Development of a Novel Egg Surface Decontamination Method via Electro-nano-spray

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By

Shiva Aminian

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Canada

OR

Dean

College of Graduate and Postdoctoral Studies

University of Saskatchewan

116 Thorvaldson Building, 110 Science Place

Saskatoon, Saskatchewan S7N 5C9 Canada

Abstract

Chicken eggs and their products are a widely consumed and important source of nutrients for people worldwide. However, they can also be vehicles for pathogens like *Salmonella* and *Escherichia coli (E. coli)* that can cause foodborne illnesses. Commercially processed eggs in North America are typically washed with hot water and a chemical solution to decontaminate the surface of eggshells. Although the washing process is effective, this approach also removes the egg cuticle, which acts as a natural barrier to bacterial intrusion. In addition, the use of large amounts of water and washing chemicals produces significant amounts of chemically contaminated wastewater, making this approach environmentally unsustainable. Therefore, exploring alternative methods and innovative technologies that are both effective in preserving food and environmentally friendly would be important to the egg industry.

Recently, a new and innovative technique based on nanotechnology called Engineered Water Nanostructures (EWNS) has been developed as a chemical-free solution for disinfection processes. EWNS are formed by electrospraying and ionizing water to create highly charged nanoscale water droplets that possess unique physicochemical properties. It means EWNS are electron-rich water shells that contain a variety of reactive oxygen species (ROS) including hydroxyl radicals, superoxide, and hydrogen peroxide generated during the electrospray process which has been proven to effectively deactivate bacteria. Researchers have explored the effectiveness of EWNS against food-related microorganisms on the surface of various fruits and vegetables. The consumption of eggs is common in Canada, with an average person consuming about 242 eggs per year. However, it has not yet been tested whether EWNS could effectively decontaminate egg surfaces, which could potentially serve as an alternative disinfection method in the egg industry.

To evaluate the effectiveness of EWNS on eggshell decontamination, this research project was conducted in three phases. In Phase 1, an electro-nano-spray system was developed to generate EWNS, and lab-based experiments were conducted to assess the effectiveness of the process against *E. coli* inoculated on the eggshell surface. The parameters investigated included exposure time, water flow rate, and electric field strength to identify the most optimal operating conditions for the EWNS system. In Phase 2, the efficacy of the EWNS method to inactivate *Salmonella* on the egg surface was investigated under the optimal operating conditions established in Phase 1. In Phase 3, the impact of the EWNS technique on the quality attributes of treated eggs was evaluated

and compared to washed and fresh eggs. Egg quality was measured based on physical properties such as eggshell specific gravity, eggshell thickness, albumen and yolk pH, yolk index, Haugh unit, and moisture content of albumen and yolk, as well as chemical components such as the main proteins of albumen.

The results of the study showed that in 5 minutes of exposure time, the optimal EWNS operating conditions that produced the highest inactivation efficiency for *E. coli* inoculated on the egg surface included a water flow rate of 1 μL/min/needle (total flow rate of 16 μL/min), and an electric field strength of 9.0 kV/cm (-4.5 kV at 0.5 cm distance). At these conditions, the system achieved the inactivation efficiency of 97.6% for *Escherichia coli W3110* with a 1.64 log reduction and 80.4% for *Salmonella enterica serovar Enteritidis* with a 0.71 log reduction. Statistical analyses of the physical characteristics of treated eggs showed that there was no significant difference in the properties compared to unwashed and washed eggs one week after treatment (20 eggs per group). Moreover, the physical characteristics of different egg groups (3 eggs per group), including unwashed, washed, and treated eggs, were analyzed over a 21-day storage period, and it was found that the quality of all groups decreased over time. However, there was no significant difference in physical properties between the EWNS-treated eggs and the control (unwashed and washed eggs). The intensity of protein bands of SDS-PAGE gel images were analyzed statistically, and the results indicated that there was no significant variation in protein features between the three sets of eggs (3 eggs per group). The research has demonstrated that the EWNS system can be a promising and environmentally friendly method for decontaminating eggshell surfaces, and may be a suitable substitute for traditional egg sanitation methods. However, the study was limited in scale, and further investigations are required to how the EWNS system can be applied for larger-scale commercial applications.

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Lastly, in honor of my father's memory, I dedicate this thesis to him, a man whose life was defined by his unwavering dedication to his family. Though he is no longer with us, his example of resilience and commitment continues to inspire me every day.

