmonella Enteritidis	2018	Frozen raw breaded chicken products	PHAC (2018)
Salmonella spp.	2017	Live poultry (chicks)	CDC (2017)
Salmonella spp.	2016	Live poultry (chicks)	CDC (2016a)
Salmonella spp.	2015	Live poultry (chicks)	CDC (2015a)
Salmonella Enteritidis	2015	Raw, frozen, stuffed chicken	CDC (2015b)
Salmonella Enteritidis	2015	Raw chicken	CDC (2015c)
Salmonella spp.	2015	Frozen raw breaded chicken products	PHAC (2015a)
Salmonella spp.	2015	Live baby poultry	PHAC (2015b)
Salmonella Heidelberg	2014	Chicken	CDC (2014b)
Salmonella spp.	2014	Live poultry (chicks)	CDC (2014a)
Salmonella Heidelberg	2014	Chicken	CDC (2014d)
Salmonella Typhimurium	2013	Live poultry (chicks)	CDC (2013a)
Salmonella Heidelberg	2013	Chicken	CDC (2013b)
Salmonella spp.	2012	Live poultry (chicks)	CDC (2012d)
Salmonella Hadar	2012	Live hatchery (chicks)	CDC (2012a)
Salmonella Montevideo	2012	Live poultry (chicks)	CDC (2012e)
Salmonella Heidelberg	2012	Kosher Broiled Chicken Livers	CDC (2012b)
Salmonella Altona and *Salmonella* Johannesburg	2011	Live poultry (chicks, chickens)	CDC (2011b)

2.3.5 Conventional disinfection methods for broiler DWS

The ideal barn temperatures (± 25 °C), low flow rates, and the presence of medicines and vitamin supplements in the drinking water provides favorable conditions for *Salmonella* proliferation and biofilm formation (Maes et al., 2019).

Chlorine and hydrogen peroxide disinfectants are used for the disinfection of the drinker lines (Maharjan et al., 2017b). Some chlorine disinfectants have sodium hypochlorite (NaOCl) formulations present in lower levels (Maharjan, Dey, et al., 2017). Hydrolysis of NaOCl can form hypochlorous acid as per the following equation (Maharjan, Dey, et al., 2017);

$$NaOCl + H_2O \rightarrow HOCl + Na^{+} + OH^{-} \quad (2.7)$$

The residual levels of chlorine and H_2O_2 in the drinking water should be in the ranges 2-5 ppm and 25-50 ppm, respectively (Maharjan et al., 2017b). The disinfectant efficacy varies based on the biofilm age, the type and concentration of the disinfectant, and the treatment time (Janjaroen et al., 2013). Moreover an inadequate disinfection of the DWS can promote the formation of resistant strains (Roberts et al., 2008).

2.3.6 Novel disinfection methods using plasma activated water

Plasma activated water (PAW) can be produced by discharging plasma either over the surface of water or an underwater discharge in the bubbles (Mai-Prochnow et al., 2021). The size of the bubbles can be adjusted to provide better dissolution of the RONS and subsequently improve the plasma antimicrobial potential (Xu & Tan, 2023). Biofilm inactivation efficacy of PAW can be enhanced by the optimization of the processing parameters, including the gas mixtures, volume of the water treated, type of plasma source, discharge frequency, and operating voltage (Mai-Prochnow et al., 2021).

The formation of various short lived ($ONOO^-$, $^{\bullet}OH$, $^{\bullet}NO$) and long lived reactive species (NO_3^-, NO_2^-, O_3, H_2O_2) contributes to the antimicrobial potential of PAW (Mai-Prochnow et al., 2021). PAW serves as an effective eco-friendly substitute to chlorine disinfectants (Patange et al., 2021). Unlike the chemical disinfectants, no toxic by products are produced in the PAW, and the reactive species produced depletes itself over time (Xu & Tan, 2023). Moreover, the oxidation potential of the various plasma reactive species including ozone (E_0 = 2.07 V) and $^{\bullet}OH$ (E_0 = 2.85 V) is higher as compared to hydrogen peroxide (E_0 = 1.77 V) and chlorine (E_0 = 1.36 V) (Mai-Prochnow et al., 2021). Plasma mediated biofilm inactivation is due to the penetration of the reactive species in the EPS matrix, followed by the resultant lipid peroxidation, protein denaturation, and DNA damage. Moreover, plasma RONS erodes the cell membrane and causes cytoplasmic oxidative stress (Zhou et al., 2019).

2.4 Summary of the research needs

Incidence of *Salmonella* in the dry and wet environments of the food industry is a major food safety concern. The survival of *Salmonella* in low-a_w foods under harsh industrial settings will vary based on the various intrinsic and extrinsic parameters. The increase in the thermal resistance of *Salmonella* post desiccation stress has been well documented. Hence, there is a need to study the role of different food matrices and water activities on the thermal kinetics of different *Salmonella* spp. (Chapter 3).

An appropriate disinfection approach should be selected based on *Salmonella's* occurrence, i.e., in low-a_w environments or in biofilms. Chemical sanitizers and disinfectants are mostly used to disinfect low-a_w food processing facilities. However, there is a need to explore other novel eco-friendly technologies for *Salmonella* control. Moreover, a comparative study is needed to assess the lethality of conventional and novel disinfectants against the dry bacteria on the contact surfaces

(Chapter 4). To mimic the industrial settings, it is important to evaluate the action of the disinfectants under hydraulic variations. Moreover, there is a need to evaluate the inactivation efficacy of plasma activated water bubbles under different hydrodynamic conditions against mixed-species biofilms (Chapter 5).

Chapter 3: Influence of drying conditions, food composition, and water activity on the thermal resistance of *Salmonella enterica*

3.1 Introduction

Salmonella enterica is a low-infectious dose organism (Scheil et al., 1998) and significantly contributes to bacterial foodborne illness. Outbreaks of salmonellosis also consistently relate to low-water activity (a_w) foods with an a_w level below 0.85 (Beuchat et al., 2013). Low-a_w foods that were implicated in outbreaks of salmonellosis includes infant formula (Louie et al., 1993), almonds (CDC, 2004), chocolate (Werber et al., 2005), spices (Van Doren et al., 2013), dog food (CDC, 2008a), dried milk products (Silva et al., 2018) and peanut butter (CDC, 2009a). Control of *S. enterica* in low-a_w foods is impeded by its long-term survival and enhanced thermal resistance in the dry state (Blessington et al., 2012; Danyluk et al., 2005; Gruzdev et al., 2011). To minimize the risk associated with *Salmonella* in low-a_w foods, improved pathogen intervention technologies are required.

Microbial viability at low-a_w conditions is dependent on many factors, including heat or acid stress during drying, temperature and time of exposure, type of the strains used, medium composition and the nature of the solutes present in the food matrix (Doyle & Mazzotta, 2000). Thus, for improved microbial reduction in low-a_w foods it is essential to understand the interactions between the intrinsic factors including water content, water activity and food composition, the extrinsic factors including the relative humidity, treatment time and temperature (Wesche et al., 2009). The water activity of foods is temperature dependent and the water activity at the treatment temperature determines the resistance of *Salmonella* and other organisms (Jin et al., 2020; Xu et al., 2019). The differences in *Salmonella's* resistance in different foods at the same water activity were attributed

to the specific moisture sorption isotherms (Syamaladevi et al., 2016; Xu et al., 2019). However, past studies that determined whether differences in moisture sorption isotherms of different foods impact the heat resistance of *Salmonella* were confounded by experimental protocols that dried and equilibrated the bacterial inoculum in different food matrices prior to heat treatment (Gautam et al., 2020; Xu et al., 2019).

The heat resistance of bacterial endospores is largely attributable to the exclusion of water (Setlow, 2006) and is based on a mechanism termed "preferential exclusion" or "preferential hydration". High concentrations of co-solvents which are excluded from the hydration shell of proteins prevent protein denaturation by heat and/or pressure (Canchi et al., 2012; Herberhold et al., 2004). Compatible solutes that are accumulated in the bacterial cytoplasm in response to heat, osmotic stress or desiccation thus protect proteins, ribosomes and other biological macromolecules against heat denaturation (Lamosa et al., 2000; Pleitner et al., 2012; Ramos et al., 1997). Desiccated vegetative cells of *Salmonella* and *Escherichia coli* exhibit heat resistance that is comparable to the resistance of bacterial endospores (Li & Gänzle, 2016; Syamaladevi et al., 2016). Owing to the importance of compatible solutes to bacterial heat resistance at low a_w, their accumulation during osmotic stress is a key determinant of bacterial heat resistance (Kilimann et al., 2006). The composition of compatible solutes in desiccated *Salmonella* cells is dependent on the drying conditions and the composition of the food matrix (Finn, Condell, et al., 2013b) and thus provides an alternative explanation for the impact of the food matrix on the survival of desiccated *Salmonella*.

Thermal processing is widely employed for pathogen reduction in food products, including low-a_w foods (Bermúdez-Aguirre & Corradini, 2012), because of the ease of operation and the absence of the chemical residues post-treatment. However, the use of high temperatures for microbial

inactivation can adversely affect the quality of the products. Thus, the thermal processing time should be optimized to achieve the required level of reduction of the target pathogen while maintaining the quality of the products.

This study aimed to assess the effect of complex food matrices and the strain-specific differences on the elimination of *Salmonella* in low-a_w foods. Specifically, the study (i) investigated the combined effect of food matrix and a_w on the desiccation tolerance and thermal resistance of the different *Salmonella* strains in the selected low-a_w foods by using wet inoculation technique; (ii) quantitatively modeled the survival of *Salmonella* in different low-a_w foods during thermal treatments, and (iii) explored the interactions between the food components on the resistance of *Salmonella* by using dry inoculum.

3.2 Materials and methods

3.2.1 Selection of low-a_w foods

Three low-a_w foods with different size, shape and compositions were selected for this study; pet food (Adult small breed dog food procured from a local store), a carbohydrate-based burger binder (GLIB Burger seas and binder, Griffith Foods, Toronto, ON, Canada) and skim milk powder (SMP) (No name, Loblaw Companies Limited, Edmonton, AB, Canada) with an initial a_w of ~ 0.57, 0.32 and 0.29, respectively. The moisture content was determined using a convection oven (Heratherm, Thermo Scientific, Waltham, MA, USA) and the initial a_w of the selected foods were analyzed using a water activity meter (Aqualab 4TE Meter group, Pullman, WA, USA).

3.2.2 Proximate analysis

The proximate composition of low-a_w foods was determined using standard AOAC protocols. The dry basis water content was analyzed by drying 2 g of the sample in a convection oven (Heratherm,

Thermo Scientific, Waltham, MA, USA) at 105 °C for 8 h until no successive difference in the sample weight was observed (AOAC, 2000). The ash content was determined by incinerating 1 g of the sample in an electric muffle furnace (Model F-A1730, Thermolyne Corporation, Dubuque, IA, USA) at 550 °C for overnight (AOAC, 2000). The test tube method was used for estimation of the fat, and protein was estimated using the Leco TruSpec nitrogen analyzer (Model FP- 428, Leco Instruments Ltd., Mississauga, ON, Canada). The total carbohydrates were calculated by deducting the sum of the moisture, ash, fat, and protein content from 100. All analyses were replicated two times.

3.2.3 Enumeration of the background microbiota

Viable microorganisms in the three products were enumerated by surface plating on the tryptic soy agar (Difco, BD, Franklin Lakes, NJ, USA) plates supplemented with 0.6% yeast extract (TSAYE). An aliquot (0.3 ± 0.01 g) of each sample was added into 3 mL of 0.1% peptone water. Samples were soaked for 30 min, mixed thoroughly and serially diluted in 0.1% peptone water and duplicate spread plated onto TSAYE plates.

3.2.4 *Salmonella* strains used in the study

Three strains of *Salmonella enterica* were used in this study, *S.* Typhimurium ATCC13311, *S.* Senftenberg ATCC43845, a strain resistant to wet heat (Mercer et al., 2017) and *S.* Enteritidis FUA1946, a wastewater isolate resistant to dry heat (Schultze et al., 2020). Stock cultures were maintained in 70% (v/v) glycerol at -80 °C. Working cultures were maintained on the TSAYE plates and stored at 4 ± 2 °C. One colony from the TSAYE plates was inoculated into 5 mL tryptic soy broth (TSB; Difco, BD, Franklin Lakes, NJ, USA) supplemented with 0.6% (w/v) yeast extract (TSBYE) and incubated aerobically at 37 °C for 24 h. A second transfer was made into the TSBYE

and incubated overnight (~18 h) at 37 °C and 0.1 mL of this culture was spread evenly on a TSAYE plate and incubated for 24 h at 37 °C.

Cells harvested from a lawn have a higher heat resistance than cells from broth-based cultures (Wiertzema et al., 2019), therefore, a lawn-based liquid inoculum technique (Danyluk et al., 2005) was used for the preparation of culture inoculum. Bacterial cells were harvested from the TSAYE plates with 1.5 mL of 0.1% peptone water (BD Difco™, Franklin Lakes, NJ, USA) and centrifuged at 9632 x g for 5 min. The pellet was washed in 0.1% peptone water, vortexed and cells were harvested by a second centrifugation step. The pellet was resuspended with 0.1% (w/v) peptone water and adjusted to a final volume of 1 mL. Before inoculation into the food samples, each strain was enumerated on TSAYE to obtain 9 - 10 log CFU/mL of the test organisms.

3.2.5 Experimental design

3.2.5.1 Wet inoculation of foods

The flat side of the pet food pellets was inoculated with 20 µL of the culture suspension prepared above. The powder samples were inoculated as described by Du et al. (2020). In brief, 10 g of SMP or burger binder were mixed with 1.25 mL of inoculum in a sterile polyethene bag. The inoculated powders were hand massaged for 30 s and ground for 1 min to break remaining lumps (Hamilton Beach coffee grinder, Belleville, ON, Canada). Following culture inoculation, all the three foods were air-dried for 1 h in a biological safety cabinet, mixed, and further dried for 18-24 h.

The air-dried samples were equilibrated to a_w of 0.33 and 0.75 in sealed containers containing saturated salt solutions of $MgCl_2$ and NaCl (Sigma-Aldrich, Oakville, ON, Canada) for 10 days. Following the equilibration, a_w was checked in triplicates using a water activity meter (Aqualab

4TE Meter group, Pullman, WA, USA). Equilibrated samples were enumerated, and cell viability was determined before the thermal treatment.

3.2.5.2 Dry inoculation of foods

The resistance of *Salmonella* to dry heat is known to depend on the drying conditions. Therefore, a second series of experiments was conducted with *S.* Enteritidis FUA1946 by inoculating dry foods with bacterial cultures that were dried in peptone water. Moreover, the selected foods differed in their physical structure and shapes, i.e., pet food was in pellet form, the burger binder was a coarse powder, and SMP was a fine powder. To eliminate an effect of the food structure in experiments with dry inoculum, pet foods were ground with grinder (Hamilton Beach coffee grinder, Belleville, ON, Canada). Ground pet food, burger binder and the skim milk powder (50 g each) were then mechanically sieved using a horizontal sieve shaker (Ro-Tap® RX-812, Haver & Boecker OHG, Germany) for 10 min. The fraction of the sample collected on each sieve was weighed and the percentage particle size was calculated. The fractionated foods with a particle size of 362.5 ± 62.5 µm was used for the experiments.

The bacterial suspension prepared above was transferred into clear glass vials (2 mL per vial) and dried in the biosafety cabinet for approximately 4 - 5 d at ambient conditions. After air drying, bacteria were dehydrated using silica gel to an a_w of less than 0.1. Before inoculation, both the dried culture and the ground foods were separately conditioned to a_w 0.75 in saturated salt solutions for approximately 6 d. The ratio of the dried culture to powder samples was standardized to 10 mg of culture per 1 g of the sample, corresponding to about 10 - 11 log(CFU/g). Bacterial homogeneity was ensured by mixing the dried bacteria thoroughly with the food sample. Microbial counts were determined immediately after the inoculation and heat treatment was performed on the same day.

3.2.6 Thermal treatment

Inoculated samples were treated in aluminium-based thermal-death-time cells (Chung et al., 2008). Each test cell was loaded with 0.3 ± 0.01 g of the sample and heated in a 70 °C water bath (Fisher Scientific Isotemp GPD10 Water Bath, Fisher Scientific, Ottawa, ON, Canada). Samples were drawn after 5, 10, 15, 20 and 25 min of heating. The come-up time (CUT) for all the samples was determined using a T-type thermocouple (Fisher Scientific, Ottawa, ON, Canada) and was taken as time zero. Following treatment, the test cells were transferred to an icebox for 45 s and samples were diluted with 3 mL 0.1% peptone. Multiple samples were drawn from the powder formulations to ensure homogeneity. The homogenized suspension obtained was spread plated in duplicate on the TSAYE plates and incubated at 37 °C for 24 h.

3.2.7 Modelling of *Salmonella* inactivation kinetics

The Weibull model has been extensively used to fit microbial death-time curves (Peleg & Cole, 1998; Van Boekel, 2002) and can be expressed as:

$$\log\left(\frac{N}{N_0}\right) = -\frac{1}{2.303}\left(\frac{t}{\alpha}\right)^{\beta} \tag{3.1}$$

where N_0 is the initial microbial count (log CFU/g), N is the microbial count at the time t (log CFU/g), t is the treatment time (min), α is a scale parameter corresponding to the characteristic time (min) and β is a dimensionless parameter defining the shape of the inactivation curve. From this equation t_R, which in analogy to the *D*-value corresponds to the time required for a reduction of the cell counts by 90% (Van Boekel, 2002) was calculated as:

$$t_R = \alpha\,(2.303)^{\frac{1}{\beta}} \tag{3.2}$$

The Weibull parameters α and β (Eq. 3.2) were computed by non-linear regression method with solver function of Microsoft Excel 2010 (Microsoft Corp., Redmond, WA, USA), which minimises the sum of residual squares between the experimental and predicted values. Average values of experiments were used for calculation of the Weibull parameters and t_R.

3.2.8 Water sorption isotherms of low-a_w foods

The water sorption isotherms of pet food, burger binder and SMP were determined at 20 °C and 70 °C with water activities ranging from 0.1 – 0.8 using the static gravimetric method. A thermal cell with a relative humidity sensor (Omega Engineering, Inc. St-Eustache, QC, Canada) was used for the generation of water sorption isotherms (Syamaladevi, Tadapaneni, et al., 2016). Ten g of the sample was taken in the petri-plates and pre-dried to $a_w < 0.1$ using silica gel. The dried samples obtained were then transferred to sealed containers having supersaturated salt solutions of varying relative humidity values at room temperature (25 °C) for approximately two weeks, to achieve the desired a_w. During the test, 2 g of the equilibrated sample was placed in the thermal death time cell (Syamaladevi, Tadapaneni, et al., 2016) heated to 70 °C in a water bath until equilibrium water content was achieved. The dry basis water content was analyzed by drying 2 g of the respective sample in a convection oven (Heratherm, Thermo Scientific, Waltham, MA, USA) at 105 °C for 8 h until no successive difference in the sample weight was observed. The experimental data obtained was fitted to the Guggenheim, Anderson and De Boer (GAB) model (Quirijns et al., 2005).

$$M_e = \frac{W_m Y K a_w}{(1-Ka_w)(1-Ka_w+YKa_w)} \tag{3.3}$$

where M_e is the moisture content of the food material (dry basis), W_m is the moisture content of the monolayer (dry basis), Y is strength of the binding water to the primary binding sites, K represents as the correction factor (Quirijns et al., 2005).

The experimental data for water activity and water content are shown as means ± standard deviations of three independent replicates. The equation constants were estimated by using the solver function of Microsoft Excel 2010 (Microsoft Corp., Redmond, WA, USA).

3.2.9 Statistical analysis

The log-transformed viable counts of *Salmonella* spp. are shown as means ± standard deviations of three biological replicates. Significant differences were determined in SAS® University edition (Proc Glimmax; SAS studio 9.4) by two-way analysis of variance ANOVA with Tukey's test as post-hoc analysis and an error probability of 5% ($p \leq 0.05$) denoting significant differences.

3.3 Results

3.3.1 Proximate composition

The proximate composition of the foods investigated is shown in Table A1 (Appendix 1). The proximate analysis was consistent with the label information. Pet food was labeled to contain a mixture of protein meal, grains, animal fat, thus contributing to (31%) protein and (17%) fat. The burger binder formulation contained toasted wheat crumbs, spices including mustard, autolyzed yeast extract, monounsaturated vegetable oil and NaCl. The SMP contained carbohydrates (52%), proteins (36%), sodium (0.46%) and other minerals.

3.3.2 Survival of *Salmonella* during desiccation and equilibration, and the come-up times

Wet inoculation of low-a_w foods exposed *Salmonella* spp. to different conditions during drying and equilibration. Overnight drying resulted in a < 1 log reduction of all the strains in the three food matrices (data not shown), with the exception of *S*. Typhimurium ATCC13311, which was reduced by 1.25 ± 0.13 log(CFU/g) after drying in the burger binder. Survival of the strains of *Salmonella* after drying and 10 d of equilibration at a_w 0.33 and 0.75 is shown in Tables 3.1 and 3.2, respectively. Equilibration to a_w 0.33 in any of the foods reduced *Salmonella* populations by less than 1 log (CFU/g); the reduction of cell counts during equilibration to a_w 0.75 ranged from less than 0.5 to more than 2.5 log(CFU/g). Overall, *S.* Enteritidis FUA1946 was found to be more resistant to equilibration and drying than the other two strains. Moreover, pet foods showed higher desiccation survival of *Salmonella* in comparison to burger binder and SMP, indicating that the effect of the food matrix depended on the strain and the water activity (Tables 3.1 and 3.2).

Table 3.1: Survival of *Salmonella* during drying and equilibration in pet foods, skim milk powder and burger binder equilibrated to a_w 0.33.

Food matrix	**Strain**	**Initial population (log CFU/g)**	**Post equilibration population (log CFU/g)**	**Reduction (log CFU/g)**
Pet food	*S.* Enteritidis FUA1946	9.56 (0.07)^{A}	9.30 (0.04)^{A}	0.26 (0.04)^{CB}
	S. Senftenberg ATCC43845	9.65 (0.10)^{A}	9.39 (0.14)^{A}	0.26 (0.05)^{CB}
	S. Typhimurium ATCC13311	9.26 (0.20)^{B}	8.93 (0.24)^{B}	0.33 (0.06)^{B}
Skim milk powder	*S.* Enteritidis FUA1946	8.58 (0.20)^{C}	8.49 (0.19)^{C}	0.09 (0.02)^{D}
	S. Senftenberg ATCC43845	8.48 (0.15)^{C}	8.44 (0.15)^{C}	0.05 (0.01)^{D}
	S. Typhimurium ATCC13311	8.04 (0.19)^{D}	7.35 (0.25)^{E}	0.68 (0.15)^{A}
Burger binder	*S.* Enteritidis FUA1946	8.75 (0.19)^{C}	8.58 (0.22)^{C}	0.17 (0.03)^{CD}
	S. Senftenberg ATCC43845	8.14 (0.09)^{D}	8.03 (0.09)^{D}	0.11 (0.02)^{D}
	S. Typhimurium ATCC13311	7.44 (0.19)^{E}	6.69(0.26)^{F}	0.76 (0.14)^{A}

Data shown are expressed as means (standard deviation) of three biological replicates (n = 3). Within each column, different superscript letters represent significant ($p < 0.05$) difference among the logarithmic counts.

Table 3.2: Survival of *Salmonella* during drying and equilibration in pet foods, skim milk powder and burger binder equilibrated to a_w 0.75.

Food matrix	Strain	Initial population (log CFU/g)	Post equilibration population (log CFU/g)	Reduction (log CFU/g)
Pet food	*S.* Enteritidis FUA1946	9.59 (0.09)^{A}	9.25 (0.07)^{A}	0.34 (0.04)^{C}
	S. Senftenberg ATCC43845	9.49 (0.06)^{A}	9.28 (0.06)^{A}	0.20 (0.05)^{C}
	S. Typhimurium ATCC13311	9.43 (0.04)^{A}	8.80 (0.08)^{B}	0.63 (0.12)^{C}
Skim milk powder	*S.* Enteritidis FUA1946	8.30 (0.16)^{CB}	6.23 (0.40)^{D}	2.07 (0.28)^{B}
	S. Senftenberg ATCC43845	8.43 (0.23)^{B}	5.99 (0.25)^{D}	2.44 (0.42)^{BA}
	S. Typhimurium ATCC13311	7.37 (0.32)^{D}	4.98 (0.33)^{E}	2.39 (0.46)^{BA}
Burger binder	*S.* Enteritidis FUA1946	8.52 (0.21)^{B}	7.90 (0.22)^{C}	0.62 (0.03)^{C}
	S. Senftenberg ATCC43845	7.98 (0.12)^{C}	5.36 (0.28)^{E}	2.62 (0.38)^{A}
	S. Typhimurium ATCC13311	6.68 (0.28)^{E}	4.15 (0.06)^{F}	2.53 (0.33)^{BA}

Data shown are expressed as means (standard deviation) of three biological replicates (n = 3). Within each column, different superscript letters represent significant ($p < 0.05$) difference among the logarithmic counts.

To calculate the kinetics of thermal inactivation based on isothermal treatments, the time when the geometrical center of the product reached the treatment temperature was considered as 0 min treatment time. The time required to reach 70 ± 0.5 °C in the geometrical centre of the product (come-up time) for pet food, burger binder and SMP at a_w 0.33 were 116, 56 and 53 s, respectively; at a_w 0.75, come up times of 161, 60, 68 s, respectively, were observed. The reduction of cell counts of the three strains of *Salmonella* after the come-up time in the three food matrices conditioned to a_w 0.33 and 0.75 is shown in Table 3.3. Significant ($p < 0.05$) interactions between the matrix and strain on *Salmonella* reduction were observed at both water activities and a low a_w improved survival during the come-up time.

Table 3.3: Reduction of the wet inoculated *Salmonella* after the come-up times (CUT) at 70 °C in the food matrixes equilibrated to a_w 0.33 and 0.75.

Food matrix	Strain	Reduction (log CFU/g) at a_w 0.33	Reduction (log CFU/g) at a_w 0.75
Pet food	*S.* Enteritidis FUA1946	0.05 (0.00)^{EF}	0.25 (0.04)^{E}
	S. Senftenberg ATCC43845	0.11 (0.01)^{ED}	0.48 (0.07)^{ED}
	S. Typhimurium ATCC13311	0.15 (0.03)^{CD}	0.79 (0.09)^{CB}
Skim milk powder	*S.* Enteritidis FUA1946	0.38 (0.08)^{A}	0.61 (0.09)^{CBD}
	S. Senftenberg ATCC43845	0.24 (0.03)^{B}	0.52 (0.14)^{CED}
	S. Typhimurium ATCC13311	0.04 (0.01)^{F}	1.55 (0.40)^{A}
Burger binder	*S.* Enteritidis FUA1946	0.19 (0.03)^{CB}	0.84 (0.10)^{B}
	S. Senftenberg ATCC43845	0.38 (0.06)^{A}	0.77 (0.15)^{CB}
	S. Typhimurium ATCC13311	0.34 (0.02)^{A}	0.32 (0.05)^{E}

Values are expressed as means (standard deviation) of the three biological replicates (n = 3). Reduction (log CFU/g) is calculated by subtracting *Salmonella* survivor counts before and after the CUT. Different superscript letters in the column indicate significant ($p < 0.05$) difference among the logarithmic counts.