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Abbreviations

Symbol	Definition
D	Distance between needle tips and counter plate electrode (cm)
d	Diameter of the yolk (mm)
E	Electric field strength (kV/cm)
F	Flow rate of water (µL/min) or (µL/min/needle)
h	Height of the yolk Or Height of the thick albumen (mm)
HU	Haugh unit
I	Electric current (A)
m_1	Total mass of the weighing bottle and sample (grams)
m_2	Total mass of the weighing bottle and dried sample (grams)
m_3	Mass of dried weighing bottle (grams)
Q	Quantity of electric charges (coulomb)
T	EWNS exposure time (min)
t	Contact time between the fluid and electrode (sec)
V	Applied voltage between electrode plates (kV)
w	Weight of whole egg (grams)
YI	Yolk Index

Chapter 1 – Introduction

Chicken eggs and egg products are commonly consumed all over the world in the food service and commercial food industries due to their versatility and nutrient content [1]. Eggs are an excellent source of nutrients such as essential amino acids, vitamins A, B2, B3, B6, B9, B12, E, micronutrients, and minerals such as iron, phosphorus, calcium, and potassium, which are essential for human health [2],[3],[4]. Additionally, eggs are considered functional ingredients in various food products because of their ability to stabilize emulsions, provide foaming constancy, and undergo thermal gelation [5].

However, eggs have the potential to act as vehicles for microbial growth, such as *Salmonella typhimurium, Escherichia coli (E. coli), Alcaligenes, Bacillus, Pseudomonas, Proteus, Listeria monocytogenes*, which can survive on the eggshells and even penetrate through shells and shell membranes, leading to spoilage or transmission of food-borne pathogens [6],[7]. The growth of microorganisms in eggs can be attributed to the nutrient-rich environment provided by the egg's content, which includes proteins, lipids, and carbohydrates [8].

The extent of bacterial contamination found on eggshells varies widely, ranging from zero to millions. On average, unwashed or untreated eggs contain approximately 100,000 bacteria per shell [9]. *Salmonella* and *E. coli* are the two main pathogens associated with foodborne disease outbreaks and are responsible for a significant number of illnesses [10]. *Salmonella* bacteria cause fever, stomach cramps, and diarrhea in humans and that may lead to serious illness and death [11]. While most strains of *E. coli* are harmless, emerging evidence suggests that some strains can cause disease in humans and animals; especially affecting children in developing countries. *E. coli O157:H7* is a particularly pathogenic strain that can cause illnesses, including bloody diarrhea, hemolytic uremic syndrome, urinary tract infections, pneumonia, meningitis, vomiting and low-grade fever and peritonitis in humans [12],[13]. Vinayananda et al. [14] reported that the incidence of *Salmonella spp.* and *E. coli* in the European Union and the US were 20.9 and 17.2 cases per 100,000 and 1.82 and 2.2 per 100,000, respectively.

Bacterial infection of eggshells can occur in two ways: vertically and horizontally. Vertical transmission occurs when the bacteria infect the reproductive organs of hens, such as the ovaries or oviduct tissues, and contaminate the yolk, albumen, and membranes directly as eggs are being

formed. This type of infection occurs before eggs are covered by shells. On the other hand, horizontal transmission occurs when the eggshell is contaminated after it is formed, either during oviposition or from the surrounding environment during production, processing, preparation and packaging [8],[9],[15]. Microbial contamination of poultry and poultry products, such as eggs, has a significant negative impact on the breeding poultry industry. The contamination can lead to economic losses due to various factors, including mortality of embryos, lower hatchability, and increased early chick mortality. These losses are particularly significant because they affect the entire supply chain, from breeders to consumers [9]. Overall, microbial contamination in the poultry industry can result in significant economic losses due to cleaning and containment, mortality, and risks to the health of the poultry and therefore needs to be managed and prevented effectively [16].

Although cooking and heat can be effective in killing or reducing the number of bacteria present in eggs, certain toxic microorganisms like *E. coli* are heat-stable and can still be harmful to humans [16],[17]. Therefore, it is important to recognize that relying solely on heat to eliminate all potential risks in eggs may not always be sufficient, and to ensure the safety of eggs, it is necessary for the egg industry to take measures to help mitigate the risks associated with heat-stable toxins and ensure that their products are safe for consumption.

In commercial processing, hot water containing sanitizing agents such as chlorine (sodium hypochlorite) solutions is commonly used to wash eggs, with the aim of reducing the levels of harmful microorganisms on the eggshell surface [18],[19]. Studies have shown that this process can decrease the microbial load by up to 6 log units [20]. It is worth noting that while egg washing is a common practice in the United States, Canada, Australia, and Japan, it is not allowed in the European Union for class A eggs due to potential drawbacks. One of the potential disadvantages is that washing eggs with chemicals can alter the delicate protein structure of eggs, potentially leading to a loss of quality [21]. Furthermore, the cuticle, which is an outer layer of eggs and serves as a natural barrier against bacterial access, can be damaged by chemicals used in egg washing [10],[22],[23]. The loss of the cuticle can increase the likelihood of bacteria penetrating the eggshell. Another concern associated with egg washing is production of a significant amount of

chemically contaminated waste, which could pose environmental concerns [24],[25]. As a result, the development of alternative approaches is needed to ensure egg safety.