3.3.3 Thermal inactivation kinetics after drying and heating in wet inoculated low-a_w foods

Heat resistance of *Salmonella* was determined in three different food matrices, for three strains and at two water activities (0.33 and 0.75). The Weibull model provided a good fit to the inactivation curves with correlation coefficients R^2 ranging from 0.96 to 0.998 (Table A2 and A3, Appendix 1). The fit of the model to the experimental data is depicted in Figures 3.1 and Figure 3.2 and the Weibull estimates are listed in Table 3.4. At a_w 0.33, survival curves for all the three strains in the burger binder demonstrated tailing ($\beta < 1$) while the survival curves for SMP and pet food demonstrated shouldering effects ($\beta > 1$), indicating that the remaining cells accounted for cumulative damage and have become more susceptible to heat (Peleg, 2006). Significant ($p < 0.05$) interactions on the thermal inactivation of *Salmonella* were observed between the

combinations of 'time-matrix' and 'matrix-strain'. The heat resistance of the three strains differed ($p < 0.05$). At a_w 0.75 (Figure 3.2), survival curves exhibited tailing ($\beta < 1$), corresponding to the diminishing inactivation rate of *Salmonella* to the thermal treatment. The shape of the survivor curve obtained was not only dependent on the food matrix chosen, but also on the strain type. The Weibull parameter α (scale parameter) was significantly ($p < 0.05$) influenced by the matrix and the strain type. Decreasing a_w yielded higher α values. A similar trend in the α values was observed in pet food and binder, where *S.* Enteritidis FUA1946 corresponded to the higher α value followed by *S.* Senftenberg ATCC43845 and *S.* Typhimurium ATCC13311, thus indicating the higher resistance of *S.* Enteritidis FUA1946. However, there was no significant difference ($p > 0.05$) in the α values of the strains *S.* Enteritidis FUA1946 and *S.* Senftenberg ATCC43845 in SMP and were significantly greater ($p < 0.05$) than those of *S.* Typhimurium ATCC13311.

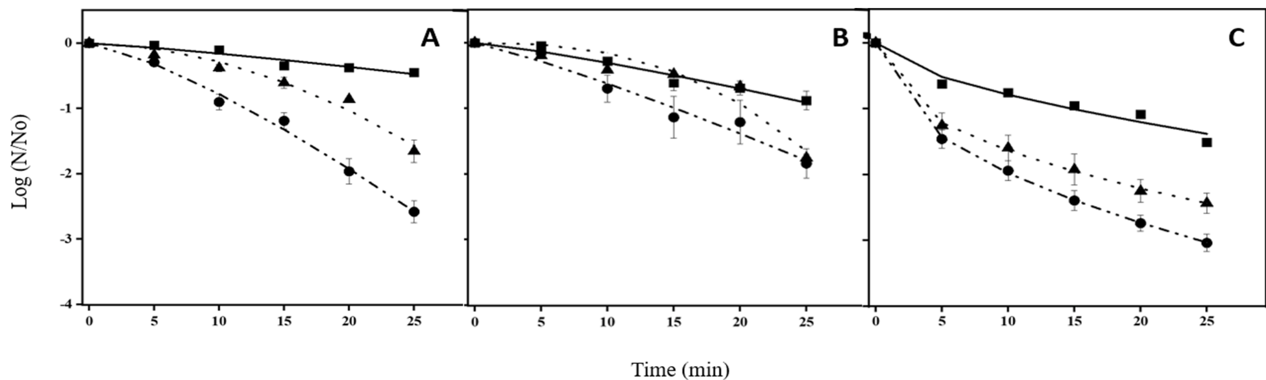


Figure 3.1: Survival curves of the *Salmonella* strains; *S.* Enteritidis FUA1946 (■), *S.* Senftenberg ATCC43845 (▲) and *S.* Typhimurium ATCC13311 (●) in (A) pet food, (B) skim milk powder and (C) burger binder, equilibrated to a_w 0.33 and heat treated at 70 °C. Data shown are expressed as means ± standard deviation of three independent trials (n = 3). Lines represent the Weibull fitting and the points without lines are not fitted. Limit of detection: 2 log(CFU/g).

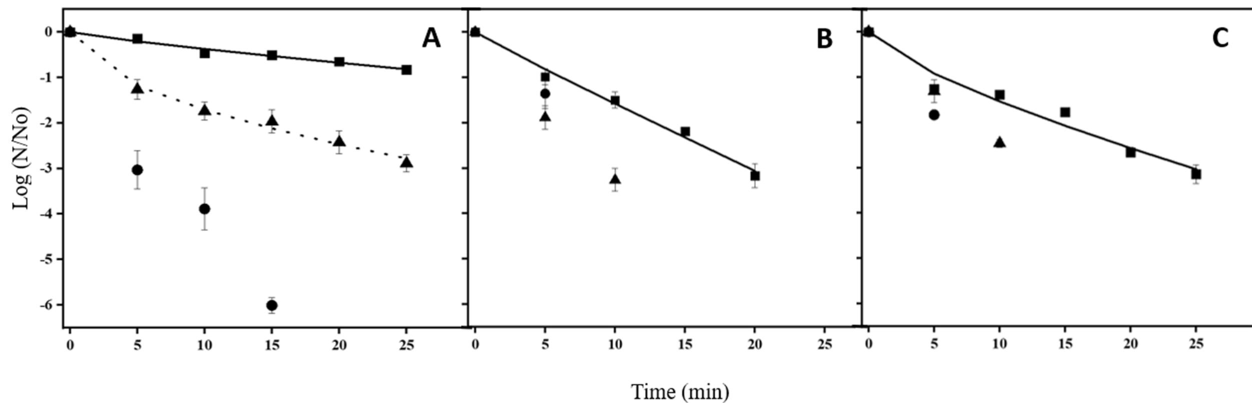


Figure 3.2: Survival curves of the *Salmonella* strains; *S.* Enteritidis FUA1946 (■), *S.* Senftenberg ATCC43845 (▲) and *S.* Typhimurium ATCC13311 (●) in (A) pet food, (B) skim milk powder and (C) burger binder, equilibrated to a_w 0.75 and heat treated at 70 °C. Data shown are expressed as means ± standard deviation of three independent trials (n = 3). Lines represent the Weibull fitting and the points without lines are not fitted. Limit of detection: 2 log(CFU/g).

Comparison of log reductions (Figures 3.1 and 3.2), and t_R values (Table 3.4) of wet inoculated *Salmonella* strains in pet food, burger binder and SMP showed that the *Salmonella* inactivation rate during thermal treatment was dependent not only on the a_w change at treatment temperature but also on the strain type, the food matrix, and their interactions. For instance, at a_w 0.33, after 25 min of thermal treatment at 70 °C, a lower reduction of 0.44 ± 0.03 log (CFU/g) in the population of most heat resistant *S.* Enteritidis FUA1946 was observed in the pet food as compared to 0.88 ± 0.14 log(CFU/g) reduction in the SMP. While under similar conditions, in the case of the most heat-sensitive *S.* Typhimurium ATCC13311, the log reduction was higher in the pet food (2.58 ± 0.17 log CFU/g) as compared to the SMP (1.84 ± 0.23 log CFU/g). For *S.* Senftenberg ATCC43845, a similar pattern of reductions in the pet food (1.65 ± 0.17 log CFU/g) and SMP (1.75 ± 0.06 log CFU/g) was observed. When compared to the pet food and SMP, the burger binder had the highest reduction of the *Salmonella* strains; *S.* Enteritidis FUA1946 (1.51 ± 0.11 log CFU/g), *S.* Senftenberg ATCC43845 (2.43 ± 0.15 log CFU/g) and *S.* Typhimurium ATCC13311 (3.04 ± 0.13 log CFU/g).

Table 3.4: Comparison of kinetic Weibull parameters for the thermal inactivation kinetics of the wet inoculated *Salmonella* strains in the low-a_w foods equilibrated to a_w 0.33 and 0.75.

Food matrix	**Strain**	**a_w 0.33**			**a_w 0.75**		
		α-value (min)	**β-value**	**t_R (min)**	**α-value (min)**	**β-value**	**t_R (min)**
Pet food	*S.* Enteritidis FUA1946	23.28 (1.64)^{a}	1.19 (0.06)^{b}	46.90 (2.93)^{a}	12.06 (1.63)	0.86 (0.05)	31.68 (3.00)
	S. Senftenberg ATCC43845	12.59 (1.19)^{b}	1.88 (0.29)^{a}	19.71 (0.87)^{b}	0.84 (0.54)	0.54 (0.08)	3.79 (1.55)
	S. Typhimurium ATCC13311	6.43 (0.84)^{c}	1.31 (0.09)^{b}	12.14 (1.11)^{c}	NA	NA	NA
Skim milk powder	*S.* Enteritidis FUA1946	13.62 (1.96)^{a}	1.20 (0.08)^{b}	27.42 (4.06)^{a}	2.61 (0.85)	0.96 (0.17)	6.19 (1.09)
	S. Senftenberg ATCC43845	14.96 (0.21)^{a}	2.60 (0.12)^{a}	20.63 (0.10)^{b}	NA	NA	NA
	S. Typhimurium ATCC13311	7.63 (2.33)^{b}	1.19 (0.19)^{b}	15.29 (3.21)^{b}	NA	NA	NA
Burger binder	*S.* Enteritidis FUA1946	3.91 (1.50)^{a}	0.62 (0.09)^{a}	14.78 (2.83)^{a}	1.84 (0.65)	0.74 (0.13)	5.57 (1.02)
	S. Senftenberg ATCC43845	0.53 (0.36)^{b}	0.44 (0.07)^{b}	3.33 (1.37)^{b}	NA	NA	NA
	S. Typhimurium ATCC13311	0.40 (0.17)^{b}	0.47 (0.04)^{b}	2.35 (0.65)^{b}	NA	NA	NA

'NA' indicates not available, the Weibull model was not fitted since the microbial counts were below the detection limit of 2 log(CFU/g) after few min of isothermal treatment at 70 °C, indicating the sensitivity in the behaviour of the strains to the conditions subjected.

Values are expressed as means (standard deviation) of the three biological replicates (n = 3).

Different superscript letters in the column indicate significant ($p < 0.05$) difference among the respective values in individual food matrix.

3.3.4 Thermal inactivation kinetics after heating in dry inoculated low- a_w foods

Because the food matrix clearly impacted the survival of strains of *Salmonella* during drying and equilibration, a second set of experiments was performed with a culture that was dried and equilibrated in peptone water and mixed with the powdered dry foods after drying and equilibration. The microbial counts of *S.* Enteritidis FUA1946 post air drying was 11.71 ± 0.20 log (CFU/g); silica drying and equilibration to a_w 0.75 did not reduce the cell counts further (data not shown). The come-up times recorded in the thermal death time cells were 70 s, 56 s, 53 s for the similar-sized pet food, burger binder and SMP, and it was taken to be 60 s for the experiments; the reduction of cell counts during the come-up time was less than 0.2 log(CFU/g) for all foods.

The isothermal inactivation kinetics of the dried *S*. Enteritidis FUA1946 in the three foods at a_w 0.75 are shown in Figure 3.3. The Weibull model described the inactivation curves with an R^2 of 0.96 – 0.98. The Weibull parameters from these survivor curves are listed in Table 3.5 and the goodness-of-fit parameters obtained are reported in Table A4 (Appendix 1). Lower β-values of < 1 have been observed for the three foods, indicating that the remaining cells became more resistant, and the survivor curves exhibited the tailing effect (Figure 3.3).

Mixing dried and equilibrated cells with powdered, dried, and equilibrated foods substantially increased the resistance of *S.* Enteritidis FUA1946 to dry heat and virtually eliminated differences in the thermal death curves on the three foods (Figure 3.3). The treatment conditions (a_w 0.75 and 70 °C temperature) observed less than 1 log(CFU/g) reduction of the cell counts. The reduction of cell counts in powdered pet food, skim milk powder and burger binder were 0.77 ± 0.04 log(CFU/g), 0.62 ± 0.01 log(CFU/g) and 0.64 ± 0.06 log(CFU/g), respectively.

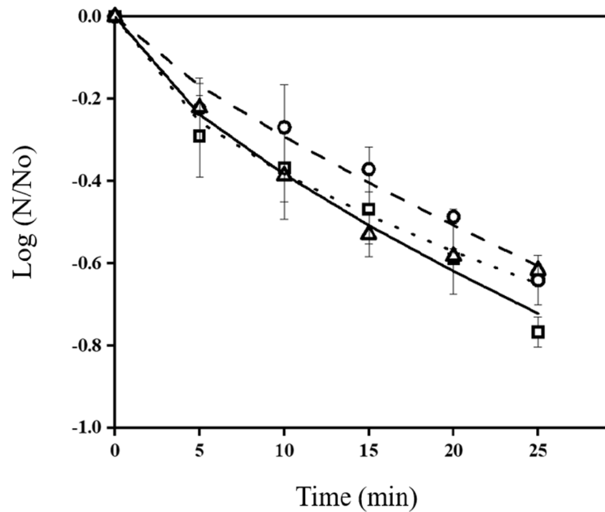


Figure 3.3: Survival curves of the dried *S.* Enteritidis FUA1946 in pet food (□), skim milk powder (△) and burger binder (○), equilibrated to a_w 0.75 and heat treated at 70°C. Data shown is expressed as means ± standard deviation of three independent trials (n = 3). Lines represent the Weibull fitting.

3.3.5. Moisture sorption moisture isotherms of pet foods, skim milk powder and burger binder

Adsorption isotherms were constructed using the GAB equation, to study the effect of temperature on a_w change and the subsequent effect on the thermal inactivation of *Salmonella* strains. The GAB parameters are listed in Table A5 (Appendix 1). Isotherms of pet food and burger binder exhibited type III Brunauer–Emmett–Teller (BET) sorption behavior (Figure 3.4, panels A and C). Isotherm of SMP showed classical sigmoidal shaped curves with type II BET classification (Štencl, 1999), with a clear inflection at an a_w of 0.2-0.4 and 0.6-0.7 (Figure 3.4B). At a constant water content, a significant increase of the a_w in response to an increased temperatures was observed in all three food matrices. This increase was most pronounced in pet food, where the a_w increased from 0.33 to about 0.58 upon heating from 20 to 70 °C (Figure 3.4A). The a_w in the burger binder increased to about 0.55 (Figure 3.4C) while no change of a_w was observed in SMP (Figure 3.4B). In

comparison, the samples equilibrated to a higher a_w of 0.75 showed no considerable change in the water activity at the treatment temperature (Figure 3.4).

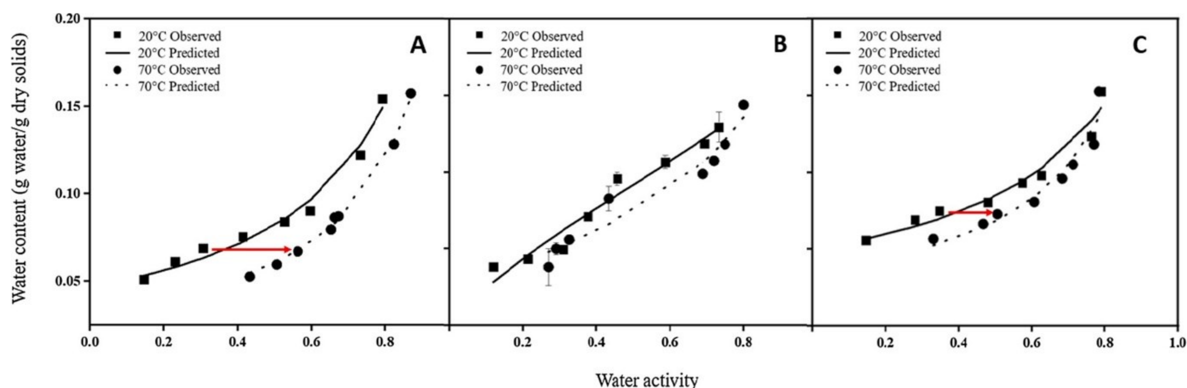


Figure 3.4: Moisture adsorption isotherms of (A) pet food, (B) skim milk powder and (C) burger binder at 20°C (■) and 70°C (●). Data shown are expressed as means ± standard deviation of three independent trials (n = 3). Curves represent the values predicted using the GAB model.

Table 3.5: The kinetic Weibull parameters for the thermal inactivation kinetics of the dry inoculated *S.* Enteritidis FUA1946 in low-a_w foods equilibrated to a_w 0.75.

Values are expressed as means (standard deviation) of the three biological experiments (n = 3).

Food matrix	$a_{w70°C}$	**Observed X_W (db)**	**α-value (min)**	**β-value**	**t_R (min)**
Pet Food	0.82	0.13	11.9 (3.70)	0.72 (0.21)	40.13 (4.19)
Skim milk powder	0.80	0.14	12.2 (2.76)	0.60 (0.22)	56.43 (16.59)
Burger binder	0.77	0.12	16.3 (1.76)	0.84 (0.42)	53.13 (19.36)

3.4. Discussion

This study determined the impact of the food matrix on the desiccation and thermal resistance of three strains of *Salmonella*. Two experimental protocols were used, one involved drying and equilibration of *Salmonella* in the different foods, and a second that employed cells of *Salmonella* that were dried and equilibrated prior to mixing with the different foods followed by thermal treatments. Regardless of the food matrix and the initial cell concentration, *S.* Enteritidis FUA1946 was more heat resistant than *S.* Senftenberg ATCC43845 and *S.* Typhimurium ATCC13311.

Schultze et al. (2020) also reported a high resistance of this wastewater isolate to high pressure carbon dioxide. The variability in the heat resistance of *S. enterica* is highly dependent on the strain specific characteristics (Lianou & Koutsoumanis, 2013) and would potentially be linked to its inherent resistance and genetic mechanism (Guillén et al., 2020).

The wet inoculation resulted in higher *Salmonella* thermal reduction as compared to the dry inoculation. This could be related to the fact that the aqueous culture may not be uniformly distributed in the multi-component foods and may create localized microenvironments, thus affecting the *Salmonella* reduction (Li et al., 2014). Addition of the liquid inoculum into low-a_w foods creates a moisture gradient, which forces the water out of the bacterial cells into the foods until a state of vapor pressure equilibrium has been achieved. This sudden expulsion of the water molecules from the bacterial cells can further contribute to its desiccation and the thermal survival in low-a_w foods. This was followed by subsequent drying, to lower the a_w levels of the foods, thus resulting in reduced water mobility. Furthermore, the inoculated foods were conditioned to the respective a_w of 0.33 and 0.75, thereby creating a moisture gradient involving the movement of the water from the saturated salt solution into the food. At equilibrium, the a_w of the bacterial cell inoculated into the foods is equivalent to the relative humidity of the surrounding environment (Syamaladevi, Tang, & Zhong, 2016). Thus, the rate of moisture diffusion in the foods depends on the relative humidity of the surrounding environment. Therefore, an understanding of the behavior of the bacterial cells under the constantly stressed environment is vital for the thermal process validation studies.

Another reason for the higher *Salmonella* reduction observed in the wet inoculated foods could be due to the interactions between the food components and the inoculation methodology. It has been reported that liquid cultures increase the a_w of the foods, thereby causing greater cell death due to

the osmotic shock (Xu et al., 2020). The higher *S.* Enteritidis FUA1946 reduction observed in the burger binder when using the wet inoculum may relate to the presence of a high concentration of NaCl, which increases osmotic stress, or the presence of antimicrobial compounds in the spices present in the burger binder (Hildebrandt et al., 2017). In contrast, the dry inoculum increased bacterial survival (Hoffmans & Fung, 1992; Xu et al., 2020). Irrespective of the inoculation methodology, no significant difference in the *Salmonella* cell counts was observed in the burger binder and SMP, whereas it was significantly different in the pet food. This indicates that the food components have a major impact on the *Salmonella* survival.

Habituation of *Salmonella* cells to desiccation stress prior to the thermal treatment may offer cross-protection to multiple stresses, including osmotic stress and high temperature (Gruzdev et al., 2011), thus contributing to an increased heat resistance (Mattick et al., 2000). The mechanism underlying the enhanced thermal tolerance of pathogens relates to a decrease in the a_w (Villa-Rojas et al., 2017). Osmotic stress contributes to the enhanced resistance of *Salmonella* (Mattick et al., 2000). Bacterial cells, when exposed to a stressful environment, accumulate or produce compatible solutes including glycine betaine, trehalose, or proline to balance the osmotic pressure without interfering with metabolism (Finn, Condell, et al., 2013), and further prevents protein denaturation, thereby supporting the integrity of the cytoplasmic membrane (Ball, 2008).

The immediate response of *Salmonella* to desiccation also induces an increased expression of the general stress sigma factors, RpoE and RpoS (McMeechan et al., 2007), biosynthesis of trehalose or import of compatible solutes (Balaji et al., 2005), the formation of Fe-S clusters (Finn, Händler, et al., 2013) and catabolism of fatty acids (Finn, Händler, et al., 2013). The transcriptome analysis of *Salmonella* spp. documented the expression of virulence profiles under the desiccation stress (Deng et al., 2012). *S. enterica* strains showed a varied and heterogenous response in the resistance

and the gene expression patterns when exposed to the same stressor (Fong & Wang, 2016b; Sherry et al., 2004). Thereby, for improved mitigation tools, there is a need to elucidate differential gene regulation patterns involved in the pathogen resistance in low-a_w environments (Jayeola et al., 2020). The response of *Salmonella* to desiccation is thus impacted by the food matrix which determines the time of desiccation, the availability of compatible solutes or of substrates of compatible solutes, and the presence of salts. The present study compared the heat resistance of *Salmonella* that were dried on different foods or added to different foods after drying in peptone water and thus demonstrated that the food matrix strongly impacts the adaptation of *Salmonella* during desiccation.

Salmonella that are present in low moisture foods exhibit increased resistance not only to heat but also to UV irradiation and sanitizing agents, thereby reducing the efficiency of these strategies for pathogen control (Gruzdev et al., 2011). Heat and desiccation are the commonly employed stressors in low-a_w food processing (Fong & Wang, 2016a). It is evident that upregulation of the genetic and virulence traits plays an important role in the enhanced *Salmonella* survival under different stress conditions (Gruzdev et al., 2012). Hence, further investigation on *Salmonella's* stress response mechanism under desiccated and thermal conditions is required for quantitative microbial risk assessment (Guillén et al., 2020). Moreover, it is of paramount importance to consider the strain-specific differences in the *Salmonella* thermal inactivation kinetics in a multicomponent food system.

Drying in the different food matrices impacted bacterial survival. This could be due to differences in the desiccation tolerance, a_w (Podolak et al., 2010), inoculation methodology (Liu et al., 2019; Li et al., 2014) and the composition of the foods (Li et al., 2014). Regardless of the differences in the physical structures of the food matrix, the inoculation methodology used did not have a

significant ($p = 0.3982$) effect on the thermal reduction of *S.* Enteritidis FUA1946 in the pet food in the pelleted or the ground form. This may be attributable to the high fat content of the pet food, which might have provided an additional protective effect on the bacteria against cell injury (Podolak et al., 2010). Moreover, there could be gradual dehydration of the bacterial cells in the lipid phase, which had a positive effect in increasing the bacterial thermal resistance (Ahmed & Conner, 1995). However, the reduction of *S.* Enteritidis FUA1946 inoculated into the burger binder and SMP was significantly ($p < 0.05$) impacted by the inoculation methods. This further implies the need to consider the differences in physical structures, inoculation methods, a_w and food composition for designing the thermal inactivation models for low-a_w foods.

To further relate the thermal resistance of the *Salmonella* strains to a_w change during heat treatment, the sorption isotherms of pet food, SMP, and binder were determined at 70 °C (Figure 3.4). If a_w change during thermal treatment was the main parameter affecting the thermal resistance of *Salmonella* serovars, then their highest inactivation rate would have been observed in the pet food (Figure 3.4A), where the change of a_w during heating was greatest, and lowest inactivation would have been observed in the SMP, where the change of a_w during heating was smallest (Figure 3.4B). However, in this study, food matrix had a major impact on inactivation of *Salmonella* on wet inoculated foods (Figures 3.1 and 3.2), but the difference was much smaller after dry inoculation (Figure 3.3). This further demonstrates that the effect of the matrix on the osmotic stress response of *Salmonella* before thermal treatments has a stronger influence on its dry heat resistance than the a_w changes during thermal treatment. The choice of the inoculation methodology thus significantly ($p < 0.05$) impacted the thermal resistance of *Salmonella*.

Generally, to simulate the worst-case scenario, thermal death time studies are performed using a cocktail culture of various strains. Since the thermal resistance (Mercer et al., 2015) varies at the

strain level, hence under ideal circumstances, screening of the *Salmonella* strains based on their relative resistance is important in the selection of the target organism for the thermal validation studies. Moreover, the differences in the drying behavior of *Salmonella* and the variations in the physicochemical properties of the multi-component food system adversely affect the *Salmonella* serovars death rates. Further research is required to characterize the organism based on its relative resistance. Since a limited number of strains have been assessed in this study, therefore continued testing of a higher number of phenotypically different *S. enterica* strains will help in validating challenge protocols for risk assessment in a low-a_w environment (Finn, Condell, et al., 2013). Presently, limited information is available on the desiccation tolerance and thermal resistance of the individual *Salmonella* strains in different low-a_w foods.

3.5. Conclusions

The results of this study provide insights into the impact of multi-component food matrices on the desiccation tolerance and thermal susceptibility of the *Salmonella* serovars. It is ideal for lethal step validation studies to simulate the natural sources of low-a_w food contamination. The thermal resistance of *Salmonella* spp. was dependent on the strain type, inoculation methodology, a_w and the nature of the food components. In the selected low-a_w foods, the wet inoculum of *S.* Enteritidis FUA1946 exhibited the highest desiccation and heat tolerance, and *S.* Typhimurium ATCC13311 exhibited the highest heat sensitivity among the selected strains. Irrespective of the inoculation methodology, no significant difference in the *Salmonella* cell counts was observed in the burger binder and SMP, whereas it was significantly different in the pet food, indicating that the food components have a major impact on the *Salmonella* survival. Overall, this study provided an understanding of the effects of different food matrices and a_w on the thermal resistance of the *Salmonella* strains.

Chapter 4: Understanding the potential of selected disinfection technologies on the inactivation of desiccated *Salmonella enterica* on stainless steel

4.1 Introduction

Salmonella is a significant public health concern organism, causing serious foodborne illnesses. Low-water activity (a_w) foods have been associated with salmonellosis outbreaks (CDC, 2009a; Podolak et al., 2010 Werber et al., 2005). *Salmonella* has been isolated from the dust (Craven et al., 1975), floors (Binter et al., 2011; Morita et al., 2006), and equipment surfaces (Gounadaki et al., 2008) of low-a_w processing plants. The bacterium establishes itself in the dry conditions of low-a_w food processing facilities and persists for considerable periods (Finn, Condell, et al., 2013; Reiji Hiramatsu et al., 2005; Werber et al., 2005).

Stainless steel is extensively used for equipment fabrication in the food industry (Schmidt et al., 2012). Bacterial adhesion onto the equipment surfaces and subsequent resistance to the dry conditions represent a potential cross-contamination route of pathogenic microorganisms during processing (Gabriel et al., 2018; Kim, Kim, et al., 2019). Moreover, improper disinfection, fluctuations in the environmental conditions, poor equipment design (Alavi & Hansen, 2013), and plant closures remain potential factors for *Salmonella's* persistence in low-a_w processing environment (Carrasco et al., 2012). Because the bacterium can adapt to environmentally stressed conditions such as desiccation, heat, and disinfectants, this signifies the need to implement appropriate disinfection control measures to avoid bacterial growth and spread in the processing environment (Scott et al., 2009).

The common disinfection procedures such as the use of chlorine dioxide (Park & Kang, 2018), sodium hypochlorite (Djebbi-Simmons et al., 2019), peracetic acid (Kim et al., 2007), hydrogen

peroxide and dry heat (Gruzdev et al., 2011), employed in low-a_w food industry may not completely remove the pathogens from the equipment surfaces (Marriott et al., 2005). Moreover, chemical disinfectants may generate hazardous by-products, and their continued use may build bacterial resistance to antimicrobials (Verraes et al., 2013). Consequently, it is vital to explore alternative sustainable approaches for treating the desiccated *Salmonella enterica* present on contact surfaces within low-a_w food industry.

Plasma-activated water bubbles (PAWB) is a novel technology that can potentially be applied for surface disinfection (Rothwell et al., 2022). PAWB has a stronger antibacterial activity due to their increased surface area to volume ratio and superior reactive oxygen and nitrogen species dissolution (Mai-Prochnow et al., 2021). The antibacterial mechanism can be related to its oxidation potential, which is associated with the generation of the reactive oxygen and nitrogen species ($OH^{\bullet}$, $NO^{\bullet}$, O_2^-, $OONO_2^-$, $ONOO^-$, H_2O_2) (Wu et al., 2020). Plasma discharge under water is a clean, efficient, and eco-friendly technology that produces no hazardous by-products and can be preferable to conventional disinfection methods (Parmar & Majumder, 2013).

Studies have highlighted the long-term persistence of *Salmonella* under dry conditions in low-a_w food processing facilities (Bashir et al., 2022; Hiramatsu et al., 2005; Margas et al., 2014) and its subsequent resistance towards various disinfection methods involving dry heat, sodium hypochlorite, hydrogen peroxide (Gruzdev et al., 2011). However, the efficacy of different disinfection techniques against *Salmonella enterica* that could be present in distinct cellular states as a result of diverse environmental conditions prevalent in low-a_w food industry remains inadequately investigated. There is a need to comparatively understand the effectiveness of oxidizing agents, membrane-active agents, and dry heat in relation to the survival of *Salmonella* in various dry conditions. The present study aimed to evaluate the effectiveness of various

disinfection techniques, including dry heat, benzalkonium chloride (BAC), conventional chemical oxidizing agents such as peracetic acid (PAA), and hydrogen peroxide (H_2O_2) as well as novel oxidizing agents including plasma activated water bubbles (PAWB) and plasma-activated hydrogen peroxide water bubbles (PAHP-WB) on the survival of *Salmonella enterica* under conditions relevant to low-a_w food industry. Furthermore, the recirculation efficacy of PAWB against the surface-attached *Salmonella* on stainless steel coupons (SSCs) was also evaluated. The results obtained from this study will provide information on the desiccation survival of *Salmonella* in response to the disinfection regimes commonly employed in low-a_w food industry and help in the development of improved strategies for *Salmonella* control.

4.2 Materials and methods

4.2.1 Surfaces for inoculation

Stainless steel coupons (SSCs type 304; 1 × 1 × 0.1 cm) obtained from a local supplier were used as test surfaces to study the air-dried, and air-dried and equilibrated survival characteristics of the attached bacteria. Prior to experimental use, the SSCs were first soaked in a detergent and rinsed with distilled water. Subsequently, they were disinfected with 70% (v/v) ethanol and air-dried for 1 h. Next, the coupons were sterilized at 121 °C for 15 min (Fernandes et al., 2015).

4.2.2 Experimental design

The experimental methodology involved both suspended and surface-attached *Salmonella* cells (Figure 4.1A).

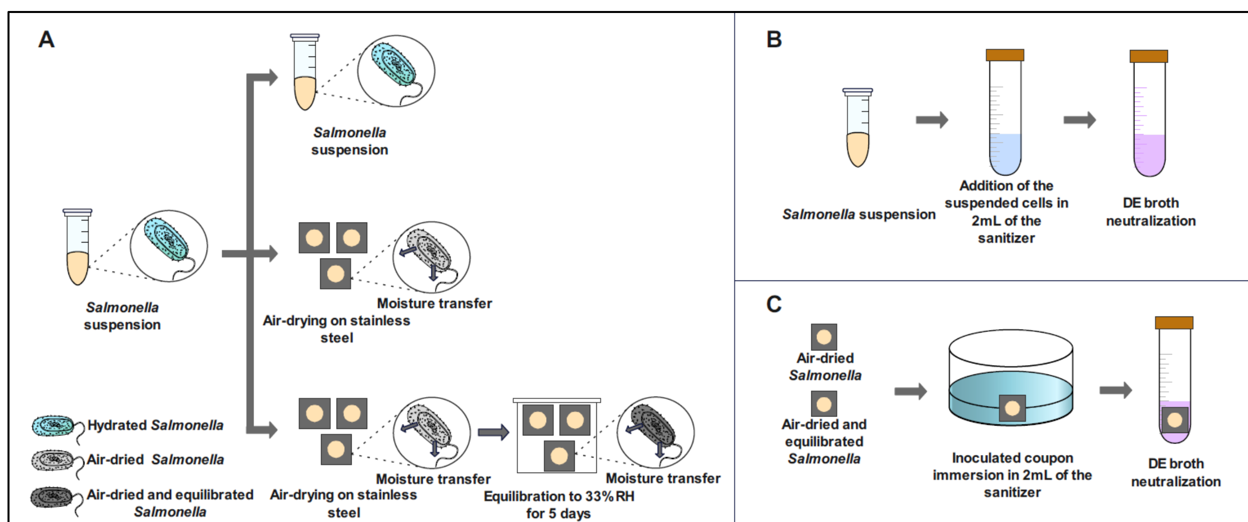


Figure 4.1: Graphical illustration of the experimental design used.

4.2.3 *Salmonella* suspension

S. Enteritidis FUA1946, a wastewater isolate was used as a relevant model pathogen because of its exhibited high heat resistance (Wang et al., 2021). The stock culture was maintained at −80 ºC in tryptic soy broth (TSB; Difco, BD, Franklin Lakes, NJ, USA) with 70% (v/v) glycerol. Prior to use, the frozen culture was activated by streaking onto the tryptic soy agar (TSA; Difco, BD, Franklin Lakes, NJ, USA) plates supplemented with 0.6% yeast extract and incubated for 24 h at 37 °C. Working cultures were prepared by inoculating an isolated colony in 5 mL tryptic soy broth and the culture was incubated for 24 h at 37 °C. Subsequently, 100 µL of the initial subculture was transferred into fresh 5 mL of TSBYE and incubated overnight under the same conditions. Cells were harvested by centrifugation at 9632 × g for 7 min, washed twice in 0.1% peptone water, and resuspended in 0.1% peptone solution to achieve inoculum with approximately 10^9 CFU/mL. Subsequently, a suspension of 20 µL was subjected to treatment with different disinfectants.

4.2.4 Air-dried *Salmonella* adhered on stainless steel

In the first set of desiccation experiments, the efficacy of the *S*. Enteritidis FUA1946 inoculated on SSCs and dried in the biosafety cabinet was assessed against the various disinfection treatments. Briefly, a 20 µL aliquot of the suspension prepared above was deposited onto the surface of sterile SSCs and evenly spread to yield an initial concentration of approximately 7 log CFU/cm^2. The inoculated coupons were dried for 1 h in a laminar flow cabinet for bacterial cell attachment. Following this, the individual coupons were withdrawn, and the bacterial counts were recorded by immersing the coupon in 2 mL of 0.1% peptone. The contents were vortexed for 2 min and 100 µL of the suspension was spread plated onto the TSAYE plates, followed by incubating the plates at 37 °C for 24 h. The number of viable cells was counted and converted to the log-transformed values/cm^2.

4.2.5 Air-dried and equilibrated *Salmonella* adhered on stainless steel

In the second series, referred to as "equilibration challenge assays", *Salmonella* cells were pre-adapted to the equilibration stress. Similar to the first set of desiccation experiments, the SSCs were inoculated with the same concentration of the *Salmonella* suspension and air-dried in the biosafety cabinet at 22–25 °C for 1 h. The inoculated coupons were then transferred to a 33% closed relative humidity (RH) chamber and were equilibrated for 5 days. Following this, an individual coupon was drawn from the desiccation chamber and immersed in 0.1% peptone water for enumeration. Subsequently, the survival response of *Salmonella* cells to the disinfection treatments was assessed.

4.2.6 Dry heat treatment

The efficacy of dry heat on the suspended, air-dried and air-dried and equilibrated *Salmonella* inoculated on SSCs was examined. Briefly, 20 μL of the suspension and the inoculated coupons prepared above were individually placed in the thermal death-time (TDT) cells (Pullman, WA, USA) and then exposed to dry heat at 70 and 80 °C for 4, 8, 12, 16, 20 and 24 min in a water bath (Fisher Scientific Isotemp GPD10 Water Bath, Fisher Scientific, Ottawa, ON, Canada). The come-up times recorded using a T-type thermocouple (Fischer Scientific, Ottawa, ON, Canada) were 30 and 35 s for 70 and 80 °C, respectively. After each treatment time, the TDT cells were immediately cooled in an ice bath for 45 s to stop the bacterial inactivation and enumerated.

4.2.7 Weibull model

The *Salmonella* inactivation data obtained by the dry heat treatment was analyzed using the Weibull model (Peleg & Cole, 1998; Van Boekel, 2002), and can be expressed as:

$$\log\left(\frac{N}{N_0}\right) = -\frac{1}{2.303}\left(\frac{t}{\alpha}\right)^{\beta} \tag{4.1}$$

where N indicates the microbial counts (log CFU/cm^2) on the SSCs at the time t, and N_0 is the initial microbial counts (log CFU/cm^2) on the SSCs at the time zero. The kinetic Weibull parameters α and β represent the scale parameter and the shape index, respectively, and were calculated using the Solver Add-in algorithm tool in Microsoft Excel 2010 (Microsoft Corp., Redmond, WA, USA) based on minimizing the residual squares errors. The goodness-of-fit indices of the Weibull model were determined (Table A6, Appendix 2).

4.2.8 Treatment with the membrane-acting disinfectants

The efficacy of benzalkonium chloride (BAC; Sigma-Aldrich, Oakville, ON, Canada) against *Salmonella* cells in suspended, air-dried, and air-dried and equilibrated cells on SSCs was determined. The benzalkonium chloride disinfectant was flushed with nitrogen for its storage.

4.2.9 Treatment with the conventional oxidative disinfectants

The efficacy of hydrogen peroxide (H_2O_2; 30% w/w in H_2O; Sigma-Aldrich, Oakville, ON, Canada) and peracetic acid (PAA; 32 wt.% in dilute acetic acid; Sigma-Aldrich, Oakville, ON, Canada), on the survival kinetics of the suspended, air-dried and air-dried and equilibrated *Salmonella* cells adhered to the SSCs was evaluated. Once opened, the peracetic acid and hydrogen peroxide was maintained under refrigerated conditions for up to six months.

4.2.10 Treatment with novel oxidative disinfectants

4.2.10.1 Plasma-activated water bubbles (PAWB)

Plasma-activated water bubbles (PAWB) were produced using a high-voltage generator (Leap100, PlasmaLeap Technologies, Sydney, Australia) as shown in Figure 4.2. The plasma bubble spark discharge was conducted using 50 mL sterile distilled water, with operating parameters of 160 V, 66 µs duty cycle, 1000 Hz, and atmospheric-pressure air (1 SLPM) as the carrier gas. Dry ice in a jacket surrounding the beaker was used to control the temperature increase of the water during plasma discharge. PAWB was generated for 10 and 30 min.

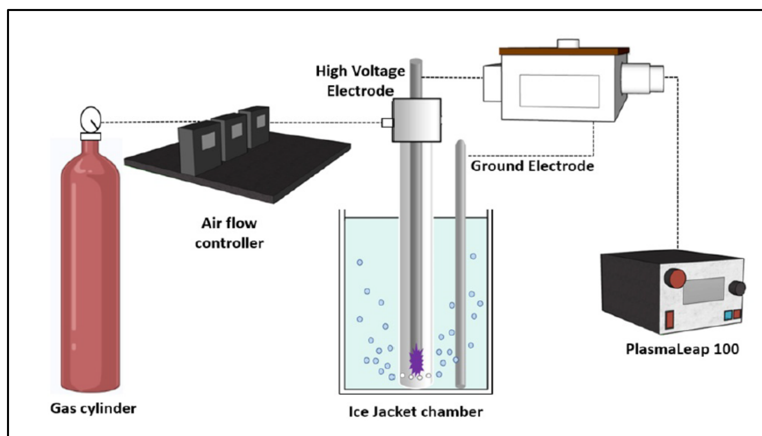


Figure 4.2: Schematic diagram for the plasma-activated water bubbles generation.

4.2.10.2 Plasma-activated hydrogen peroxide water bubbles (PAHP-WB)

Plasma-activated hydrogen peroxide water bubbles were prepared by activating 0.1 M and 1 M H_2O_2 for 10 min using the bubble spark discharge reactor. The plasma process parameters as those outlined in Section 4.2.10.1 were employed to generate PAHP-WB.

4.2.11 Plasma under water discharge characterization

The optical emission spectra (OES) of the excited species generated in the gas-phase under-water plasma discharge were measured using a spectrophotometer (Black comet C-25, StellarNet Inc., Tampa, FL, USA). A fibre optic probe (F600-UVVIS-SR, StellarNet, Inc., Tampa, FL USA) with one end attached to the spectrophotometer, was placed 1 mm from the beaker containing PAWB. The other end of the fibre optic probe was connected to the collimating lens. The spectra were gathered using a spectrophotometer with a resolution of 0.5 and an integration time of 550 ms within 200 to 900 nm of the wavelength range covering the UV and visible spectrum (Chaplot et al., 2019).

The concentrations of the major reactive oxygen and nitrogen species n the PAWB were measured using CHEMetrics test kits: hydrogen peroxide (ferrous ion oxidation by hydrogen peroxide and

formation of ferric thiocyanate, K-5543 kit), ozone (DPD (N,N-diethyl-p-phenylenediamine) oxidation by ozone, K-7423 kit), nitrate (azo dye formation by reduction of cadmium by nitrate, K6933 kit), and nitrite (azo dye formation by diazotization of sulfanilic acid by nitrite, K7003 kit) concentrations were measured as per manufacturer's instructions.

4.2.12 Continuous recirculation of plasma-activated water bubbles using a coupon holder

A benchtop PAWB reactor was built to evaluate the inactivation efficacy under different plasma hold and flow conditions against the surface-attached *Salmonella* on stainless steel. The system comprised a plasma container that was filled with 80 mL of sterile distilled water and was connected to a 3D-printed coupon holder designed using SOLIDWORKS® 2015. PAWB was generated using the operating parameters as listed in section 4.2.10.1 and was simultaneously circulated over the inoculated SSCs using a dosing pump (SIMDOS® 10 UFEM 1.10 S2, KNF Neuberger, Inc.) at a flow rate of 100 mL/min. Figure 4.3 shows the schematic representation of the continuous PAWB benchtop reactor.

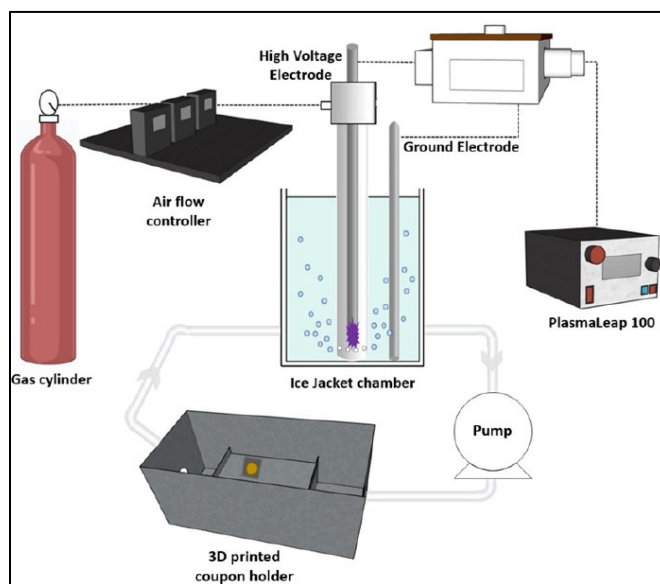


Figure 4.3: Schematic diagram for the continuous generation and circulation of plasma-activated water bubbles using a coupon holder.

4.2.13 Disinfectant treatment and bacterial enumeration

The antimicrobial activity of different types of disinfectants was evaluated against the suspended, air-dried, and air-dried and equilibrated *Salmonella* cells adhered to the SSCs (Figures 4.1B and 4.1C). *Salmonella* suspension and the inoculated SSCs were treated with the disinfectant solutions independently for the following conditions: (1) PAA and BAC solutions were prepared at concentrations of 100 and 200 ppm and subjected to treatment at temperatures of 25 and 40 °C maintained in a commercial temperature-controlled chamber (BTL-433, ESPEC North America Inc., Hudsonville, MI, USA)) for 30, 60, and 90 s, (2) 0.1 M and 1 M H_2O_2 was prepared and used for treatment times of 1, 5 and 10 min (3) PAWB was generated for 10 min and 30 min and exposed for 1, 5, and 10 min (4) 0.1 M and 1 M PAHP-WB were generated for 10 min and exposed for 1, 5 and 10 min.

After the treatment, 2 mL of Dey-Engley (DE) neutralizing broth was added and incubated for 2 min. The *Salmonella* survival counts were obtained by serially diluting in 0.1% peptone water and plating onto TSAYE plates. The concentration of disinfectants and the treatment times were determined to achieve a 1-5 log CFU/cm^2 reduction in cell counts of the surface-dried *Salmonella* on SSCs.

4.2.14 Statistical analysis

The log reductions (N/N_0) obtained for the suspended, air-dried and air-dried and equilibrated *S.* Enteritidis FUA1946 were compared using standard analysis of variance (ANOVA) using Tukey's test with $p < 0.05$ denoting the level of significance among the various disinfection techniques. The statistical interactions obtained among the environmental conditions, temperatures, concentrations, and treatment times under different experimental conditions were examined. The

results obtained were analyzed using the SAS® University edition (Proc Glimmax; SAS studio 9.4).

4.3 Results

4.3.1 Survival of *Salmonella* on SSCs

The initial number of *S*. Enteritidis FUA1946 on SSCs after one hour of air-drying in the biosafety cabinet was 7.56 ± 0.17 log CFU/cm^2. There were significant reductions in the *Salmonella* counts during the equilibration period. *Salmonella* viability after equilibration on SSCs for over 5 days of incubation at 33% RH at room temperature (25 °C) significantly ($p < 0.05$) reduced to 7.14 ± 0.15 log CFU/cm^2.

4.3.2 Efficacy of dry heat against suspended and surface-attached *Salmonella* cells

The *Salmonella* suspension demonstrated a high susceptibility to dry heat treatment, resulting in > 9 log CFU/mL reduction following the come-up time at 70 and 80 °C. The total reduction of air-dried *S.* Enteritidis FUA1946 on SSCs was 0.13 ± 0.09 log CFU/cm^2 and 0.09 ± 0.11 log CFU/cm^2 following the come-up times at 70 and 80 °C, respectively. Furthermore, the air-dried and equilibrated *S.* Enteritidis FUA1946 reduction counts observed after come-up times at 70 and 80 °C were 0.05 ± 0.06 log CFU/cm^2 and 0.10 ± 0.11 log CFU/cm^2 respectively. The Weibull model was used to describe the *Salmonella* inactivation kinetics obtained for both cellular states using dry heat (Table 4.1). The coefficient of determination (R^2) was close to 1 under all conditions (Table A6, Appendix A2), indicating the best fitting to the survival curve. The goodness-of-fit indices of the Weibull model are shown in Table A6 (Appendix A2).

Table 4.1: The kinetic Weibull parameters for the thermal inactivation kinetics of air-dried, and air-dried and equilibrated *S.* Enteritidis FUA1946 on stainless steel coupons after the dry heat treatment at 70 and 80 °C.

Temperature (°C)	Desiccation conditions	*α*-value (min)	*β*-value	t_R (min)	D-value
70	Dried Cells	11.72 (0.89)	1.95 (0.17)	18.02 (1.29)	16.87 (2.34)
	Dried and equilibrated cells	19.13 (0.82)	3.83 (0.73)	23.92 (0.96)	29.16 (7.99)
80	Dried cells	10.05 (2.61)	1.93 (0.31)	15.43 (3.12)	13.00 (3.69)
	Dried and equilibrated cells	14.60 (1.69)	2.21 (0.83)	22.25 (1.63)	23.6 (3.01)

Values are expressed as means (standard deviation) of the three independent trials (n = 3).

The dry heat treatments at 70 and 80 °C significantly ($p < 0.05$) affected the *Salmonella* reduction in SSCs. The treatments at 70 and 80 °C resulted in a steady decline in the air-dried, air-dried and equilibrated *Salmonella* counts on SSCs (Figure 4.4). Under all tested conditions, *Salmonella* survival curves displayed a tailing effect ($\beta > 1$), which means the remaining cells were more susceptible to the heat treatment (Peleg & Cole, 1998). At both treatment temperatures, air-drying and equilibration significantly ($p < 0.05$) increased the resistance of *Salmonella* to the dry heat. A dry heat treatment at 70 °C (Figure 4.4A) for 24 min resulted in a higher reduction of the air-dried cells (1.80 ± 0.03 log CFU/cm^2) in contrast to the air-dried and equilibrated cells (1.05 ± 0.18 log CFU/cm^2). A similar trend was seen at 80 °C (Figure 4.4B), where significantly ($p < 0.05$) lesser reduction of the air-dried and equilibrated *Salmonella* counts (1.26 ± 0.25 log CFU/cm^2) was observed as compared to only air-dried cells (2.33 ± 0.36 log CFU/cm^2). The detailed kinetic and goodness-of-fit and Weibull parameters are listed in Table A6, Appendix A2.

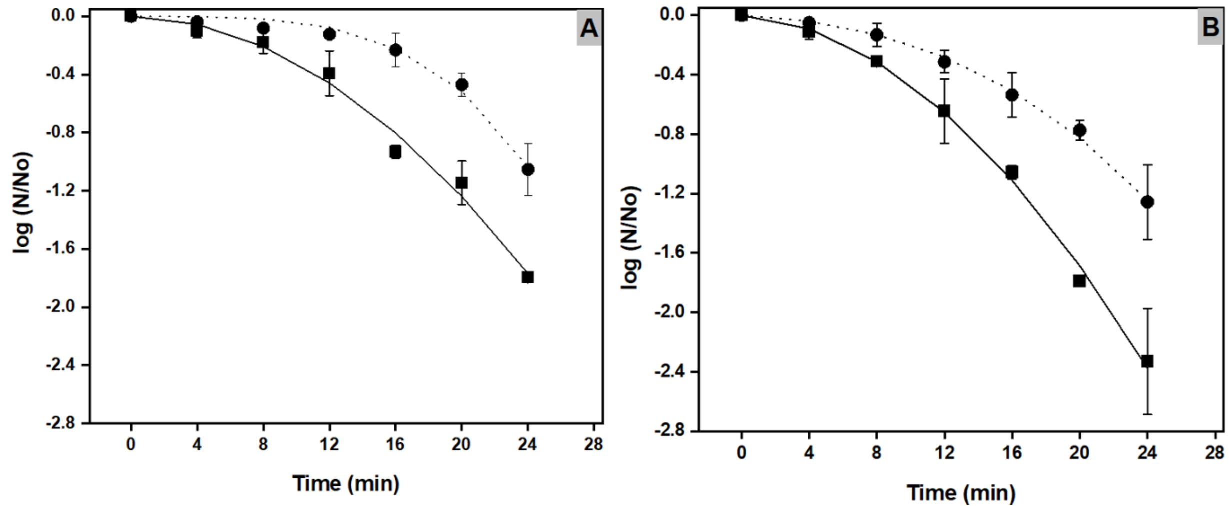


Figure 4.4: Thermal inactivation of *S*. Enteritidis FUA1946 on SSCs using dry heat at (Panel A) 70 and (Panel B) 80 ˚C for up to 24 min. Squares indicate the cells air-dried for 1 h; Circles indicate the cells air-dried for 1 h and equilibrated to an a_w of 0.33 for 5 days. Each point represents the mean ± standard deviation of three independent trials.

4.3.3 Efficacy of benzalkonium chloride against suspended and surface-attached *Salmonella* cells

At 25 and 40 °C, increasing the BAC concentration from 100 to 200 ppm resulted in a significantly ($p < 0.05$) higher reduction of both types of surface-dried *Salmonella* counts on SSCs (Figure 4.5(a)). At both concentrations, BAC for 90 s exposure was the most effective against surface-dried *Salmonella* on SSCs. However, there were no significant ($p = 0.4990$) interactions between temperature and concentration in the inactivation of suspended cells with BAC (Figure 4.5(b)). Nevertheless, an increase in temperature and time significantly ($p < 0.05$) improved the efficacy of BAC against suspended cells.

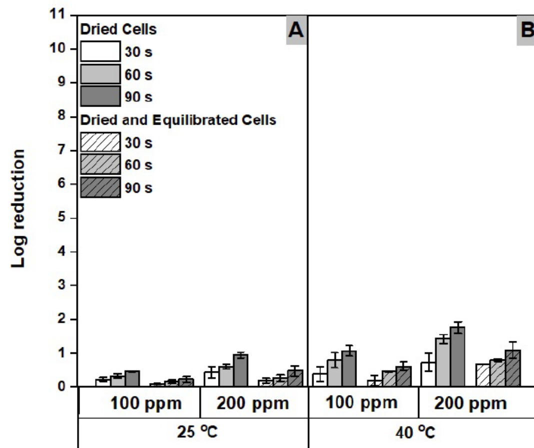


Figure 4.5(a)

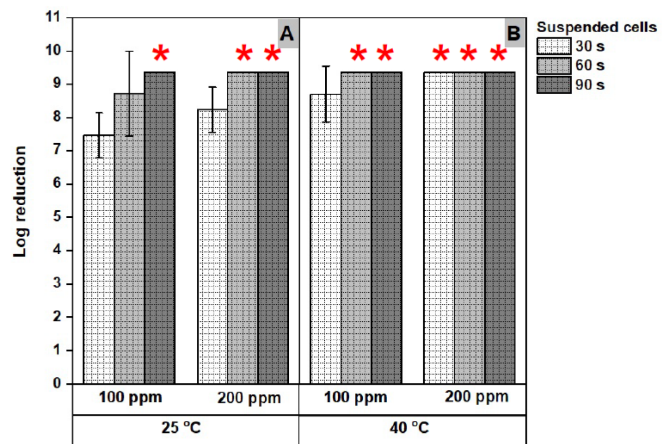


Figure 4.5(b)

Figure 4.5: Sensitivity of (a) surface-attached and (b) suspended *S.* Enteritidis FUA1946 under various conditions to BAC at (Panel A) 25 °C and (Panel B) 40 °C. Bars represent the mean ± standard deviation of three independent trials**.**

* Represents below detection limit of 1.3 log CFU/mL for the suspended *Salmonella* and 1.3 log CFU/cm^2 for the surface-attached *Salmonella* on SSCs.

Temperature significantly ($p < 0.05$) influenced the performance of BAC against the suspended, air-dried, and air-dried and equilibrated *Salmonella* on SSCs, with increasing the BAC temperature from 25 to 40 °C resulting in a higher reduction of *Salmonella*. The resistance of *Salmonella* to BAC was significantly ($p < 0.05$) affected by the various cellular states resulting from environmental conditions. *Salmonella* suspension exhibited the greatest reduction with BAC, followed by air-dried cells on SSCs, and air-dried and equilibrated cells on SSCs exhibited the least reduction.

4.3.4 Efficacy of peracetic acid against suspended and surface-attached *Salmonella* cells

The efficacy of peracetic acid was evaluated at 25 and 40 °C (Figure 4.6). Increasing the PAA concentration from 100 to 200 ppm, significantly ($p < 0.05$) affected the reduction of both suspended and surface-dried *Salmonella* counts on stainless steel.

The inactivation kinetics of air-dried, and air-dried and equilibrated *Salmonella* on SSCs significantly ($p < 0.05$) increased with increasing PAA treatment time (Figure 4.6). For instance, at 40 °C (Figure 4.6(a)), 100 ppm PAA for 30 s exposure resulted in an overall reduction of 2.07 ± 0.47 log CFU/cm^2 and 4.08 ± 0.61 log CFU/cm^2 of air-dried, and air-dried and equilibrated *Salmonella* cells on SSCs, respectively. Increasing the treatment time to 60 s at a similar concentration and temperature reduced both surface-attached *Salmonella* cells to below the detection limit (1.3 log CFU/cm^2). On the contrary, no significant ($p = 0.0954$) effect of treatment time was observed on the reduction of suspended cells (Figure 4.6(b)). For instance, at 40 °C, 100 ppm PAA treatment for 30 s and 60 s treatment resulted in an overall reductions of 8.45 ± 0.81 log CFU/mL and 8.92 ± 1.01 log CFU/mL respectively (Figure 4.6(b)).