Many different methods have been proposed for decontaminating eggshells, including thermal treatments such as hot air, microwaves, and irradiation, as well as non-thermal methods like electrolyzed oxidative water, ozone, ultraviolet light technology, pulsed light technology, and gas plasma technology. Each of these methods has its own advantages and disadvantages.

Therefore, it is important to develop a practical technique for decontaminating egg surfaces before they reach consumers. It is also important to ensure that egg constituents are not damaged during the decontamination process. To maintain the sensory quality of eggs, it is preferred to minimize the use of treatments that affect the taste, appearance, or texture of the eggs. In addition to being effective, the decontamination method should be non-corrosive, have low public health impacts, and be environmentally friendly. These factors should be taken into account when developing a suitable decontamination method for shell eggs.

Over the past 20 years, nanotechnology has emerged as a rapidly expanding area in the agri/feed/food sector. It has led to the development of various applications such as antimicrobial food surfaces, nano-enabled sensors, intelligent packaging, and novel disinfection platforms, all of which have great potential for the food industry [26]. One of the latest developments in this field is the introduction of a nanotechnology-based antimicrobial platform called Engineered Water Nanostructures (EWNS). This uniqueness of this platform is that it is chemical-free, low-cost, and relies on the generation of EWNS by combining the processes of electrospray and ionization [27],[28]. This innovative technology has the potential to revolutionize the food industry by providing an effective and sustainable solution for the decontamination of food products.

1.1 Project motivation and knowledge gap

The Engineered Water Nanostructures (EWNS) synthesized utilizing an electrospray system have been shown to be an effective, chemical-free, and environmentally friendly antimicrobial platform for disinfection. These EWNS possess unique physicochemical properties that play a crucial role in inactivating bacteria. The efficacy of this method has been studied in different scenarios, including airborne bacteria [27],[28],[29], surface bacteria (such as on stainless

steel coupons) [29],[30] and hand hygiene-related pathogens [31]. Moreover, the potential of EWNS to inactivate food-related microorganisms such as *Escherichia coli, Salmonella enterica, Listeria innocua, Mycobacterium parafortuitum*, and *Saccharomyces cerevisiae* on the surface of various foods has also been evaluated. This includes tomatoes [32],[33], blackberries [26], and spinach as a leafy vegetable model [34]. These studies have demonstrated the ability of EWNS to effectively inactivate microorganisms, making it a promising solution for disinfection without using chemicals.

However, the potential of EWNS as an antimicrobial agent has not been tested on eggshell contamination. It could be a promising alternative to the existing disinfection methods used in the egg industry. Therefore, it is important to explore the potential use of EWNS in disinfecting eggshells to ensure the safety and quality of eggs for consumption.

Overall, the main questions in this research were:

1. Can the eggshell surface be effectively disinfected using the nano-sized droplets generated through electrospray?
2. Which operating conditions can result in higher rates of microbial inactivation?
3. Is the EWNS technique capable of effectively inactivating *Salmonella* on the surface of eggs, given that *Salmonella* is the primary concern in the egg industry?
4. What are the effects of the EWNS technique on the quality of eggs that have been treated?

1.2 Research objectives

The overall objective of the present work was to the development of an eggshell surface decontamination method for the egg industry using Engineered Water Nano-Structures (EWNS) generated via electrospray, then

- evaluate the efficacy of the technology under various operating conditions to determine the optimal operating conditions for achieving the highest efficiency in inactivating *E.coli* on the egg surface using EWNS.
- investigate the efficiency of EWNS technique to inactivate Salmonella under the most optimal operating condition, and

- compare the possible changes in the quality attributes of the processed eggs to untreated eggs.

Therefore, the first part of the thesis project systematically investigated the effectiveness of the technology over three sets of flow rates (1, 2 and 3 μL/min/needle) and a wide range of electric field strengths (4, 4.5, 5, 6, 6.5, 8, 9 kV/cm) which were calculated from applied voltages (-4, -4.5, -5, -6, -6.5, -8, -9, -10, and +4.5 kV) divided by the distance of needle tips to counter electrode (0.5, 1, and 2 cm). The most effective conditions to inactivate the *E. coli* were found to be an electric field strength of 9 kV/cm (-4.5 kV in 0.5 cm) and a water flow rate of 1 μL/min/needle . These conditions were then used to test the effectiveness of the EWNS technology in inactivating *Salmonella* on the egg surface. Finally, the impact of EWNS treatment was investigated on the physical and chemical properties of the eggs by measuring the eggshell thickness, eggshell specific gravity, albumen pH, yolk pH, yolk index, Haugh unit, and moisture content, as well as the SDS-PAGE analysis for chemical characteristics (egg protein characteristics). Overall, the study aimed to develop a chemical-free and effective method for decontaminating eggshells in the egg industry while ensuring the quality of the treated eggs.