The efficacy of the PAA significantly ($p < 0.05$) increased with the temperature increase. A treatment temperature of 40 °C resulted in higher inactivation kinetics of suspended and surface-attached *Salmonella* on SSCs. Overall, environmental conditions had a significant ($p < 0.05$) effect on the resistance of *Salmonella* to PAA treatments. Specifically, air-dried *Salmonella* on SSCs exhibited the greater resistance to PAA followed by air-dried and equilibrated cells on SSCs and *Salmonella* suspension exhibited the highest reduction.

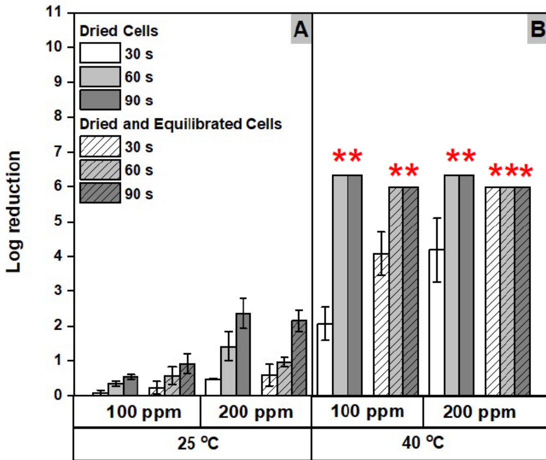


Figure 4.6(a)

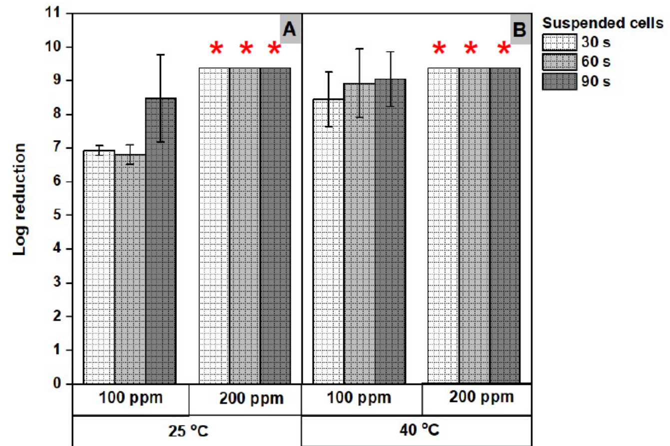


Figure 4.6(b)

Figure 4.6: Sensitivity of (a) surface-attached and (b) suspended *S.* Enteritidis FUA1946 under various conditions to PAA at (Panel A) 25 °C and (Panel B) 40 °C. Bars represent the mean ± standard deviation of three independent trials.

* Represents below detection limit of 1.3 log CFU/mL for the suspended *Salmonella* and 1.3 log CFU/cm^2 for the surface-attached *Salmonella* on SSCs.

4.3.5 Efficacy of hydrogen peroxide against suspended and surface-attached *Salmonella* cells

For both 0.1 M and 1 M H_2O_2 treatment, a significantly ($p < 0.05$) higher reduction of the suspended cells was observed, followed by air-dried, and air-dried and equilibrated cells (Figure 4.7). Significant ($p < 0.05$) interactions were observed between the concentration and treatment time. For instance, 0.1 M H_2O_2 for 10 min resulted in a lesser reduction of 3.09 ± 0.07 log CFU/mL, 1.16 ± 0.25 log CFU/cm^2 and 0.78 ± 0.04 log CFU/cm^2 of suspended, air-dried, and air-dried and equilibrated cells, respectively. However, 1 M H_2O_2 exposure for 10 min resulted in higher reductions of 5.26 ± 0.88 log CFU/cm^2 and 4.68 ± 0.96 log CFU/cm^2 for air-dried and air-dried and equilibrated cells, respectively (Figure 4.7(a)). On the contrary, *Salmonella* cells in suspension were highly susceptible to the H_2O_2 treatment, with exposure to 1 M H_2O_2 for 10 min resulting in > 9 log reduction in the counts (Figure 4.7(b)).

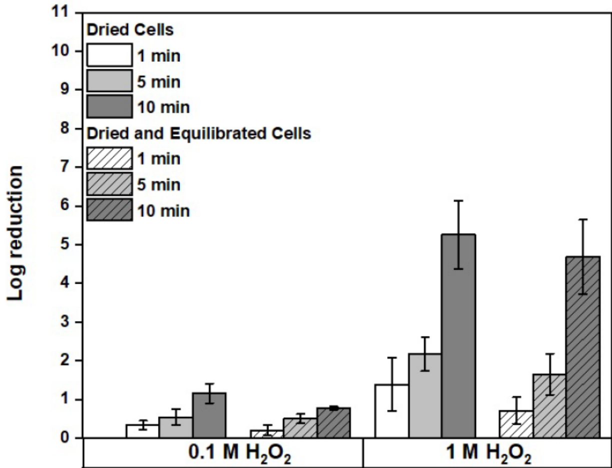


Figure 4.7(a)

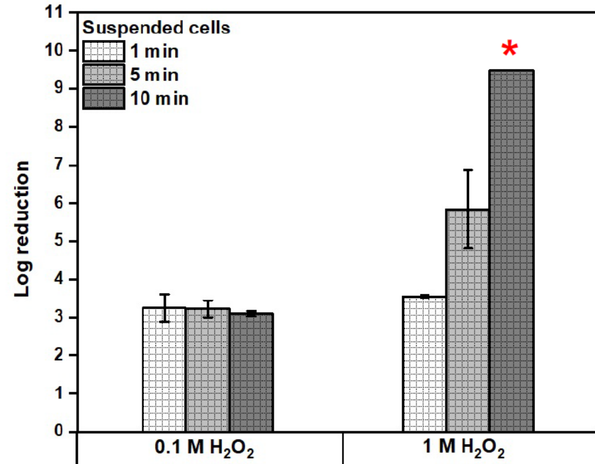


Figure 4.7(b)

Figure 4.7: Sensitivity of (a) surface-attached and (b) suspended *S.* Enteritidis FUA1946 under various conditions to 0.1 M and 1 M H_2O_2 treatment. Bars represent the mean ± standard deviation of three independent trials.

* Represents below detection limit of 1.3 log CFU/mL for the suspended *Salmonella* and 1.3 log CFU/cm^2 for the surface-attached *Salmonella* on SSCs.

4.3.6 Efficacy of plasma-activated water bubbles (PAWB) against suspended and surface-attached *Salmonella* cells

The PAWB generation time significantly ($p < 0.05$) increased *Salmonella* reduction under different environmental conditions. PAWB generation for 10 min resulted in a significantly ($p < 0.05$) lower reduction of suspended and surface-dried *Salmonella* cells than PAWB generation for 30 min (Figure 4.8). Significant ($p < 0.05$) interactions observed between the generation time and the treatment time resulted in a higher reduction of *Salmonella*. For instance, for the PAWB generated for 10 min followed by a treatment time of 10 min, the reductions of suspended, air-dried, and air-dried and equilibrated cells were 4.54 ± 0.93 log CFU/mL, 1.31 ± 0.36 log CFU/cm^2 and 0.63 ± 0.24 log CFU/cm^2 respectively (Figure 4.8). Similarly, for PAWB generated for 30 min, a 10 min treatment time resulted in a 7.08 ± 1.09 log CFU/mL reduction of suspended cells, and, the air-dried cells were reduced by > 6 log CFU/cm^2. On the contrary, the air-dried and equilibrated cells

showed a lesser reduction of 4.90 ± 0.33 log CFU/cm^2. Figures 4.10(a) and 4.10(b) shows the intensity of water-phase reactive species produced as a function of PAWB generation time. Emission spectra showed prominent peaks of OH, N_2, and N_2^+ in the UV region (280 – 400 nm). The intensity of the peaks increased with an increase in the PAWB generation time (Figures 4.10(a) and 4.10(b)). The 310–450 nm range of the spectrum was dominated by N_2 spectra, which correspond to the vibrational band of neutral and ionic emission of nitrogen molecules (Rajasekaran et al., 2012). Significant O peaks were observed at 777 and 844 nm (Yadav et al., 2019) for PAWB generated for 10 min (Figure 4.10(a)). The intensity of the O peaks also increased with an increase in the PAWB generation time of 30 min (Figure 4.10(b)).

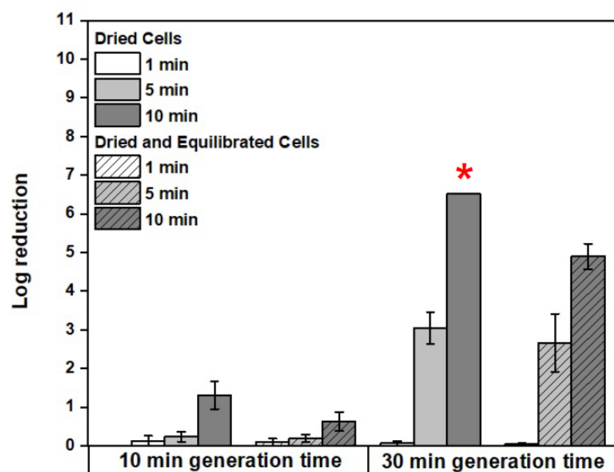


Figure 4.8(a)

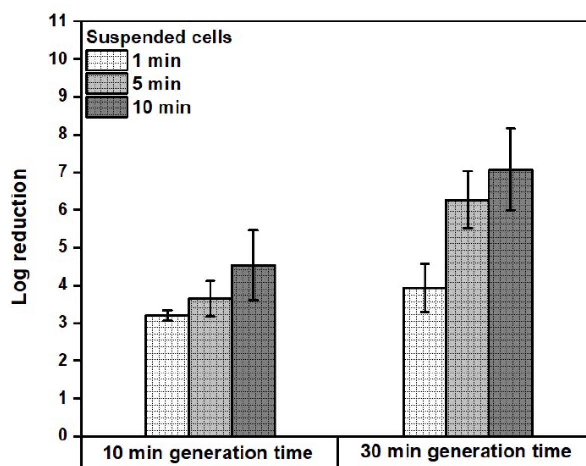


Figure 4.8(a)

Figure 4.8: Sensitivity of (a) surface-attached and (b) suspended *S.* Enteritidis FUA1946 under various conditions to plasma activated water bubbles (PAWB) generated for 10 min and 30 min. Bars represent the mean ± standard deviation of three independent trials.

* Represents below detection limit of 1.3 log CFU/mL for the suspended *Salmonella* and 1.3 log CFU/cm^2 for the surface-attached *Salmonella* on SSCs.

The concentration of RONS increased significantly and the pH of the water dropped from 7.1 to 2.64 and 2.44 after 10 and 30 min of PAWB production, respectively (Table 4.2). The ORP

increased significantly ($p < 0.05$) from 587.33 to 629.33 when the PAWB generation time was increased from 10 to 30 min. Moreover, the concentration of major RONS including, ozone (O_3), nitrate (NO_3^-), and nitrite (NO_2^-) also exhibited a significant ($p < 0.05$) increase with an increase in the generation time of PAWB (Table 4.2). Overall, regardless of PAWB generation time and treatment time, air-dried and equilibrated *Salmonella* on SSCs were significantly ($p < 0.05$) more resistant to the PAWB treatment followed by air-dried *Salmonella* on SSCs and suspended cells.

Table 4.2: Concentration of major reactive oxygen and nitrogen species produced in the PAWB generated for 10 and 30 min.

Plasma generation time	pH	ORP (mV)	Ozone (ppm)	Hydrogen peroxide (ppm)	Nitrite (ppm)	Nitrate (ppm)
10 min	2.64 ± 0.12^a	587.33 ± 0.58^b	6.70 ± 4.85^b	85.17 ± 6.73^a	19.58 ± 2.60^b	154.04 ± 26.94^b
30 min	2.44 ± 0.10^a	629.33 ± 12.58^a	57.18 ± 6.54^a	120.72 ± 29.01^a	33.08 ± 2.75^a	632.20 ± 40.54^a

Values are means ± standard deviations from n = 3 experiments.
Different superscripts indicate significant ($p < 0.05$) differences within each column.

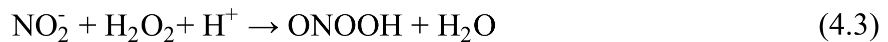
4.3.7 Efficacy of plasma-activated hydrogen peroxide water bubbles (PAHP-WB) against suspended and surface-attached *Salmonella* cells

PAHP-WB demonstrated high effectiveness against *Salmonella* in the suspension. Upon treatment with 0.1 M PAHP-WB for 1 min, suspended *Salmonella* cells were inactivated to below the detection limit of 1.3 log CFU/mL. Increasing the concentration of H_2O_2 in the PAHP-WB from 0.1 M to 1 M, significantly ($p < 0.05$) increased the reduction of both air-dried and air-dried and equilibrated *Salmonella* on SSCs (Figure 4.9). A 5 min exposure to 1 M PAHP-WB reduced *Salmonella* cells adhered to SSCs under both surface attached conditions to below detection limit of 1.3 log CFU/cm^2 (Figure 4.9). The effect of environmental conditions on the desiccation

survival of *Salmonella* on SSCs was found to be statistically insignificant ($p = 0.2682$), in relation to its reduction by PAHP-WB treatment.

Figures 4.10(c) and 4.10(d) show the intensity of water-phase reactive species produced as a function of PAHP-WB concentration. The intensity of the O peaks observed at 777 and 844 nm was higher at 1 M PAHP-WB as compared to 0.1 M PAHP-WB; however, more intense peaks for N_2 spectra were observed for 0.1 M PAHP-WB (Figure 4.10(c)). In addition, OH peaks were found to be missing with increasing concentration of the PAHP-WB (Figure 4.10(d)). The RONS characterization indicated that the ozone concentration in 1 M PAHP-WB was significantly ($p < 0.05$) greater than that in 0.1 M PAHP-WB (Table 4.3). PAWB generated for 10 min resulted in a lower concentration of ozone and hydrogen peroxide species as compared to 0.1 M and 1 M PAHP-WB (Tables 4.2 and 4.3). Furthermore, the nitrate concentration in 0.1 M PAHP-WB was significantly ($p < 0.05$) higher than that in 1 M PAHP-WB. This discrepancy could be due to nitrate decomposition and the subsequent interaction between high concentration of H_2O_2 produced in the 1 M PAHP-WB with the nitrites, thereby resulting in the formation peroxynitrous acid (ONOOH), peroxynitrite ($ONOO^-$), and peroxynitrate (O_2NOO^-) as represented in the reactions below (Bradu, 2020; Lu et al., 2009; Ostrikov et al., 2020);

$$NO_3^- + h\nu \rightarrow NO_2^- + O^\bullet \tag{4.2}$$



$$NO_2^- + H_2O_2 + H^+ \rightarrow ONOOH + H_2O \tag{4.3}$$

$$NO_2^- + H_2O_2 \rightarrow ONOO^- \tag{4.4}$$

$$ONOOH + ONOO^- \rightarrow NO_2^- + O_2NOO^- + H^+ \tag{4.5}$$

The emission spectra of PAWB generated for 10 min (Figure 4.10(a)) demonstrated comparatively lowered peaks of N_2 in relation to the emission spectra of 0.1 and 1 M PAHP-WB. The latter

displayed higher intensities and prominent peaks of N_2 spectra spanning from 280 to 400 nm (Figures 4.10(c) and 4.10(d)).

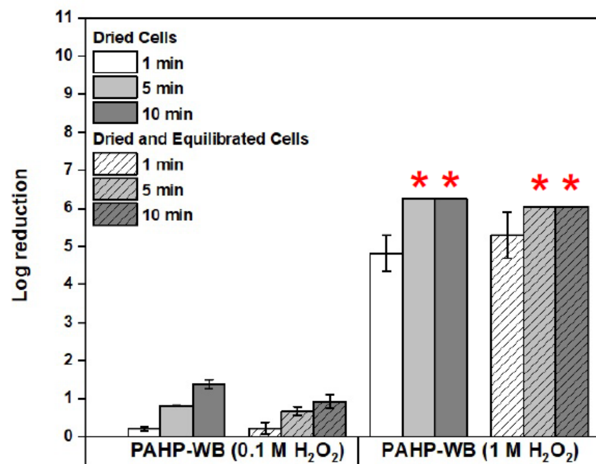


Figure 4.9

Figure 4.9: Sensitivity of *S.* Enteritidis FUA1946 on SSCs under various conditions to plasma-activated hydrogen peroxide water bubbles (PAHP-WB) generated for 10 min. Bars represent the mean ± standard deviation of three independent trials.

* Represents below detection limit of 1.3 log CFU/cm^2 for the surface-attached *Salmonella* on SSCs.

Table 4.3: Concentration of major reactive oxygen and nitrogen species produced in the 0.1 M and 1 M PAHP-WB generated for 10 min.

Conditions	**pH**	**ORP (mV)**	**Ozone (ppm)**	**Hydrogen peroxide (ppm)**	**Nitrite (ppm)**	**Nitrate (ppm)**
0.1 M PAHP-WB	2.42 ± 0.09^a	481.33 ± 2.08^a	57.69 ± 8.67^b	4523.75 ± 1292.37	2.16 ± 0.76^a	289.80 ± 36.53^a
1 M PAHP-WB	2.67 ± 0.13^a	467.67 ± 7.51^b	217.15 ± 32.09^a	Overrating	1.78 ± 0.34^a	128.07 ± 22.05^b

Values are means ± standard deviations from n = 3 experiments.

Different superscripts indicate significant differences within each column.

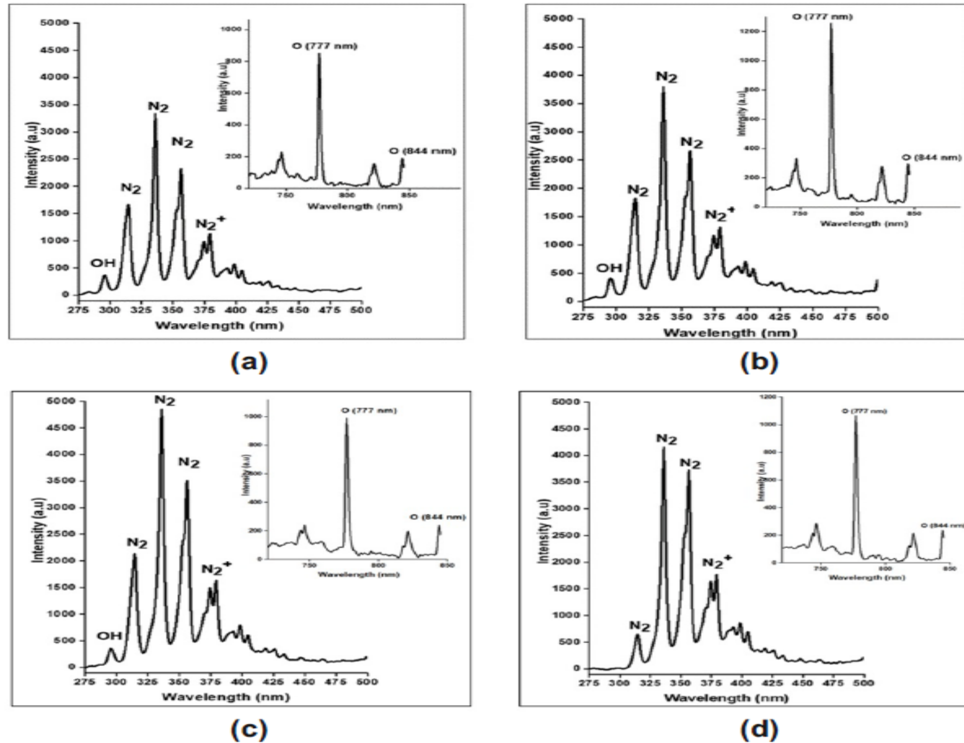


Figure 4.10: Optical emission spectra of plasma discharge under water at (a) PAWB 10 min generation, (b) PAWB 30 min generation, (c) 0.1 M PAHP-WB 10 min generation, and (b) 1 M PAHP-WB 10 min generation.

4.3.8 Efficacy of plasma-activated water bubbles recirculation under different combinations of holding and flowing times in the inactivation of desiccated *Salmonella*

A significant ($p < 0.05$) effect of increasing the PAWB flowing time on the inactivation of air-dried and equilibrated *Salmonella* on stainless steel was observed (Figure 4.11). For instance, a 15 min PAWB hold in combination with 10 min PAWB flow resulted in a lesser reduction of 1.40 ± 0.64 log CFU/cm^2. On the contrary, a continuous production and recirculation of PAWB for a similar treatment time of 25 min, significantly ($p < 0.05$) increased the reduction of the air-dried and equilibrated *Salmonella* to 3.94 log CFU/cm^2.

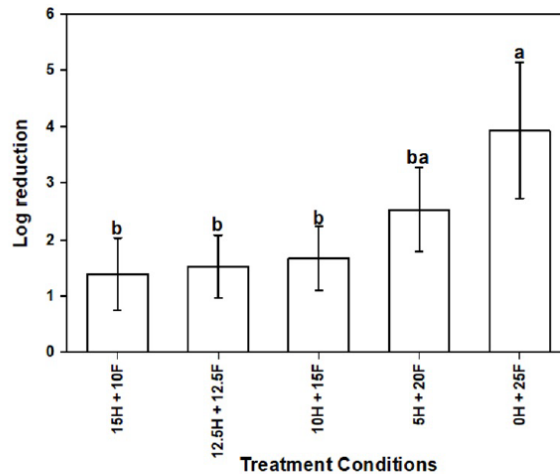


Figure 4.11: Inactivation of *S.* Enteritidis FUA1946 on SSCs by recirculation of PAWB using a coupon holder. Bars indicate the cells air-dried for 1 h and equilibrated to an a_w of 0.33 for 5 days. Each point represents the mean ± standard deviation of three independent trials. Treatment conditions used were 15 min hold (H) + 10 min flow (F), 12.5 min (H) + 12.5 min (F), 10 min (H) + 15 min (F), 5 min (H) + 20 min (F), and 0 min (H) + 25 min (F) in the coupon holder.

4.4 Discussion

This study compared different conventional and novel disinfection treatments to examine the inactivation kinetics of *Salmonella*, under conditions typical of low-a_w food industry. *S*. Enteritidis FUA1946 was selected for this study, due to its remarkable high resistance observed towards other stressors, including dry heat (Wang et al., 2021) and high-pressure CO_2 (Schultze et al., 2020). The introduction of moisture during the cleaning process may result in the removal of surface-attached bacteria and can subsequently form suspensions in the bulk liquid (Møretrø et al., 2009). The two distinct drying conditions were chosen to simulate the environmental conditions of short-term and intermediate desiccation significant to low-a_w food processing facilities. The process of air-drying and air-drying followed by equilibration on SSCs results in mass transfer, thus creating a hypertonic state in the surrounding environment (Csonka, 1989; Maserati et al., 2017; Zoz et al., 2021). This expulsion of water can potentially induce matric and mechanical stresses, leading to

changes in membrane fluidity and phase transitions that can subsequently affect the integrity of the cell membrane (Billi and Potts, 2002).

A significant ($p < 0.05$) effect of dry heat (70 and 80 °C), on the inactivation of the air-dried, and air-dried and equilibrated *Salmonella* on SSCs was observed (Figure 4.4). There were no significant interactions observed between the temperature and treatment time ($p = 0.1013$), thereby resulting in an overall lesser reduction of both types of surface-dried cells. Exposure to the desiccation stress might have resulted in an increased cross-tolerance to the heat treatment, resulting in a subsequent lesser reduction of *Salmonella* at higher temperatures (Kirby & Davies, 1990; Maserati et al., 2018). Moreover, high temperature causes the vibration of water molecules, which disrupts the protein molecular bonds and results in their denaturation (Archer et al., 1998). The resistance of dried *Salmonella* to high temperatures has been related to its low water activity and water content, which inhibits the vibration action of water molecules, thereby resulting in a decreased degree of protein denaturation (Figure 4.1A) (Archer et al., 1998). The air-dried and equilibrated *S*. Enteritidis FUA1946 on SSCs was found to be more resistant to dry heat than the air-dried cells. This could potentially be due to the genetic adaptive mechanisms involved during desiccation stress (Finn, Händler, et al., 2013; Gruzdev et al., 2012). Moreover, the potential coexistence of overlapping osmotic, oxidative, and thermal tolerances can also contribute to a higher resistance to dry heat resulting from desiccation (Maserati et al., 2018).

The effectiveness of chemical disinfectants can be increased by increasing the treatment temperature, concentration, and exposure time (Aksoy et al., 2020). Increasing the BAC concentration (100 to 200 ppm), significantly ($p < 0.05$) increased the reduction of air-dried, and air-dried and equilibrated *Salmonella* on SSCs (Figure 4.5(a)). Higher BAC concentration indicates greater electrostatic interactions between the cationic surfactants and anionic cell

membrane, thereby resulting in an increased membrane destabilization and eventually leading to cell death (Chen et al., 2018; Glover et al., 1999). However, repeated exposure to chemical disinfectants might enhance the bacterial response to antimicrobial resistance, leading to serious public health concerns (Chen et al., 2018). BAC are cationic surfactants (Tandukar et al., 2013), that are commonly used for surface disinfection in the food industry. The bactericidal mode of BAC is due to their cell wall adsorption followed by subsequent membrane disorganization (Tezel & Pavlostathis, 2012). In this study, a lesser reduction in the cell counts of air-dried and air-dried and equilibrated *Salmonella* on SSCs was reported following the BAC treatments (Figure 4.5(a)). Previous studies have reported that the gram-negative bacteria due to the presence of a higher lipid content of their outer membrane, limits the penetration of quaternary ammonium compounds (Frank & Koffi, 1990; Ghoshal et al., 2022). The potential presence of resistance genes encoding for active efflux pumps within *Salmonella's* mobile genomic island has also been hypothesized as a possible explanation for the organism's higher resistance to BAC treatments (Michael & Schwarz, 2016). However, the genes upregulated under desiccation and BAC stress might vary between the strains. The presence of desiccation-resistant and BAC-resistant strains in low-a_w food environments would pose a significant threat to food safety (Haubert et al., 2019).

The present study demonstrated that PAA at both concentrations (100 and 200 ppm) was highly effective against suspended, air-dried, and air-dried and equilibrated *Salmonella* on SSCs (Figure 4.6). This could be due to the decontamination mechanism of the disinfectants and the active components present (H. D. N. Nguyen & Yuk, 2013). PAA is an organic peroxide and is synthesized from acetic acid and hydrogen peroxide. It has a higher oxidation potential and it oxidizes the sulfhydryl and sulfur bonds present in the enzymes (Kim & Huang, 2021). Increasing PAA concentration promotes local acidification of the cell membrane and increases membrane

fluidity by disrupting lipid bilayers, resulting in greater bacterial reduction (Angelova et al., 2018). The PAA concentration used in the study was as per the US FDA guidelines, with not more than 200 ppm for the food contact surface disinfection (FDA, 2023). Increasing the PAA treatment temperature fromof 25 and 40 °C significantly ($p < 0.05$) increased the *Salmonella* reduction as compared to 25 °C (Figure 4.6). An increase in temperature is related to increased transportation of PAA, resulting in a disruption of osmotic balance and increased oxidative stress, thereby contributing to higher inactivation (Laroche et al., 2001). Shen et al. (2019) reported greater effectiveness of PAA at elevated temperatures in comparison to room temperature. The same study reported that the application of peracetic acid (PAA) at a concentration of 80 ppm for a duration of 2 min at a temperature of 43°C resulted in a significant reduction of *Listeria monocytogenes* on apple surfaces, with a decrease of 2.37 ± 0.05 log10 CFU/apple. In comparison, a reduction of only 1.7 log CFU/apple was observed at a temperature of 22°C. A significant decrease in the suspended, air-dried, and air-dried and equilibrated on SSCs was observed upon subjecting them to BAC and PAA treatments at temperatures ranging from 25 to 40 °C (Figures 4.5 and 4.6). The efficacy of bacterial inactivation is reduced at low temperatures, owing to the existence of a densely compacted lipopolysaccharide that exhibits restricted fluidity (Ioannou et al., 2007). Furthermore, a greater reduction was observed in suspended cells. This may be attributed to the effective dispersion of the disinfectant among them, thereby resulting in membrane disruption, lipid peroxidation, and DNA damage (Kunigk & Almeida, 2001).

Studies have indicated that different responses to desiccation might occur based on the type of strains, desiccation period, experimental conditions, and selected surfaces (Finn, Händler, et al., 2013; Gruzdev, et al., 2012; Li, Bhaskara, et al., 2012). Therefore, to ensure food safety, it is essential to analyze the impact of various desiccation conditions on the genetic expression of

bacteria. Increasing the concentration of H_2O_2 (0.1 to 1M) considerably accelerated the inactivation of suspended and surface-dried *Salmonella* on SSCs (Figure 4.7). The greater reduction is attributable to oxidative stress caused by the formation of major reactive oxygen species (O_2^- and $^\bullet OH$). Hydroxyl radical ($^\bullet OH$) is a powerful oxidant that disrupts the electron transport chain, causes membrane damage, lipid peroxidation, and DNA damage and ultimately leads to cell death (ImLay & Linn, 1988).

This study also assessed the effectiveness of two novel oxidizing disinfectants, PAWB and PAHP-WB, against the dried *Salmonella* on SSCs (Figures 4.8 and 4.9). The antibacterial efficacy of PAWB is due to the oxidative stress caused by the synthesis of short-lived ($^\bullet OH$, $^\bullet NO$, O_2^-, $OONO_2^-$, $ONOO^-$) and long-lived (NO_2^-, NO_3^-, H_2O_2, O_3) reactive species (Tables 4.2 and 4.3) (Mai-Prochnow et al., 2021).

PAWB contains electrons, ions, UV photons, charged particles, and reactive oxygen and nitrogen species; its antimicrobial efficacy can be improved by combining it with H_2O_2 (Wu et al., 2017). Plasma activation of H_2O_2 generates greater quantities of both short- and long-lived (HNO_2, NO_3, H_2O_2) reactive species (Table 4.3), which can contribute to its enhanced inactivation efficiency (Figure 4.9). The oxidative stress produced by PAHP-WB could influence the inactivation of microorganisms. Wu et al. (2017) observed that combining plasma-activated water (PAW) and H_2O_2 resulted in a higher *S. aureus* inactivation as compared to PAW alone. H_2O_2 is a potent ROS that can be generated in the gaseous phase during plasma discharge (Mai-Prochnow et al., 2021). In addition, $^\bullet OH$ radicals produced by PAHP-WB diffuse into the liquid phase and form H_2O_2 (Scally et al., 2021). Additional plasma radical reactions are depicted in the following equations (Cui et al., 2018; Scally et al., 2021).

$$^{\bullet}OH + {}^{\bullet}OH \rightarrow H_2O_2 \quad (4.6)$$

$$O_3 + OH \rightarrow HO_2 + O_2 \quad (4.7)$$

$$O_2 + {}^{\bullet}H \rightarrow HO_2 \quad (4.8)$$

$$H_2O_2 + O_3 \rightarrow {}^{\bullet}OH + O_2 + HO_2 \quad (4.9)$$

$$^{\bullet}H + HO_2 \rightarrow H_2O_2 \quad (4.10)$$

$$2H_2O \rightarrow H_2O_2 + H_2 \quad (4.11)$$

$$H_2O_2 + {}^{\bullet}H \rightarrow H_2O + {}^{\bullet}OH \quad (4.12)$$

The high concentration of RONS (Table 4.3) generated in 1 M PAHP-WB, resulted in a higher reduction of surface-dried *Salmonella* on SSCs. A similar study by Wu et al. (2017) produced PAW containing H_2O_2 (0.001, 0.01 and 0.1 M) and reported an increase in the generation of H_2O_2, $^{\bullet}OH$, and $^{\bullet}NO$ radicals, which subsequently increased its reduction potential and resulted in a greater leakage of intracellular components in *S. aureus*.

The environmental conditions had a significant ($p < 0.05$) impact on increasing the resistance of *S.* Enteritidis FUA1946 to H_2O_2, PAA, PAWB, BAC, and dry heat treatments. The resistance of air-dried and equilibrated *Salmonella* on SSCs to H_2O_2, PAWB, BAC, and dry heat treatment was significantly ($p < 0.05$) higher than that of air-dried cells on SSCs. On the contrary, air-dried cells were found to be more resistant than air-dried and equilibrated cells to the PAA treatment. however, no effect ($p = 0.4087$) of the desiccation method was observed in the reduction of the surface-dried *Salmonella* cells by PAHP-WB treatment.

The results of this study indicated that the suspended cells showed greater susceptibility to all the disinfection treatments. Several studies have demonstrated that suspended cells exhibit a greater susceptibility to the effects of various disinfectants in comparison to surface-attached cells (Carballo & Araújo, 2012; Kusumaningrum et al., 2003; Lineback et al., 2018). Moreover, the

surface-dried cells on SSCs demonstrated a higher susceptibility to oxidizing agents, while the least inactivation of both surface-dried cells was observed using dry heat and benzalkonium chloride (BAC) treatments. The food industry uses air-drying techniques after cleaning and disinfection procedures to eradicate the cold spots and reduce moisture content, to eventually prevent the growth and proliferation of microorganisms (Esbelin et al., 2018; Zoz et al., 2016). However, insufficient cleaning and disinfection protocol can result in the survival of microorganisms, which may encounter various environmental stressors due to changes in the humidity levels of food processing facilities (Zoz et al., 2016; Zoz et al., 2021).

The desiccation mechanism responsible for *Salmonella's* survival under low-a_w conditions remains elusive and is dependent on the experimental design. *Salmonella* encounters various lethal changes in the environment and thus responds by modifying its physiological and structural characteristics (Spector & Cubitt, 1992). Moreover, bacteria can synthesize various low-molecular-weight compatible solutes (proline, betaine, and trehalose) to sustain the turgor pressure in response to matric stress generated due to air-drying (Finn, Condell, et al., 2013). The desiccation response of 15 *Salmonella* strains on stainless steel was investigated by Margas et al. (2014). *Salmonella* strains were subjected to a drying process at 30 °C for 80 min followed by equilibration to 33% RH. A decrease in cell viability was observed within the first 72 h, after which no further reduction was observed until the end of 30 days. The study reported that the length of the desiccation period had no significant effect on *Salmonella* reduction. Margas et al. (2014) indicated that *Salmonella* can adapt to different environmental stressors over a period of time, leading to the development of subpopulations. Moreover, the initial stress may result in a more substantial reduction in cell counts, whereas subsequent adaptation to stress may lead to a comparatively lesser reduction (Margas et al., 2014). This hypothesis holds significance for this

study, as for most of the stressors a greater disinfection resistance for air-dried and equilibrated cells was observed, as compared to air-dried *Salmonella* on SSCs. The potential correlation between the extended survival of foodborne industry isolates in dry production environments and their persistence over time demands further investigation (CDC, 2011a; CDC, 2012f; CDC, 2013c). For future research, it is crucial to evaluate the disinfection efficacy, using strains isolated from low-a_w food industry, as they might be well adapted to the desiccation stress.

Spray and immersion methods are used for the disinfection of food contact surfaces (Holah et al., 1998). In this study, to simulate the food industry disinfection protocol, the effectiveness of PAWB recirculation against the surface-attached *Salmonella* on SSCs was investigated. Air-dried and equilibrated *Salmonella* was selected due to its demonstrated high resistance observed towards various other stressors (BAC, dry heat, PAWB and hydrogen peroxide treatment) as compared to the air-dried *Salmonella*. Under a similar treatment time (25 min), increasing the PAWB flow time resulted in a higher reduction of air-dried and equilibrated *Salmonella* on SSCs (Figure 4.11). This potentially could be either due to the high shear stress generated (Xu & Tan, 2023) or due to the increased interaction of RONS with the surface-attached bacteria at higher PAWB flow treatment times.

A better understanding of the transcriptome of air-dried, and air-dried and equilibrated *Salmonella* post-exposure to different disinfection treatments is required to prevent the spread of the bacterium. In this study, a heat-resistant *Salmonella* strain was used to understand its resistance to the common disinfection treatments used in low-a_w food industry. However, to mimic the worst-case scenario, future work should incorporate the use of multiple desiccation-resistant strains in a cocktail. Furthermore, there is a possibility of overlapping between the stress responses to osmotic

stress and desiccation stress, making it difficult to identify the mechanisms specific to each of these stresses (Gandhi & Chikindas, 2007).

4.5 Conclusions

The results of this study demonstrated the significant effect of the environmental conditions, treatment temperature, disinfectant concentration, and exposure time on the survival of suspended, air-dried, and air-dried and equilibrated *Salmonella* on SSCs. However, the effectiveness of these parameters will differ based on the experimental conditions employed. For instance, under tested conditions, desiccation significantly ($p < 0.05$) increased the resistance of air-dried and equilibrated *Salmonella* to H_2O_2, PAWB, BAC, and dry heat treatments than the air-dried cells. On the contrary, air-dried cells observed a higher resistance to the PAA treatment. Furthermore, no effect of desiccation was showed for the PAHP-WB treatment. This discrepancy observed in comparison to other disinfection methods may be due to the environmental conditions selected. The research holds significant importance as low-a_w food industry generally uses various oxidizing and membrane-active agents to ensure adequate disinfection. The introduction of moisture in low-a_w food industry can facilitate bacterial proliferation. The process of drying and subsequent equilibration to the humidity levels commonly found in low-a_w food processing environments can lead to a subsequent increase in *Salmonella's* resistance to disinfectants. This necessitates the importance of selecting appropriate disinfecting agents. Moreover, the study signifies the importance of understanding the diverse conditions of desiccation resistance of *Salmonella* against the commonly employed disinfection techniques in low-a_w food industry.

Chapter 5: Continuous production and recirculation of plasma-activated water bubbles under different flow regimes for mixed-species bacterial biofilm inactivation inside pipelines

5.1 Introduction

Biofilms are composed of complex bacterial communities, embedded in extracellular polymeric matrix substances (EPS) consisting of polysaccharides, nucleic acids, and proteins (Liu et al., 2016). EPS provides mechanical stability (Zheng et al., 2021) and protection against various antimicrobial treatments (Davies, 2003; Simões et al., 2005a; Simões et al., 2009).

Salmonella spp., *Pseudomonas* spp., and *Campylobacter jejuni* are frequently isolated from the broiler drinking water systems (DWS) (Chia et al., 2009; Jafari et al., 2006; Maes et al., 2019; Maes et al., 2020; Zimmer et al., 2003). *Aeromonas* is also recognized as an important biofilm-forming waterborne pathogen, commonly prevalent in the DWS (Aravena-Román et al., 2013; Van Der Wielen & Lut, 2016). Biofilm colonization of the DWS (Hahne et al., 2022) serves as a microbial reservoir and increases the load of waterborne pathogens (Mohammed & Attia, 2022). The frequent association of *Salmonella* in broiler chickens, eggs, and raw poultry meat is a major public health concern and can lead to significant economic losses in the broiler industry (Cosby et al., 2015; Oliveira et al., 2014).

Biofilm formation on the inner surfaces of the DWS can limit the action of various disinfectants (Wang et al., 2023) due to restricted water flow rates and clogged drinker lines (Maes et al., 2020). Moreover, biofilm dispersal and subsequent attachment on clean surfaces serve as a waterborne transmission of pathogens and can increase the risk for animal and human infections (Gomes et al., 2018; Stoodley et al., 2002). This signifies the need for effective disinfection of the drinker

lines to reduce the microbial load and minimize the associated risks involving animal health (Liu et al., 2016).

Harsh mechanical operations such as brushing, water flushing, and pigging are relatively cheap, and are effectively used to control the growth of biofilms (Lehtola et al., 2004). Various cationic surfactants, oxidative disinfectants and chlorine-based compounds are conventionally employed for the disinfection of the drinker lines (Gomes et al., 2018; Maes et al., 2019; Simões et al., 2005a). The use of chemical disinfectants pose a detrimental risk to the environment (Fraise, 2002) as their interaction with the biofilm organic matter might result in the formation of hazardous by-products (Pechaud et al., 2012). Moreover, chemical disinfection increases the risk of antimicrobial resistance among the bacterial species(Cosby et al., 2015). Furthermore, the harmful chemicals that remain in the bulk fluid can subsequently be transmitted to the broilers . It is therefore of utmost importance to use sustainable approaches for biofilm control in the DWS.

Plasma-activated water bubble (PAWB) technology is a promising, sustainable approach for the inactivation and control of biofilms (Mai-Prochnow et al., 2021; Zhou et al., 2019). Studies have investigated the use of PAWB as a green sanitizer for inactivating the *E. coli* and *Klebsiella michiganensis* biofilms grown on stainless steel surfaces (Zhou et al., 2019) and PVC pipes (Xu & Tan, 2023), respectively. The gas phase discharge exhibits a reactive environment composed of free electrons, charged particles, UV photons, ions, radicals, and various reactive oxygen and nitrogen species (RONS) (Hong et al., 2021; Hu et al., 2022). A high antimicrobial potential of PAWB is due to the formation of ozone (O_3), superoxide (O_2^-), singlet oxygen (O), hydrogen peroxide (H_2O_2), hydroxyl radical (OH), nitrate (NO_3^-), nitrite (NO_2^-), peroxynitrite (ONOOH) and nitric oxide (NO) (Xu & Tan, 2023; Zhou et al., 2019). Plasma discharge in the gas and the subsequent dissolution of the reactive species in the bubbles provide enhanced mass transfer and

increase their residence time in the aqueous environment (Hong et al., 2021; Katsaros et al., 2023; Zhou et al., 2019). Moreover, bubbles upon bursting create mechanical agitation, and cause localized heating, thereby resulting in a higher biofilm inactivation. PAWB technology can be effectively used for DWS disinfection as it is an eco-friendly approach and does not generate any hazardous chemical residues on surfaces (Los et al., 2020).

Previous literature has evaluated bacterial adhesion and biofilm formation on various surfaces under different hydrodynamic flow regimes (Brugnoni et al., 2011; Chang et al., 2020; Gomes & Mergulhão, 2021; Horn et al., 2003; Percival et al., 1999; Perni et al., 2006; Shen et al., 2015; Zheng et al., 2021). In a fluid flow system, the hydrodynamic fluctuations also play a key role in governing biofilm dispersion (Oder et al., 2018). Previous research has explored the impact of shear stress using plasma-activated water and plasma-activated microbubble water on the inactivation of surface-adhered biofilms (Tan & Karwe, 2021; Xu & Tan, 2023), yet an integrated study determining the effect of varying hydrodynamic fluctuations experienced in the DWS is rarely studied. PAWB disinfection under hydrodynamic conditions provides better transportation of the reactive species and imparts shear stress (Xu & Tan, 2023) that could alter biofilm structures and contribute to its dispersal (Gomes et al., 2018; Lemos et al., 2015). This study aimed to investigate the influence of different flow regimes (laminar, transitional, and turbulent)that may impact the efficacy of PAWB against mixed species biofilms developed on the inner surfaces of the PVC pipes. The effect of the PAWB volume, concentration of the major plasma RONS, and treatment time was also explored for biofilm inactivation, under hydrodynamic conditions relevant to the broiler DWS.

5.2 Material and methodology

5.2.1 Bacterial strains

The bacterial strains, *Salmonella* Typhimurium ATCC13311 and *Aeromonas australiensis* 03-09 (beef processing plant isolate) (Visvalingam, Wang, et al., 2019) were used. A loopful of the culture from the glycerol stocks maintained at -80 °C, was revived on Lennox agar with no salt agar plates (LA-NS). The formulation of the LA-NS agar medium included tryptone (10 g/L), yeast extract (5 g/L), and agar (10 g/L). An individual colony was aseptically inoculated in 5 mL Lennox broth with no salt broth (LB-NS broth) and incubated for 24 h at 37 °C. Following this, 500 µL of the individual culture was separately added to 25 mL of LB-NS and incubated at 37 °C for 18 h.

5.2.2 PVC test surfaces

Polyvinyl chloride (PVC) is the preferred material for the fabrication of broiler drinking water lines (pipelines, feeders, drinking fountains) (Trachoo et al., 2002). PVC pipes, PVC lock couplers, and PVC lock elbows were purchased from a local supplier. The diameter of all the components was ¾ inch (19.05 mm). PVC pipes were laser cut to make 90 mm sections. All the parts were washed with a detergent and submerged in 70% ethanol overnight and dried in the biosafety cabinet (BSC) for 1 h before use.

5.2.3 Mixed-species biofilm formation in a magnetic rotating biofilm reactor

Biofilms in the DWS generally exist as mixed cultures or co-cultures (Liu et al., 2016; Tan et al., 2017). The stationary phase individual cultures prepared as previously described in section 5.2.1 were mixed in equal volumes. The prepared mixed-species culture was further diluted in a 1:10 ratio using sterile LB-NS to obtain an initial bacterial suspension of approximately 10^8 CFU/mL.

The diluted inoculum was dispensed in a 1 L glass beaker containing a magnetic stirrer and a 3D-printed pipe section holder made using SOLIDWORKS® 2015. The outer surfaces of the PVC pipe sections were covered using a parafilm and the pipe sections were mounted over the holder as shown in Figure 5.1A. Mixed-species biofilms were formed on the inner surfaces of PVC pipe sections during incubation under dynamic conditions at 23 °C for 6 and 10 days.

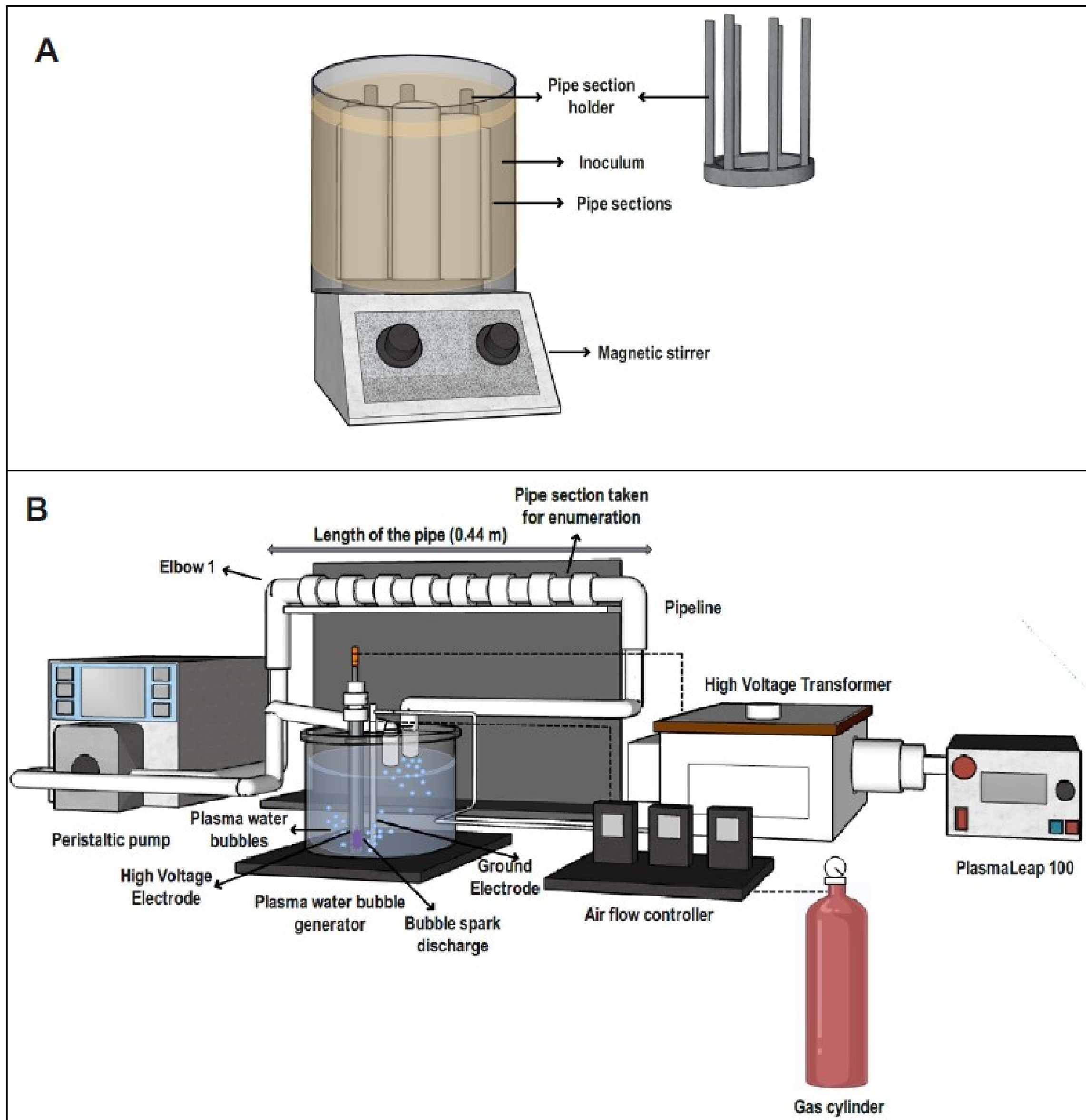


Figure 5.1: Schematic representation of (A) a magnetic rotating biofilm reactor with a 3D-printed pipe section holder, and (B) continuous plasma-activated water bubbles (PAWB) generation and circulation in a benchtop pipeline model.

5.2.4 Bacterial enumeration

After 6 and 10 days for biofilm formation, the PVC pipe sections were aseptically removed from the holder, and the parafilm covering was removed. The inner surfaces of the pipes were gently rinsed using 3 mL 0.1% peptone water to remove loosely attached cells. Following that, the outer section of the pipe was wiped using 70% ethanol and air-dried in the BSC for 5 min. The pipe sections were then suspended in 0.1% peptone water along with glass beads and vortexed for 120 s to ensure the removal of the surviving biofilm cells. Serial dilutions were done in 0.1% peptone water and then drop-plated (100 µL) onto LA-NS plates and incubated overnight at 37 °C for 24 h.

5.2.5 Crystal violet binding assay

The pipe sections after 6 and 10 days of incubation were removed from the biofilm reactor and were prepared as described in the section 5.2.4. After that, the pipe sections were visibly dried in the biosafety cabinet. Biofilms were assessed using the CV staining protocol as previously described by Xu et al. (2021) with some modifications. The inner surfaces of the pipes were stained using 1% crystal violet (Fischer Chemical^{TM}, Ottawa, ON, Canada) in 95% ethanol and incubated at room temperature (25 °C) in the dark for 20 min. The excess dye was rinsed using 0.1% peptone water. The pipe sections were then submerged in 100 mL sodium dodecyl sulphate buffer (SDS; Fischer BioReagents^{TM}, Geel, Belgium) and kept on a shaking incubator at 23 °C for 25 min. A 200 µL of the dissolved dye was taken in two technical repeats and added in a 96-well-flat bottom microtiter plate and optical density at 570 nm (OD_{570}) was measured in a plate reader (Varioskan Flash, Thermo Scientific, Nepean, ON, Canada). The non-inoculated pipe sections kept in the sterile broth served as the negative control.

The biofilm-forming ability was determined as previously described by Stepanović et al. (2000) and were categorized as; "strong" ($4OD_c \leq OD$), "moderate" ($2OD_c < OD \leq 4OD_c$), "weak" ($OD_c < OD \leq 2\ OD_c$) and "non-biofilm" ($OD < OD_c$) former. The OD_c refers to the cut-off value and was measured as; OD_{mean} negative control + 3 x $OD_{standard\ deviation}$ negative control.

5.2.6 Continuous generation and recirculation of PAWB in a benchtop pipeline model

PAWB was generated using a high-voltage power supply (PlasmaLeap 100, PlasmaLeap Technologies, Sydney, Australia) and employed bubble spark and dielectric barrier discharges in a quartz tube. Air was used as the carrier gas at 1 SLPM (standard liter per min), and 160 V, 1000 Hz frequency and 66 µs duty cycle were used as the standard operating parameters. Underwater plasma discharge provides better transportation of the reactive species in the form of bubbles (Asimakopoulou et al., 2022; Xia et al., 2023).

The benchtop pipeline model consisted of a re-circulating vessel that was filled with 700 mL of sterile distilled water and was connected to a PVC pipeline and an AIP peristaltic pump (300–6000 mL/min). The pipeline consisted of removable PVC push connectors. On day 6 and day 10, inoculated pipe sections were removed from the biofilm reactor and were individually attached to the removable benchtop pipeline model. PAWB was generated and continuously circulated over the inoculated pipe sections. The schematic representation of the plasma benchtop pipeline model is shown in Figure 5.1B. The three flow regimes were chosen corresponding to the laminar (*Re* 1000), transitional (*Re* 2500) and turbulent (*Re* 4000) flow, simulating the various flow variations in the disinfection operations.

The flow rate (Q) was calculated using the following formulas (Brading et al., 1995).

$$V = \frac{NRe\ \mu}{\rho D} \qquad (5.1)$$

$$A = \frac{\pi D^2}{4} \quad (5.2)$$

$$Q = vA \quad (5.3)$$

where *Re* is Reynold's number; ρ is the fluid density of pure water (997 kg/m^3), v is the flow velocity (m/s), D represents the inner diameter of the PVC pipe (0.02 m), μ indicates the fluid dynamic viscosity of pure water (10^{-6} m^2s) and A is the area of the inner pipe surface (m^2). Table 5.1 represents the various flow conditions tested in this study.

The head loss (*hf*) in the elbow 1 was calculated using the Darcy-Weisbach equation (Larock et al., 1999).

$$hf = f \frac{Lv^2}{D\,2g} \quad (5.4)$$

$$f = \frac{64}{Re} \quad (5.5)$$

$$RPR = \frac{\varepsilon}{D} \quad (5.6)$$

where *f* means the friction factor, L indicates the length of the PVC pipe (0.44 m), v indicates the velocity of the fluid in the PVC pipe (m/s), D is the diameter of the PVC pipe (0.02 m), and g indicates the acceleration due to gravity (9.81 m/s^2).

RPR refers to the relative pipe roughness and was calculated using the equation 5.6 , where ε denotes the PVC pipe roughness (0.0015 mm) (Larock et al., 1999). The friction factor resulting from the laminar flow was calculated using the equation 5.5. The moody chart (Moody, 1944) was used to calculate the frictional factor resulting from the transitional and the turbulent flows. The head loss (*hf*) resulting from the different pipe flows has been shown in the Table 5.2 and was considered as negligible.

Table 5.1: Hydrodynamic flow regimes used in the study.

SET 1: Effect of flow rate (flow regimes i.e., laminar, transitional, and turbulent) and circulated volume at the same treatment time				
Conditions	Velocity (m/s)	Flow rate (mL/min)	Time (min)	Volume (L)
Re 1000	0.05	942	5	4.71
Re 2500	0.125	2355	5	11.78
Re 4000	0.2	3768	5	18.84
SET 2: Effect of treatment time and circulated volume at laminar and turbulent flow regimes				
Conditions	Velocity (m/s)	Flow rate (mL/min)	Time (min)	Volume (L)
Re 1000	0.05	942	2.5	2.36
Re 1000	0.05	942	5	4.71
Re 1000	0.05	942	7.5	7.07
Re 4000	0.2	3768	2.5	9.42
Re 4000	0.2	3768	5	18.84
Re 4000	0.2	3768	7.5	28.26
SET 3: Effect of flow rate (flow regimes i.e., laminar, transitional, and turbulent) and treatment time at the same circulated volume				
Conditions	Velocity (m/s)	Flow rate (mL/min)	Time (min)	Volume (L)
Re 1000	0.05	942	20	18.84
Re 2500	0.125	2355	8	18.84
Re 4000	0.2	3768	5	18.84

Table 5.2: Head loss resulting from different pipe flows.