1.3 Organization of thesis

The thesis is organized into five chapters. The introduction is presented in Chapter 1, along with the knowledge gap, objectives, and thesis organization. In Chapter 2, the literature review provides current decontamination technologies for shell eggs, background on the principles of an electrospray system, operating parameters affecting the generation of EWNS including liquid flow rate, applied voltage, and distance between the needle tip and counter electrode, as well as the mechanism of inactivating bacteria using EWNS and egg quality measures. Chapter 3 focuses on determining the effects of different operating conditions for EWNS on the decontamination of egg surface including the treatment time, electric field strength, and sprayed liquid flow rate to determine the optimal operating conditions with the highest efficiency in inactivating *E. coli*. Then, investigate the efficacy of EWNS technology in treating inoculated *Salmonella* on the egg surface under optimal operating conditions. Chapter 4 presents the results of evaluating the physical properties of the treated egg compared to washed and unwashed (control) egg including eggshell thickness, eggshell specific gravity, albumen pH, yolk pH, yolk index, Haugh unit in short and

long storage periods as well as the protein content of albumen as chemical characteristics. Chapter 3 and Chapter 4 were prepared as manuscript format, respectively. Chapter 5 presents a summary of results, conclusions, and recommendations.

Chapter 2 – Literature review

In the following sections, current decontamination technologies for shell eggs, operating principles of electrospray system, operating parameters affecting the generation of EWNS including liquid flow rate, applied voltage and distance between the needle tip and counter electrode, mechanism of inactivating bacteria using EWNS and egg quality measures are provided.

2.1 Current decontamination technologies for shell eggs

The methods used to decontaminate eggshells can be categorized as either thermal or non-thermal [35].

2.1.1 Thermal methods

2.1.1.1 Egg washing

The commercial egg-washing process typically involves four stages: wetting, washing, rinsing, and drying. It is important to prevent eggs from being immersed in water during the process to avoid contamination, and the wash water must be maintained at a minimum temperature of 32.2 °C and at least 6.7 °C warmer than the internal temperature of the eggs to reduce the influx of water into the egg pores [35]. This helps to reduce the microbial load on the eggshell surface from 1 to 6 log units and prevent cross-contamination [35]. Class A eggs are not allowed to be washed in the European Union, whereas in the United States, Canada, Japan, and Australia, eggs are washed before being stored in a chilled environment [35]. One of the potential disadvantages is that washing eggs with chemicals can alter the delicate protein structures of eggs, potentially leading to a loss of quality [21]. Furthermore, the cuticle, which is the outer layer of the egg and serves as a natural barrier against bacterial access, can be damaged by chemicals used in egg washing [10],[22],[23]. The loss of the cuticle can increase the likelihood of bacteria penetrating the eggshell. Another concern associated with egg washing is the production of a significant amount of chemically contaminated waste, which could pose environmental concerns [24],[25].

In the United States, Canada at a licensed establishment, eggs undergo a series of processes including receiving, washing, candling, weighing, grading, and packaging into containers to ensure compliance with each country's specific food safety standards and regulations [36],[37]. In

Australia, where egg washing is not mandatory, the Food Standards Australia New Zealand (FSANZ) prohibits the sale of dirty eggs. The shell eggs, whether transferred automatically or manually, are then placed in reusable trays to undergo sorting, washing, candling (crack detection), grading, and packaging as they progress along the supply chain [38]. However, in European Union and the UK, egg washing is prohibited by law. In Europe, chickens on farms are vaccinated against salmonella, which helps to maintain the intact cuticle of the eggs when they are sold. In the UK, Class A eggs are not subjected to preservation treatments or chilled in facilities where the temperature is artificially kept below 5°C, except for limited periods during chilled transport or temporary storage on retail premises. It is important to store eggs in clean, dry conditions away from direct sunlight at all times [39],[40]. For example, in Italy eggs are carefully wiped clean to remove any debris before they are packaged and made available for sale and are never subjected to a washing process [41].

2.1.1.2 Hot air treatment

The use of hot air pasteurization is a potential method for decontaminating shell eggs, and it is both inexpensive and easy to apply on an industrial scale before packaging. Hot air pasteurization techniques can be classified into two categories: forced convection (FC) and natural convection (NC) methods. The former is particularly effective in surface decontamination, while the latter is effective in the interior of the egg. Convection ovens are commonly used for NC methods, but their application on an industrial scale is limited due to their long treatment time. In recent years, researchers have explored alternative methods such as hot air guns and prototypes. Using one-shot techniques can help increase the initial log reduction of bacteria immediately after treatment, but it may not prevent the surviving bacteria from regrowing during storage. To minimize regrowth, hot air pasteurization can be combined with long-term techniques, such as refrigeration and modified atmosphere packaging, that can maintain their decontamination effect throughout the supply chain [42],[43].