Reynolds number (*Re*)	**Velocity (m/s)**	**Friction factor (*f*)**	**Head loss (*hf*) (m)**
1000	0.05	0.064	0.00017959
2500	0.125	0.047	0.00082710
4000	0.2	0.040	0.00179592

The cleaning and disinfection of the drinker lines involve the application of disinfectant in the hydrodynamic motion to reach all the pipe parts. DWS experiences varying hydraulic conditions of high and low flow rates contributing to inadequate disinfection (Chen et al., 2023). Thus, it is important to compare the hydrodynamic conditions of the circulating fluids in the DWS. Immediately after the PAWB treatment, the pipe sections (90 mm) were extracted from the pipeline and enumerated as described in section 5.2.4. The surviving population recovered from the inner surfaces of the pipes was expressed as log CFU/cm^2. Positive control samples received no PAWB treatment. The reaction vessel was filled with water and the circulation was resumed under different flow rates.

For the analysis of the survival bacterial cells in wash water, 1 mL aliquot of the positive control and the PAWB treated sample were 10-fold serially diluted in 0.1% peptone water and plated onto LA-NS plates. Surviving cells in the wash water were presented as log CFU/mL.

5.2.7 Optical emission spectroscopy (OES) of the gas phase plasma discharge

OES was used to identify the excitation of the major reactive species produced in the gas phase underwater plasma discharge. The spectra were captured using a fibre optic probe (F600-UVVIS-SR, StallerNet, Inc., Tampa, FL, USA), with one end connected to the collimating lens and the other end connected to a spectrophotometer (Black comet, C-25, S/N 17060712, StallerNet Inc., Tampa, FL, USA) having a resolution of 0.5 nm/pixel. The spectrum was obtained over 190-850 nm of ultraviolet-visible region (Chaplot et al., 2019).

5.2.8 Determination of physicochemical properties of PAWB

Various physicochemical properties of the bulk water, post-recirculation with water (positive control) and PAWB were analyzed. The pH of the PAWB was measured using a benchtop pH

meter (Fisherbrand™ accumet™ AE150, Ottawa, ON, Canada) and oxidation-reduction potential (ORP) was determined using an ORP meter (ST20R, Ohaus Corporation, Parsippany, NJ, USA). Measurements were taken immediately after circulation. The concentration of the major reactive oxygen and nitrogen species (RONS) including ozone (based on DPD assay; K-7423), hydrogen peroxide (based on ferric thiocyanate assay; K-5543), nitrite (based on an azo dye formation; K-7003) and nitrate (based on cadmium reduction assay; K-6933) were determined using CHEMetrics test kits (CHEMetrics, LLC, Midland, VA, USA) based on the manufacturer's recommendations.

5.2.9 Scanning electron microscopy characterization of biofilms attached to the inner surfaces of the pipes

The cell morphology and adhesion of the mixed-species biofilms on the inner surfaces of the PVC pipes before and after PAWB treatment was visualized using SEM. The inoculated pipes were cut into representative sections (8 mm x 8 mm) using a fine saw and an electric cutter. The loosely attached cells on the prepared pieces were removed using 0.1% peptone water and air-dried in the BSC for 5 min. Furthermore, the air-dried pieces were attached to aluminum stubs and sputter-coated with gold particles and were visualized using a ZEISS EVO 10 Scanning Electron Microscope (Zeiss Group, Jena, Germany).

5.2.10 Statistical analysis

Three biological replicates of the experiments were conducted ($n = 3$), and values were presented as mean ± standard deviation. Statistical analysis was performed using SAS 9.4 (Proc Glimmax), by three-way ANOVA using Tukey's test with $p < 0.05$ indicating a statistical significance within different treatment groups.

5.3. Results

5.3.1 Biofilm formation in the PVC pipes

Crystal violet (CV) analysis revealed a significant difference ($p < 0.05$) in biomass production on the inner surfaces of PVC pipes, following 6 and day 10 incubation (Figure 5.2). Based on the OD_{570nm} values, no detectable biofilms were formed after 6 days of incubation and was considered as a surface-attached bacterium. On the contrary, biomass formation on the inner surfaces of the pipes increased significantly ($p < 0.05$), after 10 days of incubation at 23 °C and formed very weak biofilms (Figure 5.2). The bacterial counts reported on the PVC pipes after 6 and 10 days of incubation were 5.56 ± 0.17 log CFU/cm^2 and 5.74 ± 0.06 log CFU/cm^2, respectively. There was no significant ($p = 0.1526$) effect of the incubation period on the viable cell numbers.

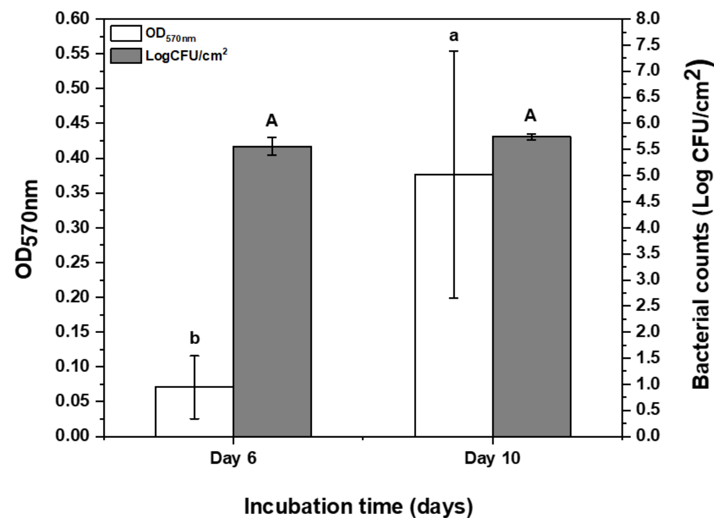


Figure 5.2: Mixed-species attachment on the inner surfaces of the PVC pipes after 6 and 10 days of incubation and analyzed with crystal violet staining assay (white bars) and log enumeration (dark gray bars).

Superscripts with different lowercase letters denotes significant differences ($p < 0.05$) among the OD_{570nm} values. Superscripts with different uppercase letters denote significant differences ($p < 0.05$) among the Log CFU/cm^2 counts. Data are presented as the mean ± standard deviation of three biological replicates ($n = 3$).

5.3.2 Effect of continuous recirculation of water under different flow regimes on biofilm inactivation

The inactivation of mixed-species biofilms grown at different incubation times was assessed by recirculating the water under different flow regimes (laminar, transitional, and turbulent). The reduction in the counts achieved on the pipe walls after recirculation with the water at a *Re* 1000 for 5 min, was 0.60 ± 0.14 log CFU/cm^2 and 0.88 ± 0.16 log CFU/cm^2 for day 6 surface-attached cells and day 10 weak-biofilms, respectively (Figures 5.3A and 5.4A). Increasing Reynold's number of water circulation resulted in a significantly ($p < 0.05$) contributed to a higher inactivation of the attached bacterium. For instance, for the surface-attached bacterium grown for 6 days, recirculation of water at transitional (*Re* 2500) and turbulent (*Re* 4000) flow regimes for 5 min resulted in reductions of 1.10 ± 0.31 log CFU/cm^2 and 1.22 ± 0.43 log CFU/cm^2, respectively (Figure 5.3A). Similarly, for day 10 weak biofilms, the water flow regimes produced using a higher Reynolds number resulted in a higher bacterial biofilm inactivation from the pipe walls (Figure 5.4A).

The contamination of the circulating water at different flow regimes could be due to the detachment of the bacteria from the pipe walls induced by the high shear stress (Xu & Tan, 2023). In this study, increasing Reynold's number of water recirculation showed no significant (p = 0.2713) effect in the inactivation of the detached bacteria in the bulk water (Figures 5.3B and 5.4B).

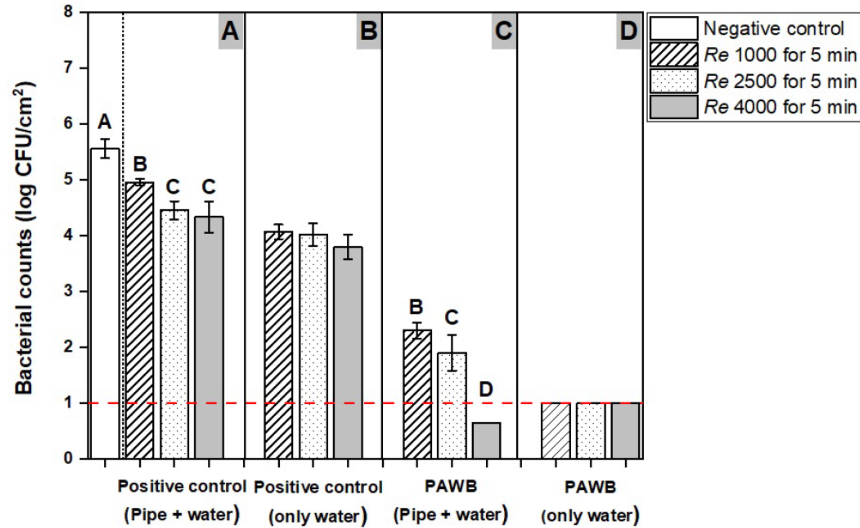


Figure 5.3: Mixed-species attachment on the inner surfaces of the PVC pipes after 6 days of incubation (white bar). Panels A and C represent the log counts observed on the pipe walls under different flow regimes after circulating with (A) water and (C) PAWB. Panels B and D represent the bacterial counts observed in the bulk water, post-circulating with (B) water, and (D) PAWB. Data are presented as the mean ± standard deviation of three biological replicates (n = 3).

Different superscript letters in the panel A and C denotes significant differences in the same panel and the negative control.

Dotted red horizontal line denotes below the detection limit of 0.65 log CFU/cm^2 for the attached bacterium on the pipe walls and 1 log CFU/mL for the detached bacteria suspended in the bulk water.

5.3.3 Application of continuous generation and recirculation of PAWB under different flow regimes on biofilm inactivation

PAWB-mediated biofilm inactivation increased significantly ($p < 0.05$) under high *Re* flow regimes. Using PAWB at laminar flow condition (*Re* 1000) for 5 min resulted in a lower reduction of 3.26 ± 0.08 and 1.44 ± 0.42 log CFU/cm^2 for day 6 surface-attached cells, and day 10 weak biofilms, respectively (Figures 5.3C and 5.4C). Furthermore, PAWB recirculation at turbulent flow regime (*Re* 4000) for 5 min resulted in the highest reduction with the cells being below the detection limit (0.65 log CFU/cm^2) for day 6 surface-attached bacteria (Figure 5.3C). On the contrary for the day 10 weak biofilms, a lower reduction of 2.96 ± 0.51 log CFU/cm^2 was obtained

using PAWB recirculation at the turbulent regime (Figure 5.4C). The PAWB treatment of the circulating fluid under the selected flow regimes for 5 min resulted in a reduction of the bacterial counts to below the detection limit (1 log CFU/mL) (Figures 5.3D and 5.4D). There were no significant interactions between the incubation day and the flow regimes.

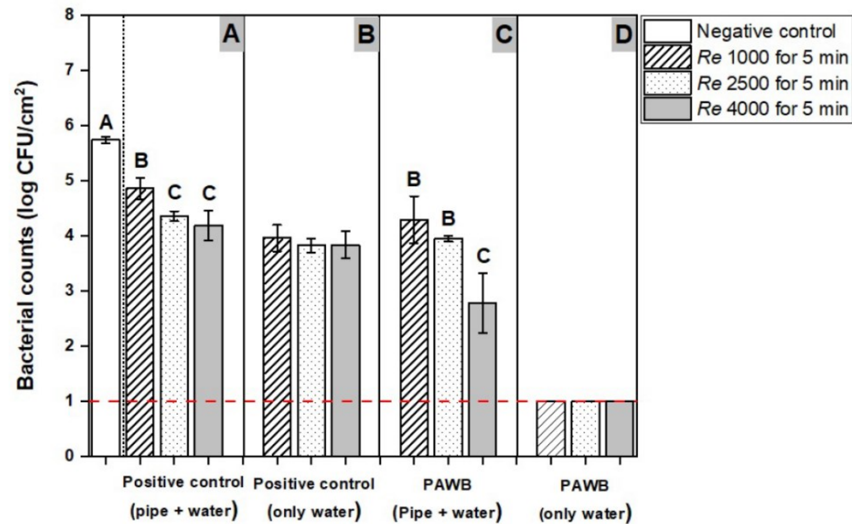


Figure 5.4: Mixed-species attachment on the inner surfaces of the PVC pipes after 10 days of incubation (white bar). Panels A and C represent the log counts observed on the pipe walls under different flow regimes after circulating with (A) water and (C) PAWB. Panels B and D represent the bacterial counts observed in the bulk water, post-circulating with (B) water, and (D) PAWB. Data are presented as the mean ± standard deviation of three biological replicates (n = 3).

Different superscript letters in the panel A and C denotes significant differences in the same panel and the negative control.

Dotted red horizontal line denotes below the detection limit of 0.65 log CFU/cm^2 for mixed-species biofilms on the pipe walls and 1 log CFU/mL for the detached bacteria suspended in the bulk water.

5.3.4 Effect of treatment time under different flow regimes on biofilm inactivation

The laminar (*Re* 1000) and turbulent (*Re* 4000) flow regimes were selected based on the above experiments to understand the effect of increasing the treatment time in the inactivation of the day-10 weak- biofilms.

At a particular flow regime of water, increasing the treatment time did not significantly ($p = 0.1983$) result in a higher inactivation of the biofilms from the inner surfaces of the PVC pipe sections (Figures 5.5A and 5.6A). For instance, circulating water at a *Re* 1000 for 2.5 min resulted in a biofilm reduction of 0.88 ± 0.30 log CFU/cm^2 from the pipe walls and increasing treatment time to 7.5 min resulted in a reduction of 1.44 ± 0.52 log CFU/cm^2 (Figure 5.5A). Similarly, water recirculation at *Re* 4000 for 2.5 min and 7.5 min resulted in reductions of 1.38 ± 0.61 log CFU/cm^2 and 1.82 ± 0.07 log CFU/cm^2, respectively (Figure 5.6A). The analysis of the circulating fluid also showed no significant difference in the bacterial counts under the specific flow conditions (Figures 5.5B and 5.6B).

However, increasing the PAWB recirculation time resulted in higher reductions in the bacterial biofilms (Figures 5.5C and 5.6C). For instance, PAWB under the laminar flow regime (*Re* 1000) for 2.5 min resulted in a reduction of 1.36 ± 0.19 log CFU/cm^2. At the similar flow regime, increasing the PAWB recirculation time to 7.5 min, resulted in a significantly ($p < 0.05$) higher reduction of 2.64 ± 0.47 log CFU/cm^2 (Figure 5.5C). Moreover, increasing the Reynold's number significantly ($p < 0.05$) increased the inactivation of bacterial biofilms. At *Re* 4000, increasing the treatment from 2.5 to 7.5 min resulted in a significantly ($p < 0.05$) higher reduction of 2.48 ± 0.44 and 3.53 ±1.00 log CFU/cm^2 respectively (Figure 5.6C). The bacterial counts in the circulating fluid post PAWB treatment were found to be below the detection limit (1.0 log CFU/mL) under all the tested conditions (Figures 5.5D and 5.6D).

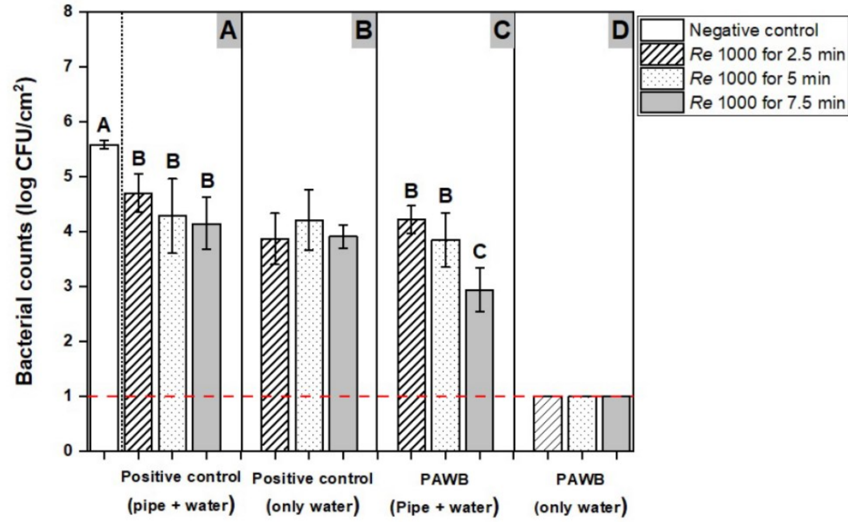


Figure 5.5: Mixed-species biofilm attachment on the inner surfaces of the PVC pipes after 10 days of incubation (white bar). Panels A and C represent the log counts observed on the pipe walls after circulating with (A) water and (C) PAWB at *Re* 1000 at different times. Panels B and D represent the bacterial counts observed in the bulk water, post-circulating with (B) water, and (D) PAWB. Data are presented as the mean ± standard deviation of three biological replicates (n = 3).

Different superscript letters in the panel A and C denotes significant differences in the same panel and the negative control.

Dotted red horizontal line denotes below the detection limit of 0.65 log CFU/cm^2 for mixed-species biofilms on the pipe walls and 1 log CFU/mL for the detached bacteria suspended in the bulk water.

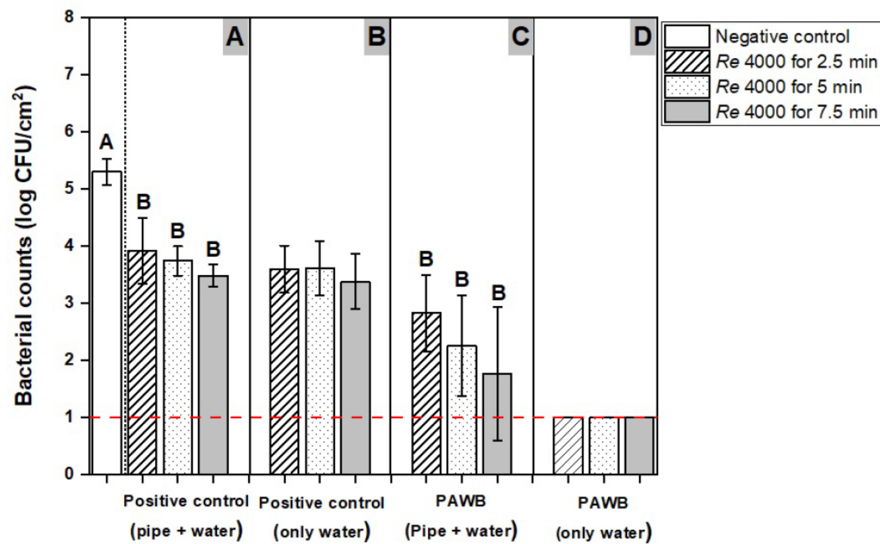


Figure 5.6: Mixed-species attachment on the inner surfaces of the PVC pipes after 10 days of incubation (white bar). Panels A and C represent the log counts observed on the pipe walls after circulating with (A) water and (C) PAWB at *Re* 4000 at different times. Panels B and D represent

the bacterial counts observed in the bulk water, post-circulating with (B) water, and (D) PAWB. Data are presented as the mean ± standard deviation of three biological replicates (n = 3).

Different superscript letters in the panel A and C denotes significant differences in the same panel and the negative control.

Dotted red horizontal line denotes below the detection limit of 0.65 log CFU/cm^2 for mixed-species biofilms on the pipe walls and 1 log CFU/mL for the detached bacteria suspended in the bulk water.

5.3.5 Effect of volume of circulated PAWB on the biofilm inactivation at different flow regimes

Next, different combinations of flow regimes and treatment times were selected to keep the volume of water and PAWB circulated through the pipe sections constant. Recirculating water under different hydraulic regimes (laminar, transitional, and turbulent) of *Re* 1000 for 20 min, *Re* 2500 for 8 min, and *Re* 4000 for 5 min, showed no significant ($p = 0.1187$) difference in the reduction of day 10 weak-biofilms by 1.04 ± 0.37, 0.78 ± 0.62, and 1.63 ± 0.16 log CFU/cm^2 respectively (Figure 5.7A). Analysis of the circulating fluid showed no significant ($p = 0.2210$) difference in the bacterial counts post-transmission of the detached bacteria in the bulk water (Figure 5.7B).

Furthermore, PAWB recirculation under different flow regimes at a constant volume did not significantly ($p = 0.3034$) affect the inactivation of the weak- biofilms on the pipe walls (Figure 5.7C). The bacterial counts in the circulating fluid were found to be below the detection limit (1 log CFU/mL) following PAWB circulation (Figure 5.7D) under these conditions.

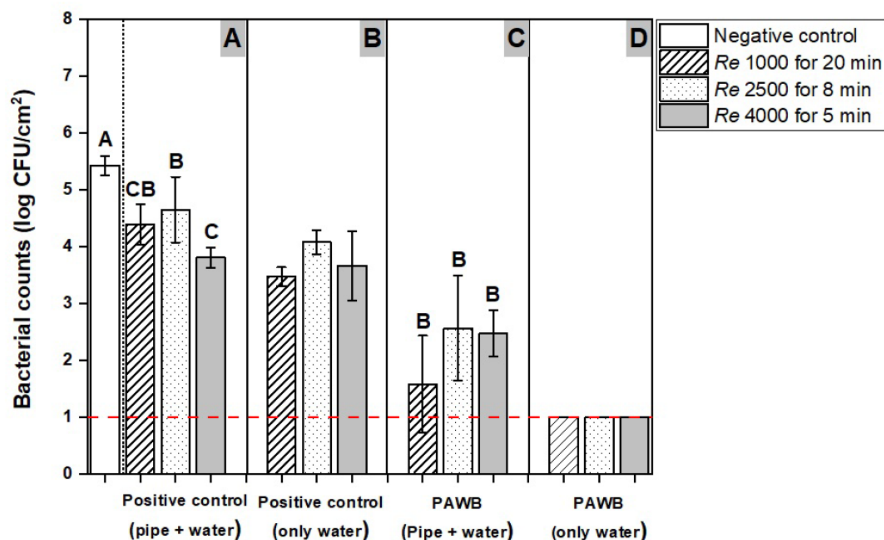


Figure 5.7: Mixed-species attachment on the inner surfaces of the PVC pipes after 10 days of incubation (white bar). Panels A and C represent the log counts observed on the pipe walls at different flow conditions of constant volume after circulating with (A) water and (C) PAWB. Panels B and D represent the bacterial counts observed in the bulk water, post-circulating with (B) water, and (D) PAWB. Data are presented as the mean ± standard deviation of three biological replicates (n = 3).

Different superscript letters in the panel A and C denotes significant differences in the same panel and the negative control.

Dotted red horizontal line denotes below the detection limit of 0.65 log CFU/cm^2 for mixed-species biofilms on the pipe walls and 1 log CFU/mL for the detached bacteria suspended in the bulk water.

5.3.6 Characterization of gas-phase plasma discharge

OES was used to provide information on the atomic transitions of the major reactive species produced in the gas phase plasma discharge in the UV-visible region, ranging from 190-850 nm (Qayyum et al., 2005). The emission spectrum was recorded to compare the different flow regimes including *Re* 1000 for 5 min, *Re* 2500 for 5 min, and *Re* 4000 for 5 min. As shown in Figure 5.8, the emission spectrum for the air-plasma dominated with the N_2 2nd positive band heads ($N_2(C^3\Pi_u \rightarrow B^3\Pi_u)$ (0,0)) at 380, 399, 405 nm (Thomas-Popo et al., 2019; Qayyum et al., 2005). The band heads of the N_2 first negative system ($N_2(B^2\Sigma_u^+ \rightarrow X^2\Sigma_g^+)$ (0,0)) were recorded at 393 and 426 nm

(Qayyum et al., 2007; Thomas-Popo et al., 2019). These emission peaks correspond to the electronically excited states produced from the molecular transitions of the ground state of N_2 ($X^1\Sigma_g^+$) (Zhou et al., 2018; Qayyum, et al., 2005). Several O (O_2 ($b_1\Sigma_g^+ \rightarrow X_3\Sigma_g^-$) (0,0)) molecular peaks (Eq. 5.12) (Thomas-Popo et al., 2019; Zhou et al., 2018) at a lower intensity were recorded at 745.5, 771, 774 nm at *Re* 1000. At *Re* 2500, O atom transitions were noted at 733.5, 772 and 789.5 nm. However, a relatively lower O emission intensity was recorded for higher *Re* 4000 at 772 nm. Moreover, the OES spectra for *Re* 4000 for 5 min showed a spectral emission intensity of NO_γ system around 274 nm (Eqs. 5.7-5.10) (Girard et al., 2016). Nitric oxide ($^{\bullet}NO$) in the air-plasma can be produced due to the interaction between the N_2 and O derivatives (Bradu et. al., 2020).

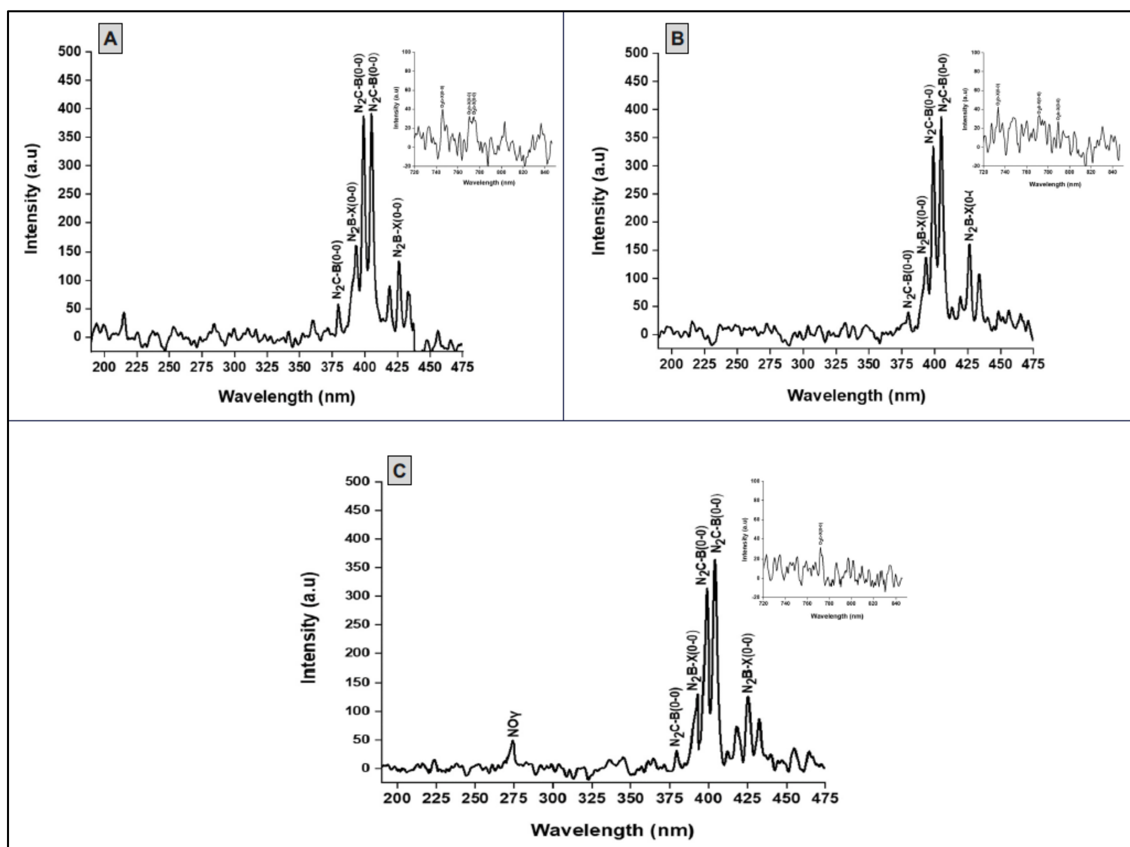


Figure 5.8: Gas-phase optical discharge spectra obtained under (A) *Re* 1000 for 5 min, (B) *Re* 2500 for 5 min, and (C) *Re* 4000 for 5 min.

5.3.7 Physicochemical properties of PAWB at the selected flow regimes

The potential generation of the reactive species in the distilled water post circulation in the pipeline under different flow regimes was analyzed (Figure 5.9). The pH and other physicochemical properties, including ozone and hydrogen peroxide did not significantly affect the bacterial reduction. The concentration of ozone and hydrogen peroxide was < 1 ppm under all hydrodynamic conditions (Figure 5.9B). However, the ORP of the water at a high *Re* of 4000 for 7.5 min increased significantly ($p < 0.05$) to 453.67 ± 67.21, as compared to the ORP of the distilled water 325 ± 19.67 (Figure 5.9A).

The circulating fluid analysis post-PAWB treatment under all the flow conditions tested in the study demonstrated a significant ($p < 0.05$) decrease in the pH (Figure 5.10A). The nitrite concentration was < 1 ppm under all hydrodynamic conditions tested (Figure 5.10C). Moreover, the hydrogen peroxide concentration was found to be < 0.2 ppm under different combinations of flow and treatment conditions. The ozone concentration significantly ($p < 0.05$) inreased upon increasing PAWB treatment time under different hydrodynamic conditions (Figure 5.10B).

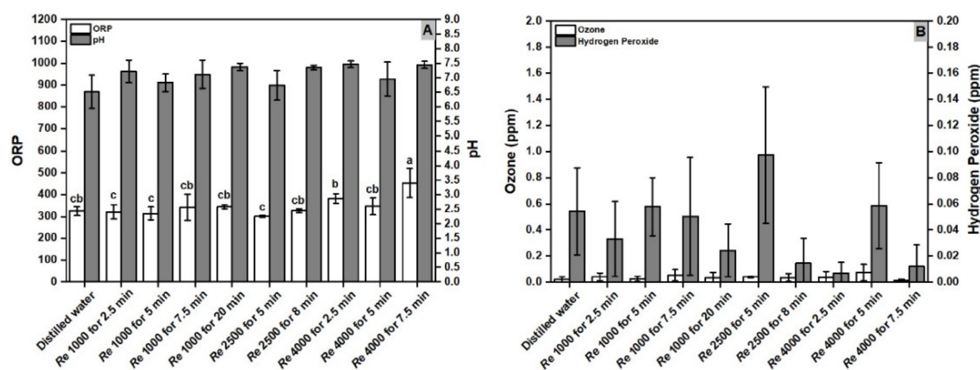


Figure 5.9: Measurement of the various physicochemical properties of the circulated distilled water including (A) pH and ORP, and (B) ozone and hydrogen peroxide concentration obtained under different flow regimes.

Superscripts with different lowercase letters denote significant differences ($p < 0.05$) among the same-coloured bars. Data are presented as the mean ± standard deviation of three biological replicates ($n = 3$).

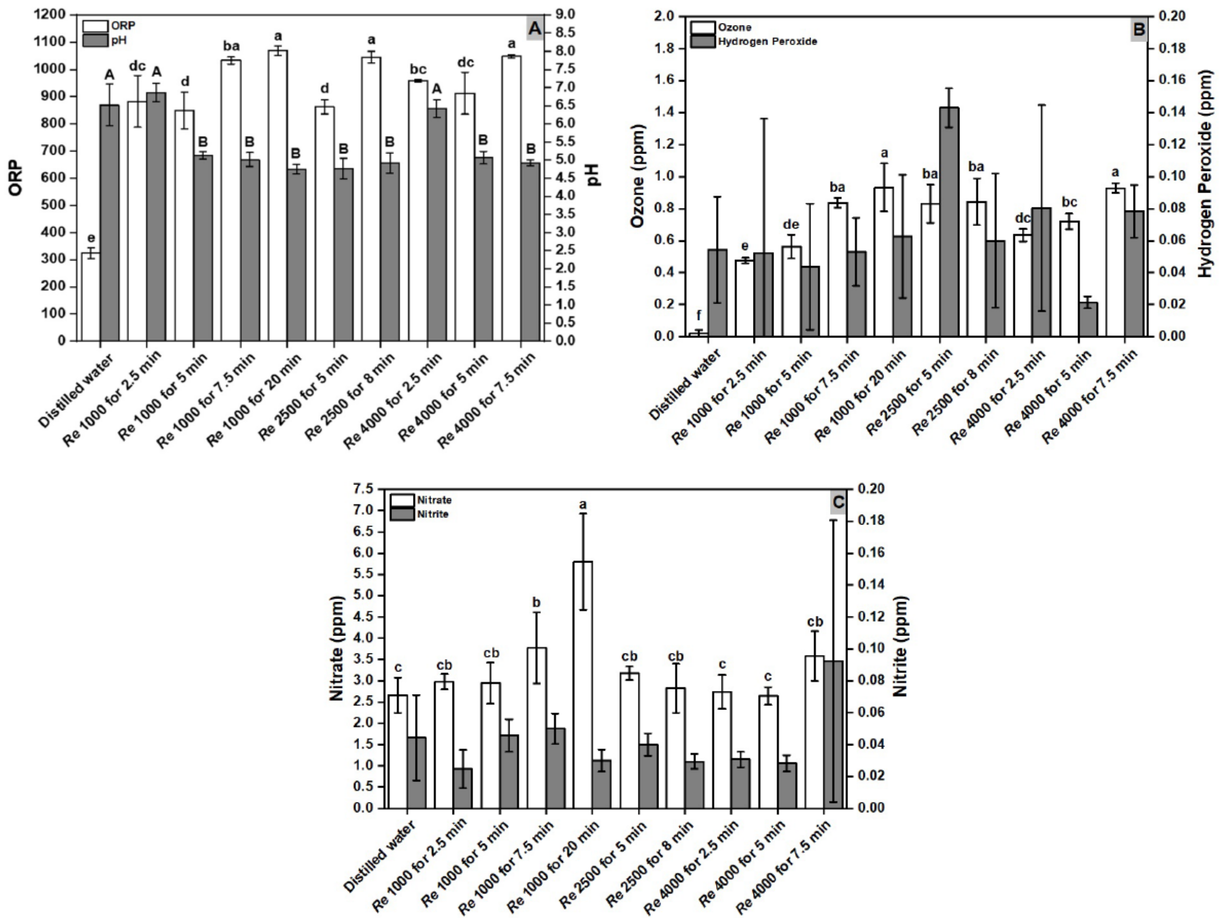


Figure 5.10: Measurement of the various physicochemical properties of the circulated PAWB including (A) pH and ORP, (B) ozone and hydrogen peroxide, and (C) nitrate and nitrite concentration obtained under different flow regimes.

Superscripts with different lowercase and uppercase letters denote significant differences ($p < 0.05$) among the same-coloured bars. Data are presented as the mean ± standard deviation of three biological replicates (n = 3).

5.3.8 SEM analysis

Scanning electron microscopy images of *S.* Typhimurium and *A. australiensis* weak biofilms after the circulation with water (positive control) and PAWB are shown in Figure 5.11. Representative images of the non-inoculated PVC pipe (Figure 5.11A,B) revealed the presence of surface irregularities, possessing larger cavities, and holes.

Water recirculation at a *Re* 4000 for 5 min (positive control) resulted in a disruption of the connective EPS matrix resulting in a weak biofilm architecture (Figure 5.11E,F). However, no other significant cellular morphological modifications were observed. Recirculation with PAWB at a *Re* 4000 resulted in a visibly greater reduction of the biofilms (Figure 5.11G,H). The treatment with an oxidative disinfectant visibly resulted in higher biomass removal and a compromised EPS.

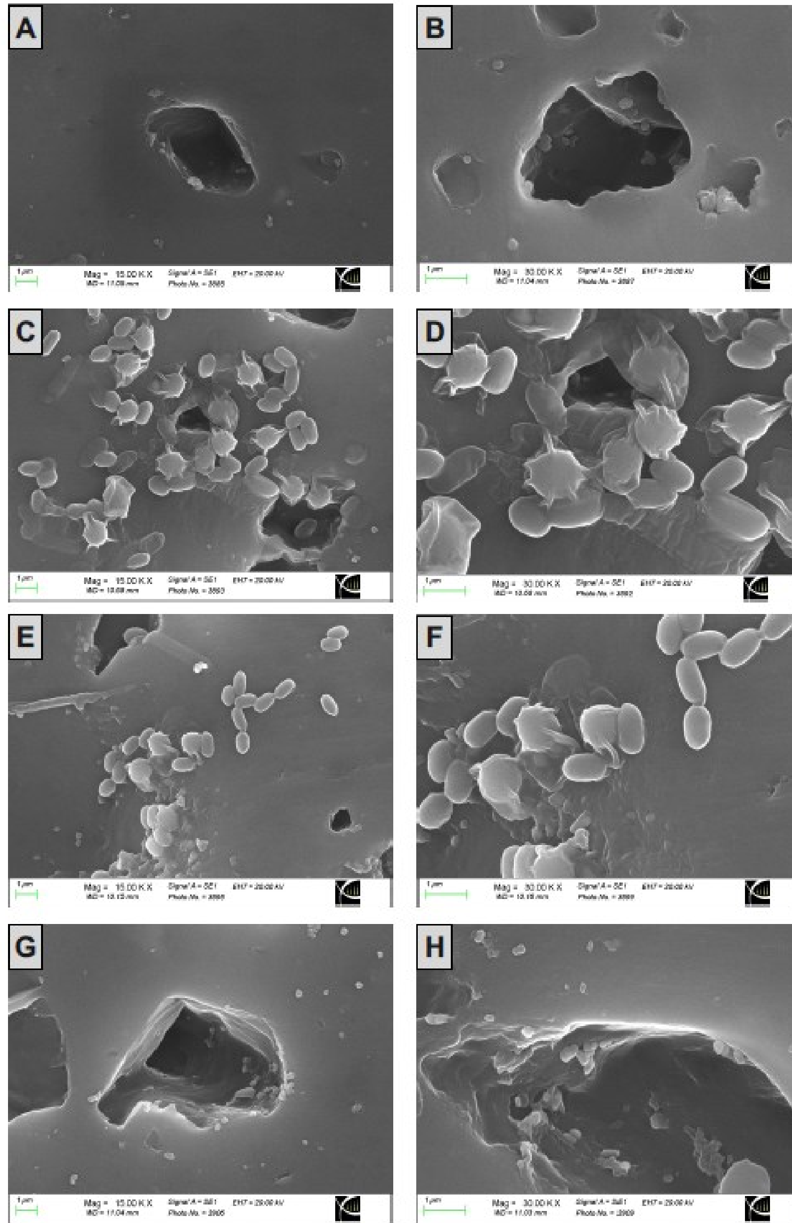


Figure. 5.11: SEM micrographs of the mixed-species biofilms of *Salmonella* Typhimurium ATCC13311 and *Aeromonas australiensis* 03-09 subjected to different treatments; (A, B) un-inoculated pipe, (C, D) negative control (day 10 mixed-species biofilms), (E, F) positive control with water at *Re* 4000 for 5 min, and (G, H) PAWB treatment at *Re* 4000 for 5 min. The scale bar is 1 µm. (A magnification of 15 KX was used for A, C, E and G, and 30 KX was used for B, D, F, and H).

5.4. Discussion

The study analyzed the effectiveness of PAWB in the inactivation of attached bacteria on the PVC pipe walls. Day 10 produced significantly ($p < 0.05$) higher biomass than day 6 surface-attached cells and were categorized as weak-biofilms. Several studies have reported a direct relationship between the incubation time and biomass production (Ma et al., 2021; Prasad & Roopesh, 2023; Singla et al., 2014; Visvalingam, Zhang, et al., 2019; Yang et al., 2018). An increase in the biomass production of the mixed-species consortia has also been positively correlated with disinfection resistance (Burmølle et al., 2006). However, increasing the incubation time did not significantly increase the cell counts on the pipe walls (Figure 5.2). This revealed that the biofilm formation ability of the mixed species as analyzed using CV assay cannot be positively correlated with the CFU determined by plating. This discrepancy reported among the CV staining values and viable numbers has been observed in published literature (Borucki et al., 2003; Kadam et al., 2013).

At similar treatment times, recirculating water under different flow regimes had a significant ($p < 0.05$) effect on the biofilm inactivation from the pipe walls. The turbulent flow regime (*Re* 4000 for 5 min) significantly ($p < 0.05$) resulted in a higher inactivation of biofilms than the transitional and laminar flow regimes (Figures 5.3A and 5.4A). Before disinfection, water flushing at turbulent flow regimes is applied to remove the organic matter and sediment deposits accumulated in the DWS (Simões & Simões, 2013). The bacterial counts in water did not significantly differ, post-circulation of water under different flow regimes at similar treatment times (Figures 5.3B and 5.4B). It can be concluded that mechanical circulation of water at high Reynold's number can effectively result in the inactivation of the attached bacteria due to high shear forces. Several studies have reported a direct relationship between the fluid flow and the resultant shear in the potential shedding of bacterial biofilms from the inner surfaces of the pipes (Lemos et al., 2015;

Stoodley et al., 2002; Tan & Karwe, 2021; Xu & Tan, 2023). The transmission of bacterial cells into the bulk water can potentially cross-contaminate the broiler drinker lines and can lead to serious public health consequences (Simões & Simões, 2013).

PAWB treatment resulted in a significantly ($p < 0.05$) lower reduction of the day 10 weak-biofilms as compared to the day 6 surface-attached bacterium (Figures 5.3C and 5.4C). This could be explained by the fact that day 10 grown weak-biofilms on the pipe walls significantly ($p < 0.05$) produced higher biomass (Figure 5.2), which resulted in a lesser penetration of the reactive species into the biofilm matrix as compared to the day 6 surface attached bacteria (Patange et al., 2021). The presence of microcolonies, water channels and pores in the biofilm layers also restricts the entry of the reactive species. Furthermore, the diffusion of reactive species in the biofilm layers is also dependent on their concentration and the subsequent generation of chemical gradients (Mai-Prochnow et al., 2021). Similarly, Xu & Tan (2023) also demonstrated that washing PVC pipes using plasma-activated microbubble water at high flow velocities of 1 m/s and 2 m/s resulted in a high reduction of *Klebsiella michiganensis* biofilms by approximately 4 log. They indicated that high shear stress resulting from the turbulent flow contributed to better accessibility of RONS and resulted in increased oxidative stress in the biofilm matrix (Xu & Tan, 2023). The interaction between the plasma water bubbles and the adjacent biofilm layer can induce physical agitation due to surface etching or erosion (Sen & Mutlu, 2013). Moreover, the removal of the boundary layer of the biofilms, increases the susceptibility of the inner layer to the RONS, thus contributing to a higher biofilm inactivation (Tan & Karwe, 2021; Xu & Tan, 2023). Additionally, the presence of the ions, radicals, and reactive species in the PAWB modifies the surface tension of the bulk fluid and eventually decreases the adhesive forces between the bacteria and the substratum, thereby contributing to their subsequent detachment. The water bubbles mediated bacterial detachment can

be enhanced due to the combined interaction of the capillary forces and the surface tension (Khodaparast et al., 2017).

The substantial biofilm inactivation reported under different PAWB flow regimes (*Re* 1000 for 5 min, *Re* 2500 for 5 min, *Re* 4000 for 5 min) could be due to the generation of atomic nitrogen and oxygen derivatives, which upon diffusion in the water may have led to the generation of various RONS (Thomas-Popo et al., 2019; Misra et al., 2015; Zhou et al., 2018). The reported air-plasma ionization reactions are listed below (Bradu et al., 2020; Qayyum et al., 2005; Zhou et al., 2018);

$$N_2 + e^- \rightarrow N^+ + N + 2e^- \quad (5.7)$$

$$N_2 + e^- \rightarrow N_2^+ + 2e^- \quad (5.8)$$

$$N + e^- \rightarrow N^+ + 2e^- \quad (5.9)$$

$$N + O_3 \rightarrow NO + O_2 \quad (5.10)$$

$$N + O \rightarrow NO \quad (5.11)$$

$$N_2 + O \rightarrow NO + N \quad (5.12)$$

$$N + O_2 \rightarrow NO + O \quad (5.13)$$

$$NO + O \rightarrow NO_2 \quad (5.14)$$

$$O_2 + e^- \rightarrow 2O + e^- \quad (5.15)$$

$$O + O_2 \rightarrow O_3 \quad (5.16)$$

$$NO + O_3 \rightarrow NO_2 + O_2 \quad (5.17)$$

The presence of fissures and cavities on the PVC pipes (Fig. 5.11(A, B)), can provide a greater surface area for bacterial attachment and can potentially prevent them from the action of the various disinfectants (Percival et al., 1999). Inadequacy in the cleaning and disinfection of the DWS could result in the survival and subsequent transmission of bacteria (Carvalho et al., 2023). Biofilms formed on the inner surfaces of the pipes after 10 days of incubation (negative control)

displayed less dense 3D biofilm architecture with cell aggregates covered in a self-secreted sheath (Fig. 5.11(C, D)). The untreated biofilm was sporadically scattered cells that appeared as rod-to-ovoid with smooth exteriors. SEM revealed that EPS interconnected the cells with each other and with the substratum.

The study demonstrated that increasing the treatment time of recirculating water at the selected flow regimes did not significantly increase the biofilm inactivation (Figures 5.5A and 5.6A). It has been reported that the integrity of the biofilm matrix is maintained due to equilibrium within the inherent stress tension and the outside shear stress generated due to different water flow regimes (Simões et al., 2005b). This indicates that a high outside shear stress, as compared to its inherent stress will aid in the biofilm inactivation. It can be postulated that within a specific flow regime, increasing the treatment time alone did not generate a higher shear stress, due to which the inactivation process did not increase with time. However, combining the mechanical phenomenon of recirculation with an oxidative disinfectant (PAWB) resulted in a significant reduction of biofilm with increasing treatment time (Figures 5.5C and 5.6C). This possibly could be due to an increase in the oxidative stress in the biofilm matrix upon increasing the PAWB treatment time. Similarly, Simões et al. (2005b) that synergistic action of mechanical stress and chemical treatment (oxidizing and non-oxidizing compounds) contributed to higher removal of *Pseudomonas fluorescens* biofilms from the stainless steel cylinder.

A constant volume flow regime indicates that irrespective of the flow regimes selected, the concentration of the reactive species circulating over the adhered biofilm will be relatively similar (Figure 5.7). PAWB circulation under different flow regimes did not significantly affect the biofilm inactivation (Figure 5.7C). It is possible that even though the concentration of RONS is similar, their subsequent interaction with the biofilm matrix under the flow regimes (laminar,

transitional, and turbulent) will be different. The chaotic movement of the RONS in turbulent flow (*Re* 4000 for 5 min) could have resulted in high shear stress and possibly a higher interaction of RONS with the biofilm layers. Whereas, under a laminar flow regime (*Re* 1000 for 20 min), irrespective of the smooth flow movement, a higher treatment time might have resulted in a higher interaction of the biofilm with the reactive species. This indicates that a complex interplay between the RONS concentration, treatment time, and shear stress produced under different flow regimes plays a major role in the biofilm inactivation.

It was found that increasing the PAWB treatment time under different flow regimes resulted in an acidic environment (Figure 5.10A). The pH drop is reported due to the formation of NO_x species (Xia et al., 2023). Nitric oxide ($^{\bullet}NO$) is a biological signalling free radical that induces the transition of biofilm to planktonic cells thus making them highly susceptible to the action of RONS (Liu et al., 2016). The acidification of the water due to the formation of various reactive nitrogen species could be as per the below-listed reactions (Wu et al., 2020);

$$NO_2^{\bullet} + {}^{\bullet}OH \rightarrow HNO_3 \quad (5.18)$$

$$NO^{\bullet} + {}^{\bullet}OH \rightarrow HNO_2 \quad (5.19)$$

$$NO_2^{\bullet} + H_2O \rightarrow HNO_2 + HNO_3 \quad (5.20)$$

Irrespective of the flow regimes, increasing the treatment time had a significant effect on the formation of the nitrates. For instance, PAWB at *Re* 1000 for 20 min resulted in a higher nitrate formation of 5.80 ± 1.13 ppm (Figure 5.10C). Patange et al. (2021) reported that under acidic conditions, an increase in the treatment time results in the formation of more nitrates. The lower concentration of hydrogen peroxide and nitrite reported could be due to a simultaneous reaction between the hydrogen peroxide, nitrite, and nitrate, thus resulting in the formation of nitric oxide

($^{\bullet}NO$), and peroxynitrite species ($ONOOH$, $ONOO^-$) (Xu & Tan, 2023). The reported air-liquid ionization reactions are listed below (Zhou et al., 2019);

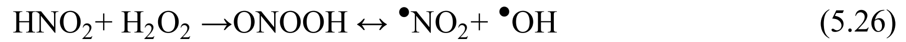
$$^{\bullet}N + {}^{\bullet}OH \rightarrow {}^{\bullet}NO + {}^{\bullet}H \quad (5.21)$$

$$^{\bullet}H + NO_2^- \rightarrow {}^{\bullet}NO + OH^- \quad (5.22)$$

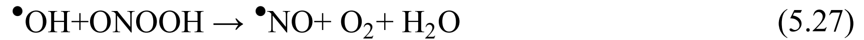
$$^{\bullet}NO_2 + {}^{\bullet}O \rightarrow {}^{\bullet}NO + O_2 \quad (5.23)$$

$$HNO_2 + {}^{\bullet}H \rightarrow {}^{\bullet}NO + H_2O \quad (5.24)$$

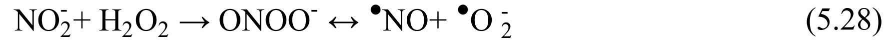
$$^{\bullet}NO + {}^{\bullet}HO_2^- \rightarrow ONOOH \quad (5.25)$$



$$HNO_2 + H_2O_2 \rightarrow ONOOH \leftrightarrow {}^{\bullet}NO_2 + {}^{\bullet}OH \quad (5.26)$$



$$^{\bullet}OH + ONOOH \rightarrow {}^{\bullet}NO + O_2 + H_2O \quad (5.27)$$



$$NO_2^- + H_2O_2 \rightarrow ONOO^- \leftrightarrow {}^{\bullet}NO + {}^{\bullet}O_2^- \quad (5.28)$$

$$^{\bullet}NO + {}^{\bullet}O_2^- \rightarrow ONOO^- \quad (5.29)$$

A significant ($p < 0.05$) increase in the ozone concentration (Figure 5.10B) could have significantly contributed to the higher reduction of the biofilm (Lu et al., 2018). Ozone can also contribute to the formation of $^{\bullet}OH$ and HO_2 species (Xu & Tan, 2023). Moreover, ozone interaction with the other ROS species might have resulted in the formation of O_2, $^{\bullet}O_2^-$, ONOO, $^{\bullet}NO$, HO_2^-, $^{\bullet}OH$ as per the following reactions (Liu et al., 2021; Zhou et al., 2019);

$$^{\bullet}NO_2 + O_3 \rightarrow {}^{\bullet}NO + O_2 \quad (5.30)$$

$$HO_2^- + O_3 \rightarrow {}^{\bullet}OH + {}^{\bullet}O_2^- + O_2 \quad (5.31)$$

$^{\bullet}OH$ radical can abstract the H atom from the lipids, and can cause lipid peroxidation, ultimately disrupting the biofilm matrix (Khosravian et al., 2015). Moreover, PAWB reactive species can also cause localized reactions with varying oxidative stress response between the subpopulations, thereby resulting in a higher biofilm inactivation (Mai-Prochnow et al., 2021).

The PAWB activity was further characterized by determining its ORP. The ORP increased significantly ($p < 0.05$) with increasing the PAWB treatment time (Figure 5.10A). ORP increase is related to the formation of RONS, ions, and radicals and it corresponds to high antimicrobial activity (Xia et al., 2023). Even though a lower concentration of RONS was reported under different hydrodynamic conditions, the continuous shear stress imparted due to different flow rates (de Beer et al., 1994) could increase the mass transport rate and their subsequent penetration and could eventually compromise the integrity of the biofilm matrix (Liu et al., 2016). Moreover, prolonged exposure to the RONS could result in biofilm inactivation (Lu et al., 2018).

The bacterial counts in the water post circulation with PAWB under all the flow regimes tested were below the detection limit (Figures 5.3D, 5.4D, 5.5D, 5.6D and 5.7D). Previous studies have reported a higher susceptibility of the planktonic cells to the PAW treatment as compared to their biofilm counterparts (Fernández-Gómez et al., 2023; Hozák et al., 2018; Smet et al., 2019). PAWB contains a complex mixture of RONS, ions, electrons, and radicals, that causes surface erosion and matrix disruption resulting in a physical removal of the cells from the biofilm interior (Asimakopoulou et al., 2022; Mai-Prochnow et al., 2021). This induces the physiological change in the cellular state, resulting in a shift from the biofilm cells to their planktonic free-flowing form. Furthermore, planktonic cells in bulk water are highly susceptible to the action of the reactive species, thereby resulting in a higher inactivation (Liu et al., 2016).

It is noteworthy to mention that the biofilm inactivation does not necessarily correlate with its removal (Simões et al., 2007), indicating that the adhered bacteria on the pipe walls following PAWB treatment could re-seed and re-grow and can further cross-contaminate the bulk water. This necessitates the proper optimization of the process parameters to completely inactivate and subsequently remove the biofilms from the pipe walls. The presence of a thin layer of mixed

species formed on the pipe walls after 10 days of incubation indicates the absence of a complex biofilm matrix. The sparse attachment of the bacterial cells as a monolayer possibly indicates the absence of a compact EPS structure. The biofilm EPS provides a barrier (Vu et al., 2009) and protects the embedded bacterial cells against the action of various disinfectants (Frank & Koffi, 1990; Wang et al., 2020). Moreover, several studies have reported a higher disinfectant resistance of the bacteria residing in mixed-species biofilms as compared to their monocultures (Burmølle et al., 2006; Norwood & Gilmour, 2000; Simões et al., 2010; Visvalingam, Zhang, et al., 2019). This signifies the need to assess the effectiveness of PAWB against the strong biofilm forming strains.

Overall, the tested flow and treatment parameters i.e., flow regimes, PAWB generation and circulation time, and total volume of the PAWB circulated in the pipeline unit influenced the mixed-species biofilm inactivation. The application of PAWB for biofilm inactivation is a potential approach for industrial application; however, the important process parameters should be carefully selected and optimized to achieve the required level of microbial biofilm inactivation in drinking water systems.

5.5 Conclusion

The disinfection potential of PAWB against the surface-attached cells and weak-biofilms grown in the PVC pipes was evaluated under selected flow regimes relevant to the broiler drinking water systems. The study found that increasing Reynold's number of water circulation significantly resulted in the higher inactivation of the surface-attached cells into the drinking water supply. However, under similar flow conditions, there was no significant difference in the bacterial counts reported in the bulk water. This indicates that the shear stress generated due to the turbulent flow regimes in the pipeline does not necessarily contribute to the reduction of the bacteria in the bulk water. Furthermore, the combined turbulent flow and oxidative stress generated using PAWB

recirculation resulted in a higher inactivation of the weak biofilms from the PVC pipe walls. Moreover, PAWB significantly resulted in a higher inactivation of detached bacterial cells in the bulk water. The inactivation effectiveness of PAWB was enhanced under a high Reynold's number indicating the synergistic action of shear stress and plasma reactive species. The disinfection effectiveness of PAWB was also dependent on the circulated volume of PAWB, treatment time, and the concentration of major reactive species generated. This study shows the potential application of PAWB for disinfection of the drinking water pipelines and emphasizes the importance of understanding different hydraulic conditions of the drinker lines for designing a disinfection protocol for biofilm control.