2.1.1.3 Microwaves

Microwaves are electromagnetic waves with frequencies ranging from 300 MHz to 300 GHz. These waves can have thermal and nonthermal effects on pathogens. Thermal inactivation occurs due to the heat generated during the microwave application process, leading to changes in

important components of the pathogen's structure, including denaturation of enzymes, proteins, nucleic acids, and disruption of membranes. Nonthermal effects include selective heating, electroporation, cell membrane rupture, and magnetic field coupling, all leading to cell death [43]. Microwaves can be used to reduce bacterial load on eggshells including *S. Enteritidis*. While this heat-based decontamination method is effective at reducing microbial contamination, it can have adverse effects on the physical-chemical properties, nutritional content, and sensory characteristics, including color and texture, of eggs. This can make the eggs less appealing to consumers [44].

2.1.1.4 Irradiation

Irradiation is a process that uses ionizing radiation to sanitize food. The three types of ionizing radiation allowed for use are high-energy gamma rays, X-rays, and accelerated electrons. Gamma rays are produced by radioactive substances like cobalt-60 and cesium-137, while electron beams are produced in linear accelerators, and X-rays are produced by interposing a metal target between the electron beam and the food product. The electrons in these types of radiation pick electrons from the atoms of the food product, which can destroy DNA molecules in living microorganisms, effectively eliminating them. Thus, electron-beam processing could be a good option for eggshell decontamination because it does not alter the temperature of the egg and allows for high dose rates [43]. Although irradiation with X-rays and γ-rays could be a potential decontamination method, there is a public fear of the potential radioactive effects on foodstuffs [45]. Scientific studies have shown that irradiation can effectively eliminate bacterial contamination in shell eggs with specific doses depending on the pathogen and the inoculum level [43].

2.1.2 Non-thermal methods

2.1.2.1 Electrolyzed water

Nowadays, electrolyzed water (EW) is used in various fields and is considered a promising non-thermal treatment for hygiene control. It is an environmentally-friendly option, as it does not produce harmful by-products or residues [46]. Electrolyzed water is obtained by passing a diluted salt solution through an electrolytic cell with an anode and cathode separated by a membrane,

resulting in an acidic and alkaline component. The acidic EW has some characteristics of pH from 2 to 3, oxidation-reduction potential (ORP), and free available chlorine concentration, while the alkaline EW has a pH of 6.8 to 11.6. Numerous studies have shown that EW has antimicrobial activity on various microorganisms due to pH, ORP, and hypochlorous acid (HOCl). EW can modify metabolic fluxes and adenosine triphosphate (ATP) production by changing the electron flow in cells. Bacteria grow in a pH range of 4 to 9, so a low pH and HOCl kill the microbial cell by inhibiting glucose oxidation by chlorine-oxidizing sulfhydryl groups of certain enzymes which are important in carbohydrate metabolism [42],[43]. Thus, acidic EW is effective in reducing the populations of pathogenic microorganisms on the surface of shell eggs, but the pH level is a crucial factor for its effectiveness and its use is limited when low pH values are observed [10],[46].

2.1.2.2 Ozone

Ozone is a powerful sanitizer that can eliminate all types of microorganisms at low concentrations. It is considered safe for use as an antimicrobial agent in food processing, storage, and treatment. It is generated by ultraviolet radiation or an electrical field and has a bactericidal effect on various organisms. Ozone destroys bacteria by attacking their membrane glycoproteins and/or glycolipids, causing cellular components to leak and leading to cell death. It is considered a nonpolluting sanitizer for decontamination of egg surfaces due to its decomposition into oxygen [35],[43]. However, due to its short half-life, ozone cannot be stored and needs to be produced upon request, making it more costly compared to other treatments [35]. In addition, the effectiveness of this treatment can be influenced by the type of environment in which it is applied, with dry and moist ozonated air being only partially effective [43]. According to recent studies, exposure to ozone leads to respiratory issues for humans by reducing lung function and inflammation, even at low concentrations of 60 ppb after 6.6 hours of exposure. In order to prevent workers from potential harm, it is crucial to eliminate the off-gases produced by the contactor [47].