Chapter 6: Conclusions and Recommendations

6.1 Overall conclusions

The persistence of *Salmonella* spp. in the food industry is a major public health concern as evidenced by the foodborne illnesses and outbreaks associated with them (Beuchat et al., 2013). In this PhD thesis, the survival of *Salmonella* in response to the adverse environmental conditions relevant to the food industry were evaluated. Furthermore, various conventional and novel technologies were employed to study the inactivation of *Salmonella* in low-a_w systems and biofilms.

The desiccation and thermal survival of *Salmonella* in low-a_w foods is dependent on the strain type, water activity, food composition and the inoculation methodology selected.

The effect of various intrinsic and extrinsic parameters on the desiccation and thermal resistance of *Salmonella* was explored in Chapter 3. Three low-a_w multicomponent foods (skim milk powder, pet food and burger binder) were wet inoculated with three different strains of *Salmonella* and equilibrated to a_w of 0.33 and 0.75 for 10 days. Equilibration to an a_w 0.33 resulted in a significantly ($p < 0.05$) higher desiccation survival of the *Salmonella* strains in the selected low-a_w foods. Overall, a high desiccation reduction of the *Salmonella* strains was observed in the skim milk powder, followed by the burger binder. Pet food, being rich in lipids demonstrated a significantly ($p < 0.05$) higher desiccation survival of *Salmonella* strains. The thermal inactivation kinetics of *Salmonella* in low-a_w foods obtained at 70 °C was significantly ($p < 0.05$) dependent on the interaction effect between the a_w at the treatment temperature, food composition, and the type of the strain.

On the contrary, for dry inoculation, the resistant *Salmonella* strain and low-a_w foods were separately equilibrated to a_w 0.75 and then mixed. A thermal reduction of < 1 log CFU/g was observed in the three dry inoculated foods. Moreover, irrespective of the physical structure of pet food (ground or pellets), no significant effect on *Salmonella* reduction was observed for both wet and dry inoculation methodologies.

The disinfection ability of dry heat, membrane-acting surfactants, and oxidizers varies based on the cellular state of *Salmonella*.

In Chapter 4, *Salmonella* survival under adverse desiccated conditions experienced in low-a_w processing environment was assessed. The disinfection potential of oxidizers (peracetic acid, hydrogen peroxide, plasma-activated water bubbles (PAWB) and plasma-activated hydrogen peroxide water bubbles (PAHP-WB)), membrane-acting cationic agents (benzalkonium chloride) and dry heat was evaluated against the suspended and surface-attached *Salmonella* cells on stainless-steel surfaces. The "air-drying" and "air-drying and equilibration" of *Salmonella* on stainless steel represented different desiccation conditions relevant to low-a_w food industry. It was shown that the inactivation mechanism of the disinfectants varied based on the cellular states of *Salmonella*. Suspended *Salmonella* was highly susceptible to the action of dry heat and PAHP-WB. While air-dried and equilibrated *Salmonella* demonstrated high resistance to dry heat, BAC, H_2O_2 and PAWB treatments, it was more susceptible to inactivation using PAA treatment as compared to air-dried *Salmonella*. This indicates that the inactivation of the different cellular states of *Salmonella* is dependent on various environmental conditions, including the treatment time, concentration, and the type of disinfectants.

The continuous recirculation of PAWB under different conditions of hold and flow demonstrated a significant ($p < 0.05$) effect of increasing the flow time in the reduction of air-dried and equilibrated *Salmonella* from the stainless steel (Chapter 4).

PAWB recirculation under different flow regimes affects the biofilm inactivation in the pipeline system.

PAWB demonstrated high effectiveness against the inactivation of suspensions and surface-attached *Salmonella* on the stainless steel (Chapter 4). Due to this, its efficacy was further tested against *Salmonella* in biofilms formed on the PVC pipe walls (Chapter 5). A lab-scale pipeline was developed for the recirculation of PAWB. High biomass produced by the day-10 biofilms significantly ($p < 0.05$) contributed to their lesser reduction upon exposure to PAWB circulation under different flow regimes as compared to day-6 biofilms.

The recirculation of PAWB under a turbulent flow regime (*Re* 4000 for 5 min) resulted in a higher biofilm inactivation from the pipe walls as compared to the laminar (*Re* 1000 for 5 min) and transitional (*Re* 2500 for 5 min) flow regimes. Furthermore, the effect of different combinations of flow regimes and treatment times was compared at constant volume. No significant difference in the biofilm inactivation was observed under the constant volume flow regimes (*Re* 1000 for 20 min, *Re* 2500 for 8 min, and *Re* 4000 for 5 min). A combined effect of various factors such as the volume of PAWB circulated, shear stress, treatment time, and the concentration of plasma reactive species produced, is important for the biofilm inactivation. Overall, the study provides important information on the use of PAWB under different flow regimes for drinking water system disinfection.

6.2 Future Recommendations

This research demonstrated the use of various conventional and novel interventions for *Salmonella* control in the food industry. The following suggestions can be further explored to effectively eradicate *Salmonella* from the dry and wet environments of the food industry:

- Novel oxidizing agents such as plasma-activated water bubbles (PAWB) and plasma-activated hydrogen peroxide water bubbles (PAHP-WB) demonstrated a high inactivation efficacy against the surface-attached *Salmonella* on stainless steel (Chapter 4). Furthermore, the food matrix had a significant ($p < 0.05$) effect on the desiccation and thermal survival of *Salmonella* in low-a_w foods (Chapter 3). Hence, it will be worthwhile to evaluate the disinfection ability of PAWB against surface-attached *Salmonella* on various food contact surfaces in the presence of organic residues and food soils containing carbohydrates, proteins, and fats.
- In Chapter 4, a significantly higher concentration of hydrogen peroxide, ozone, and nitrates (Table 4.3) was produced in PAHP-WB. Moreover, various other reactive species such as $^{\bullet}OH$ and $ONOO^-$ possess high antimicrobial potential (Mai-Prochnow et al., 2021). Therefore, for future research, scavengers can be incorporated into the experimental design to better understand the effect of major reactive oxygen species (RONS) in the inactivation of surface-attached bacteria. The scavengers are generally used along with the spin-trapping agents to obtain clear adducts of the desired plasma reactive species (Kwon & Jin, 2015).
- Hydrogen peroxide is commonly used for the disinfection of the drinker lines (Simões & Simões, 2013). In Chapter 4, disinfection using 0.1 M PAHP-WB resulted in a high inactivation of the desiccated bacteria on stainless steel. Similarly, PAHP-WB can also be employed to test its effectiveness against the biofilms in broiler DWS.

- In Chapter 5, focus has been given to the true biofilms grown in the drinking water pipelines. During the scheduled maintenance or under the conditions involving the temporarily shut off of the drinker lines, the developed biofilms are exposed to low-a_w conditions and results in the subsequent formation of dry biofilms. This necessitates the need to study the use of novel disinfectants against dry biofilms on contact surfaces.
- Biofilm incubation time significantly ($p < 0.05$) resulted in higher biomass production (Chapter 5). It will be interesting to analyze the EPS characterization (exopolysaccharides, extracellular protein, and extracellular DNA) (Simões et al., 2007) before and after the PAWB treatment. Moreover, the genetic expression of the virulence genes and biofilm regrowth potential pre and post-PAWB treatment has not yet been studied. Furthermore, biofilm metabolic activity, membrane integrity, and intracellular RONS formation can be determined following PAWB treatment.
- The incorporation of the strong biofilm-forming strains and evaluation of the inactivation efficacy of plasma-activated nanobubbles can also be studied in the future. Decreasing the size of the bubbles indicates a high internal pressure, which leads to a high energy dissipation upon bursting (Shiroodi et al., 2021). The individual action of the nanobubbles and the plasma-activated nanobubbles upon recirculation can be assessed against the strong biofilms attached to the surfaces.
- The benchtop pipeline model (Chapter 5) can be modified to include nipple drinkers, tees, and corners to better mimic the broiler DWS. Moreover, a venturi meter can be installed close to the pipe to increase the overall production of the bubbles for better biofilm inactivation.
- The inactivation efficacy of PAWB will also be affected based on the flow regimes selected during the biofilm formation. Hence, it is important to understand the biofilm formation under

different flow regimes (laminar, transitional, and turbulent) and to further correlate the detachment and inactivation behaviour using PAWB. Moreover, it is worthwhile to compare the influence of surface tension of PAWB and water, in the biofilm detachment under different flow regimes.

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Appendix

Appendix 1: Supplementary figures and tables for Chapter 3

Table A1: Proximate composition of low-a_w foods on a wet basis

Food matrix	Food component% (w/w)[a]				
	Moisture content	**Fat**	**Protein**	**Carbohydrates**	**Ash**
Pet Food	9.11 (0.06)^{A}	16.1 (0.47)^{A}	34.52 (0.02)^{B}	33.49 (0.42)^{C}	6.75 (0.03)^{C}
Skim milk powder	4.93 (0.02)^{C}	0.92 (0.02)^{C}	35.09 (0.03)^{A}	51.48 (0.01)^{B}	7.58 (0.01)^{B}
Burger binder	7.41 (0.02)^{B}	3.96 (0.12)^{B}	10.11 (0.07)^{C}	59.23 (0.02)^{A}	19.3 (0.22)^{A}

[a] Values are expressed as means (standard deviation) of two replicates. Different superscript capital letters in the column annotate significant ($p < 0.05$) difference in the% (w/w).

Table A2: Goodness-of-fit parameters of the Weibull model to simulate the thermal inactivation kinetics of the wet inoculum of *Salmonella* strains in the pet foods, skim milk powder and burger binder equilibrated to a_w 0.33.

Food matrix	**Strain**	**RSS**[a]	**Corr. R^2**[b]	**RMSE**[c]	***Af***[d]
Pet food	*S.* Enteritidis FUA1946	0.013	0.933	0.056	1.198
	S. Senftenberg ATCC43845	0.059	0.971	0.119	0.841
	S. Typhimurium ATCC13311	0.041	0.992	0.100	1.002
Skim milk powder	*S.* Enteritidis FUA1946	0.025	0.965	0.064	1.199
	S. Senftenberg ATCC43845	0.180	0.923	0.171	0.620
	S. Typhimurium ATCC13311	0.093	0.963	0.151	1.076
Burger binder	*S.* Enteritidis FUA1946	0.046	0.964	0.107	0.989
	S. Senftenberg ATCC43845	0.007	0.998	0.042	0.999
	S. Typhimurium ATCC13311	0.003	0.999	0.028	0.999

[a] Residual sum squares

[b] Correlation coefficient

[c] Root mean square error

[d] Accuracy factor

Values are expressed as means of the three biological experiments (n = 3).

Table A3: Goodness-of-fit parameters of the Weibull model to simulate the thermal inactivation kinetics of the wet inoculum of *Salmonella* strains in the pet foods, skim milk powder and burger binder equilibrated to a_w 0.75.

Food matrix	Strain	RSS[a]	Corr. R^{2b}	RMSE[c]	*Af*[d]
Pet food	*S.* Enteritidis FUA1946	0.017	0.966	0.065	1.042
	S. Senftenberg ATCC43845	0.044	0.991	0.104	0.996
	S. Typhimurium ATCC13311	NA	NA	NA	NA
Skim milk powder	*S.* Enteritidis FUA1946	0.088	0.986	0.152	0.983
	S. Senftenberg ATCC43845	NA	NA	NA	NA
	S. Typhimurium ATCC13311	NA	NA	NA	NA
Burger binder	*S.* Enteritidis FUA1946	0.247	0.961	0.247	0.980
	S. Senftenberg ATCC43845	NA	NA	NA	NA
	S. Typhimurium ATCC13311	NA	NA	NA	NA

[a] Residual sum squares

[b] Correlation coefficient

[c] Root mean square error

[d] Accuracy factor

'NA' indicates not available, the Weibull model was not fitted since the microbial counts were below the detection limit of 2 log(CFU/g) after few min of isothermal treatment at 70 °C, indicating the sensitivity in the behaviour of the strains to the conditions subjected. Values are expressed as means of the three biological experiments (n = 3).

Table A4: Goodness-of-fit parameters of the Weibull model to simulate the thermal inactivation kinetics of the dried *S.* Enteritidis FUA1946 in the pet foods, skim milk powder and burger binder equilibrated to a_w 0.75.

Food matrix	RSS[a]	Corr. R^{2b}	RMSE[c]	*Af*[d]
Pet food	0.010	0.973	0.048	0.984
Skim milk powder	0.007	0.977	0.041	1.014
Burger binder	0.010	0.964	0.043	0.976

[a] Residual sum squares

[b] Correlation coefficient

[c] Root mean square error

[d] Accuracy factor

Values are expressed as means of the three biological experiments (n = 3).

Table A5: GAB model parameters for adsorption isotherms of pet foods, skim milk powder and burger binder.

Food matrix	X_{mo}	ΔH_x	C_0	ΔH_c	K_0	ΔH_k	Corr. R^2	% RMSE
Pet food	0.004823	5515.251	101058.6	1340.419	1.1243	-629.205	0.980	0.468
Skim milk powder	0.000485	13266.4	655.6041	-11894.6	15.27723	-8388.09	0.935	0.901
Burger binder	0.005537	5352.486	101127.8	1340.905	2.028233	-2201.41	0.936	0.770

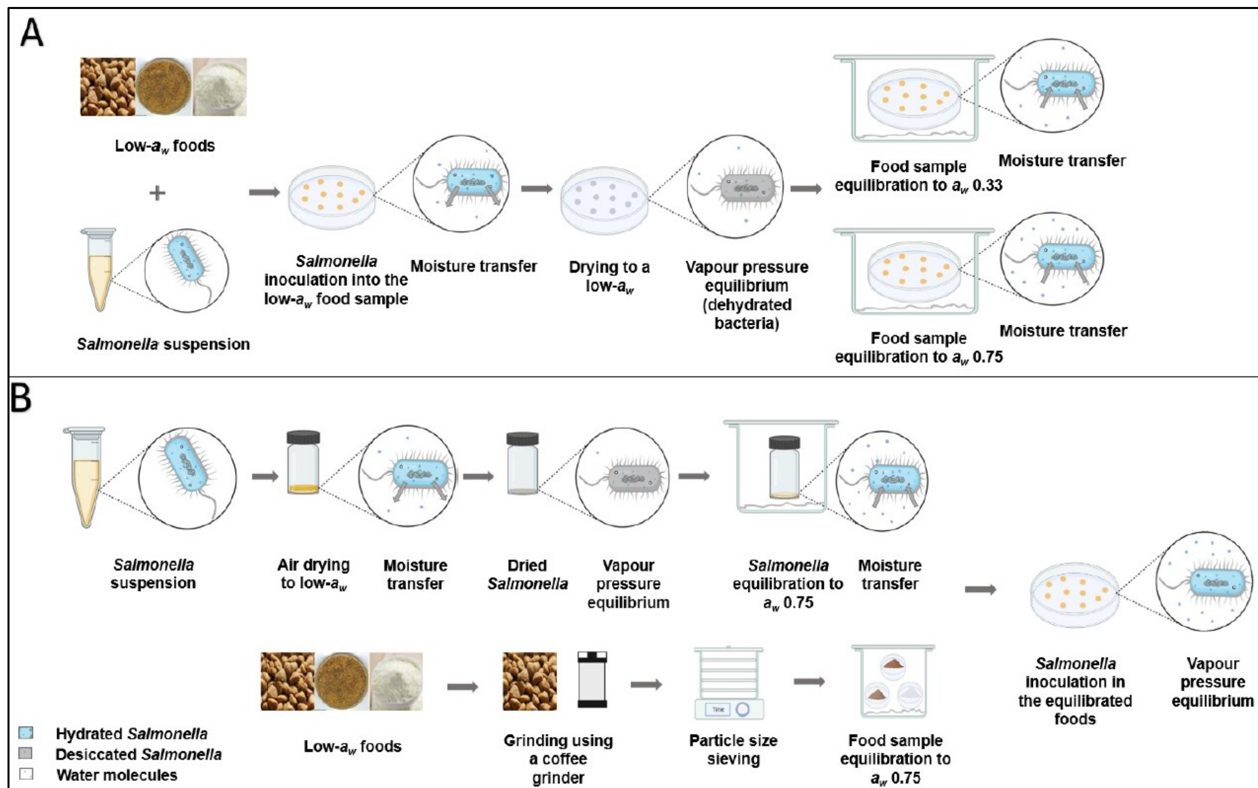


Figure A1: Graphical illustration of the inoculation methods of (A) wet and, (B) dry transfer of bacteria in low-a_w foods (Created with BioRender.com).

Appendix 2: Supplementary figures and tables for Chapter 4

Table A6: Goodness-of-fit parameters of the Weibull model to simulate the thermal inactivation kinetics of air-dried, and air-dried and equilibrated *Salmonella* on stainless steel coupons after the dry heat treatment at 70 and 80 °C.

Temperature (°C)	Desiccation	RSS[a]	Corr. R^{2b}	RMSE[c]	*Af*[d]
70	Before	0.05	0.98	0.12	0.95
	After	0.02	0.98	0.07	0.44
80	Before	0.07	0.98	0.13	0.95
	After	0.02	0.98	0.07	0.88

[a] Residual sum squares

[b] Correlation coefficient

[c] Root mean square error

[d] Accuracy factor

Values are expressed as averages of the three independent trials (n = 3).

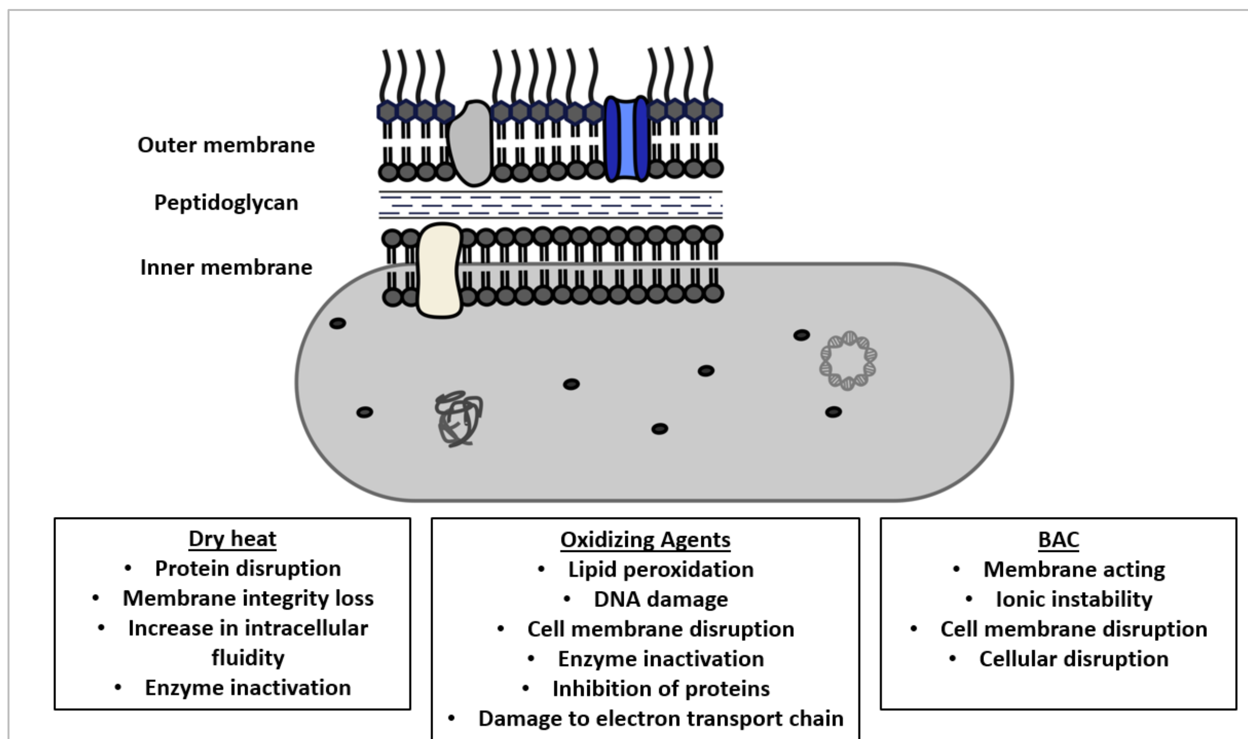


Figure A2: Schematic representation depicting *Salmonella* inactivation mechanism using dry heat, oxidizing agents, and membrane active sanitizers.

Figure A3: Setup depicting the continuous generation and circulation of PAWB on the inoculated stainless steel coupons.

Appendix 3: Supplementary figures for Chapter 5

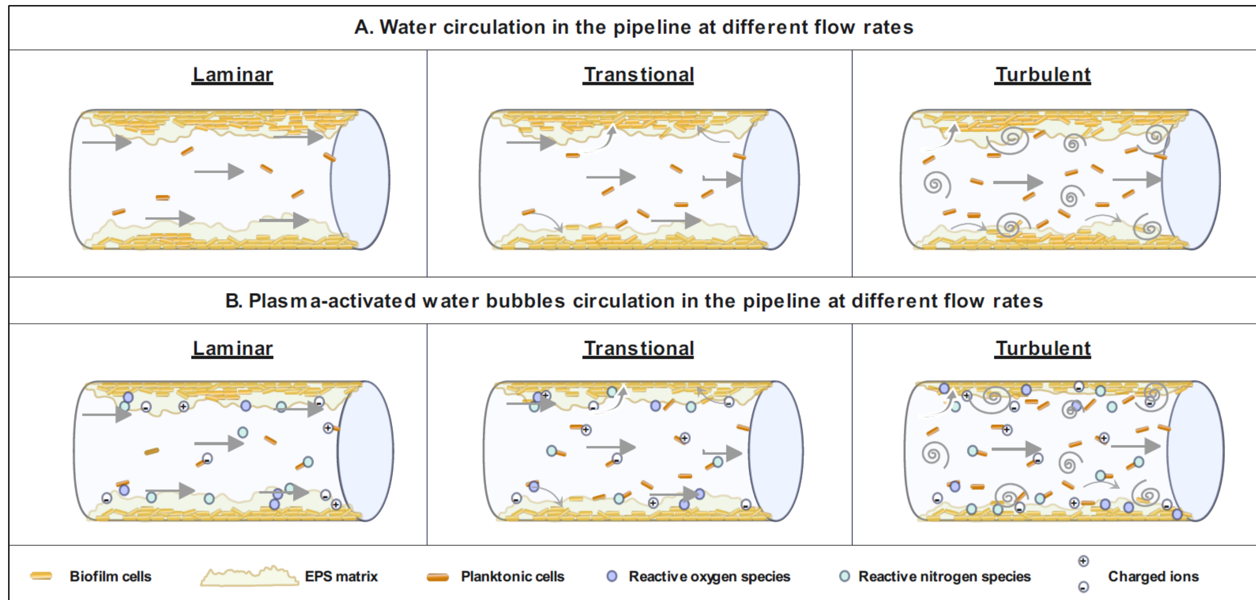
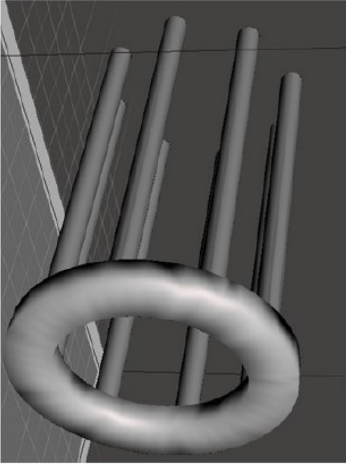


Figure A4: Graphical representation of circulating (A) water and (B) plasma-activated water bubbles (PAWB), under different flow regimes over the mixed-species biofilms grown on the inner surfaces of the pipes.



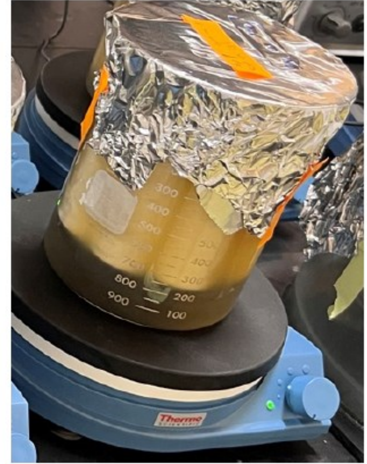


Figure A5: Representative images of (A) a 3D-printed pipe section holder, (B) glass beaker containing the inoculated broth and the PVC pipes, and (C) dynamic incubation at room temperature.

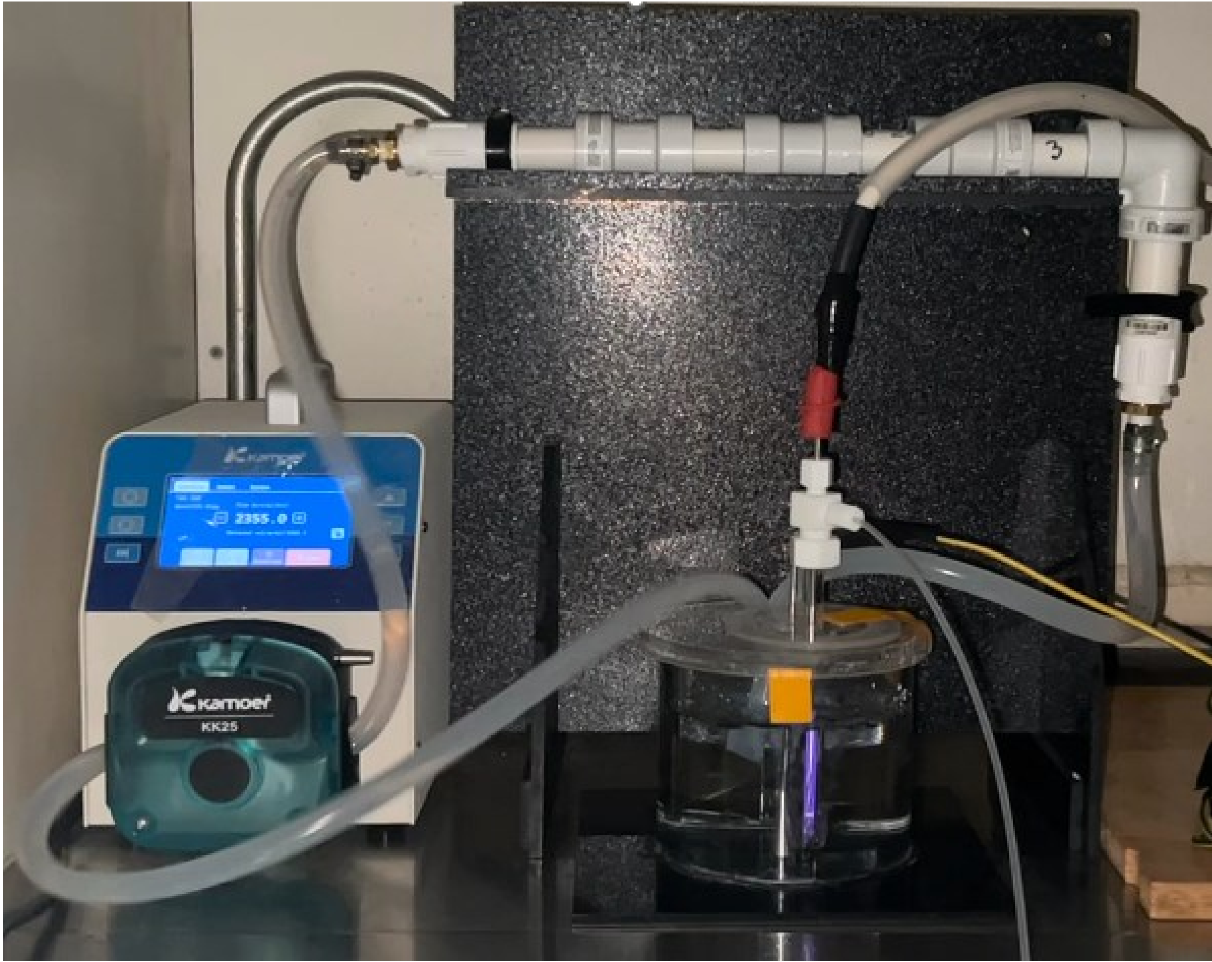


Figure A6: Setup of the continuous PAWB generation and recirculation in a benchtop pipeline system.