2.1.2.3 Ultraviolet light technology

For over 60 years, ultraviolet technology has been known to be an effective method of disinfection. There are different types of ultraviolet light, categorized by their wavelength range, including long-wave ultraviolet A (315 to 400 nm) which has minimal disinfectant properties and is present in sunlight, medium-wave ultraviolet B (280 to 315 nm) which is also present in sunlight

and has some germicidal effect, and short-wave ultraviolet C (200 to 280 nm) which has the most potent germicidal effects and is not naturally occurring, but produced by converting electrical energy using low and medium-pressure lamps [21],[35],[48]. Ultraviolet C (UV-C) has the greatest germicidal effects and is commonly used for disinfection because most microorganisms absorb ultraviolet light at 254 nm, causing damage to their DNA and hinder the transcription and replication processes of DNA and ultimately causes the death of the microbial cell on the egg surface. Furthermore, studies have shown that UV radiation impacts the integrity of the cell membrane, which modifies proteins and prevents oxidative phosphorylation [43],[49],[50]. Thus, UV-C radiation is a favorable method for disinfecting and sterilizing food due to its cost-effectiveness, dry process, lack of by-products, and absence of sensory changes or radioactivity [44],[45],[51]. However, Indiarto et al. [45] noted that prolonged exposure to UV radiation can cause damage to human health such as eye damage, burns and skin cancer. The effectiveness of the treatment is dependent on several factors such as the length of exposure time, the intensity of the light, the wavelength of the illumination, and the ability of the microorganism to withstand the UV exposure [23],[44].

2.1.2.4 Pulsed light technology

Pulsed light technology is a method that employs brief, high-energy pulses of light across a wide range of wavelengths, from 100 to 1100 nm, to remove bacteria and sanitize the surfaces of food products like eggs [50]. The treatment's lethal effects are primarily due to two mechanisms: photochemical and photothermal. Photochemical damage is primarily induced by the UV-C region of the spectrum (200 to 290 nm) and targets bacterial DNA, while photothermal damage is caused by light absorption, which leads to temporary overheating, water vaporization, and membrane rupture [35],[43]. Pulsed light technology offers several advantages, including being a non-thermal technology with a higher energy input and shorter exposure time than UV-based treatments. However, it also has some limitations, such as a limited effect due to the shadow effect and a reduction in efficiency when the cuticle's integrity is compromised [18],[42],[43]. In addition to bacteria, damage to the cuticle can lead to the formation of filamentous and bunch-shaped structures. These structures have the ability to cover certain cells and create shadowed areas, effectively protecting bacteria from pulsed light [20].

2.1.2.5 Gas plasma technology

Plasma is a collection of interacting particles, including photons, electrons, ions, atoms, free radicals, and excited molecules. It can be classified as thermal or non-thermal, depending on the conditions under which it is created. Non-thermal plasma, which is generated at lower pressures and uses less power, has a higher electron temperature than gas [22]. Furthermore, it is considered to be an environmentally-friendly option due to its lack of harmful by-products or residues [22]. During plasma treatment, microorganisms are bombarded with OH and NO radicals, causing surface lesions that the cell cannot repair quickly. Plasma treatment can also form molecular fragments and volatile compounds in the cells that lead to complete cellular destruction by absorption of plasma components onto their surface or by perforating their membranes and acidifying the environment [52]. Nonthermal gas plasma can be used as an alternative decontamination method for food products that cannot be sanitized by conventional methods [43]. Indiarto et al. [45] reported that non-thermal plasma is energy efficient but the cost of using noble gases in the process can be a limiting factor for the widespread use of this technology.

Therefore, in light of the benefits and drawbacks associated with various eggshell decontamination methods, it would be ideal to identify a practical approach that effectively disinfects egg surfaces while preserving the quality of the egg's constituents. Minimizing any sensory changes is also important, and the treatment should only be as extensive as necessary to achieve maximum bacterial reduction. Additionally, it is important for the decontamination method to be non-corrosive, have no potential worker health impacts, and be environmentally friendly.

2.2 Operating principles behind EWNS based on electro-nanospraying techniques

The Engineered Water Nanostructures (EWNS) synthesis process involves two significant phenomena, namely electrospray and ionization through an electrospray system [27],[31]. Electrospraying is a commonly used technique to convert liquids, particles, and fibers into aerosols for various industrial and environmental applications. This process is also used to synthesize polymeric and ceramic particles and for delivering DNA to elements, including a high voltage

power source, a syringe containing a solution with a metallic capillary acting as an electrode, and a counter electrode plate. The syringe is connected to a syringe pump controlling the liquid flow rate. The electrospray technique utilizes a powerful electric field created by the fine capillary and the counter electrode to transform the liquid into an aerosol. To generate the required high electric field, a high voltage (usually negative) is applied between the two electrodes. This high electric field results in the accumulation of negative charges at the water-air interface at the emitter's tip, causing the formation of a conical meniscus known as the Taylor cone, which is maintained by the surface tension of the liquid, the electrostatic force, and gravity [28],[33]. The high electric field also causes the liquid to break up into highly charged and unstable droplets, as described by the Rayleigh theory [53],[54].

As the surface charge density increases, the distance between the charges decreases, leading to an increase in the electrostatic interactions. This phenomenon ultimately causes the droplets to break. However, the surface tension force works in opposition to this and tries to maintain the droplets' integrity. There is a critical diameter, known as the Rayleigh diameter, beyond which the surface tension force is no longer sufficient to counteract the electrostatic interactions. This leads to the breakage of the droplets into smaller ones, typically at the nanoscale level, with lower surface charges [32],[53],[54].

The high electric field generated during electrospray causes ionization, splitting water and air molecules, particularly oxygen, and resulting in the formation of a significant number of reactive oxygen species (ROS), including hydroxyl and superoxide radicals. These ROS have a very short lifespan in the order of nanoseconds and become embedded in the droplets produced during electrospray, which are responsible for the biocidal and oxidizing properties of the generated electrospray water nanodroplets (EWNS) [26],[27],[31]. EWNS possess a unique structure characterized by a water shell rich in electrons that contains various ROS generated during the electrospray process [53]. Since EWNS have a nanoscale size, they are highly mobile, and their electric charge, including electrons and ions, increases their surface energy and reduces their evaporation rate [28],[32]. Moreover, the larger surface-to-volume ratio in nano-sized particles allows for greater surface exposure to microbes when compared to micro-sized particles. This increased exposure contributes to improved antimicrobial activity [55]. As a result, EWNS have a longer lifetime and can remain airborne for an extended period. EWNS have the potential

to come into contact with both airborne pathogens and those present on surfaces, leading to the delivery of their ROS payload and ultimately resulting in the inactivation of microbes [28],[32].

2.3 Factors affecting the generation of EWNS

2.3.1 Liquid flow rate

The liquid flow rate in electrospray determines the quantity of material that passes through the nozzle per unit of time, as well as the duration of contact between the material and the charged nozzle which is connected to a high voltage. So, it affects the droplet size produced by the nozzle. A shorter residence time in the nozzle leads to a lower charge uptake by the liquid, resulting in the formation of larger droplets. On the other hand, lower liquid flow rates will have a longer residence time, which leads to less charge acquisition and the formation of smaller droplets. Thus, a certain minimum flow rate is required to get a stable cone jet in electrospraying [56]. Si et al. [30] conducted an investigation to analyze the impact of the water flow rate on the electrospray area. The study showed that as the liquid flow rate increased, the area where water droplets were deposited also increased. This phenomenon occurs because an increase in the liquid flow rate results in the generation of more droplets carrying the same charge, which causes a repulsive electrostatic force and ultimately leads to an expansion of the sprayed area.

2.3.2 Applied voltage

In electrospray process, voltage is applied to charge a liquid solution, which breaks it up into tiny droplets when the charge overcomes the liquid's surface tension (up to the Rayleigh limit). Increasing the voltage can result in a significant reduction in droplet size, but there is a limit beyond which the droplet size remains constant even with increasing voltage [56]. Several researchers investigated the impact of the applied voltage and found that the current generated by negative voltage is always greater than that of positive voltage due to the higher mobility of the negative space charge [57]. Si et al. [30] also found that negative voltage polarity results in a larger electrospray area which supports the research of Rosell-Llompart et al. [58] that the relationship between the spray cone angle and the electrical current is directly proportional. Moreover, the negative voltage polarity results in a larger electrospray area because more charges per droplet are produced, which leads to a higher electrostatic force [59]. This has been supported by recent research conducted by Si et al. [30].

2.3.3 Distance between the needle tip and counter electrode

The distance between the capillary and the counter electrode has an impact on the electric discharge process. The discharge of electricity is affected by the distance between the needle tips and the counter electrode. According to research conducted by Zheng et al. [60] and Si et al. [30], there is a direct correlation between the two. If the distance increases, the strength of the electric field decreases, leading to a reduction in the electric current. When it comes to electrospraying, the diameter of the electrospray droplets is also affected by the distance between the needle tip and the counter electrode. In particular, Si et al. [30] found that when the distance was 2 cm, the electrospray droplets had a wider area compared to other distances of 3 and 4 cm. This phenomenon could be attributed to increased water evaporation as the distance between two electrodes increases.

2.4 Mechanism of inactivating bacteria using EWNS

Experiments have been carried out to understand how the bacterial inactivation induced by EWNS occurs, specifically through the permeability of the bacterial membrane and lipid peroxidation assays [53],[54]. It is widely acknowledged that the inactivation is due to the presence of reactive oxygen species (ROS) in the EWNS. These ROS species cause damage to the lipid membrane of the bacterial cell wall through oxidation. This is supported by the detection of malondialdehyde, a byproduct of lipid peroxidation reaction of unsaturated fatty acids in bacteria exposed to EWNS [61],[54],[61].

Studies have shown that gram-negative microorganisms like *E. coli* can experience oxidative stress when exposed to high levels of ROS, which include superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2), and hydroxyl radicals (HO^*). This stress can lead to irreversible damage to cellular components, decreased cell stability, and modifications to proteins [62],[63]. As a result, it appears that the inactivation of bacteria by EWNS is achieved through oxidative damage caused by ROS. These findings have significant implications for various fields, including

medicine and water treatment, where EWNS could be applied to effectively neutralize harmful bacteria.

Figure 2.1 displays electron micrographs that illustrate the physical alterations that occurred in the cells of three different bacteria species, namely, *E. coli, S. enterica, and L. innocua*, as a result of exposure to EWNS, which led to their inactivation. The figure provides a visual comparison between the control cells and the cells that were exposed to EWNS. The control cells showed no noticeable changes and appeared to have intact cell membranes, well-defined periplasmic spaces, and intact intercellular structures. On the other hand, the cells exposed to EWNS showed visible damage to their cell membranes, such as cracks and ruptures, indicating that the exposure to EWNS caused physical harm to the cell structure of these bacteria [62].

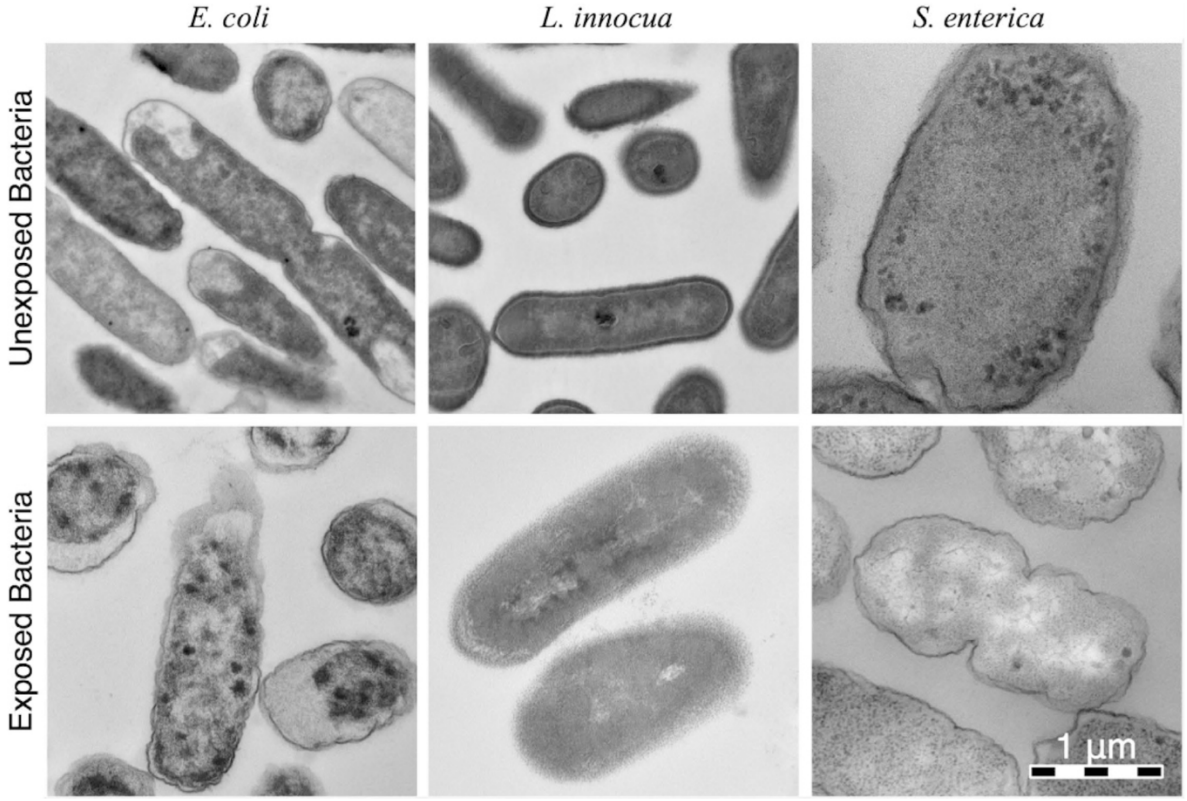

Figure 2. 1 Electron microscopy imaging of the control and exposed bacteria from G. Pyrgiotakis et al. [62], 2016, (Page 9). [Open access article]

Figure 2.2 also presents Transmission Electron Microscopy (TEM) images of *S. marcescens*. Figure 2.2 (a) displays an image of a live *S. marcescens* cell, which had a robust and

well-defined membrane structure and appeared rod-like in shape. In contrast, the exposed *S. marcescens* cell in Figure 2.2 (b) appeared to have lost its outer membrane and has an elliptical shape. Additionally, the dead bacteria in Figure 2.2 (b) exhibited a large vacuole containing a crystalline structure that may have been resulted from a disturbance in the formation of cytoskeletal proteins, such as actin and tubulin, which may have become cross-linked due to oxidation. These changes were likely the direct consequences of exposure to EWNS [64].

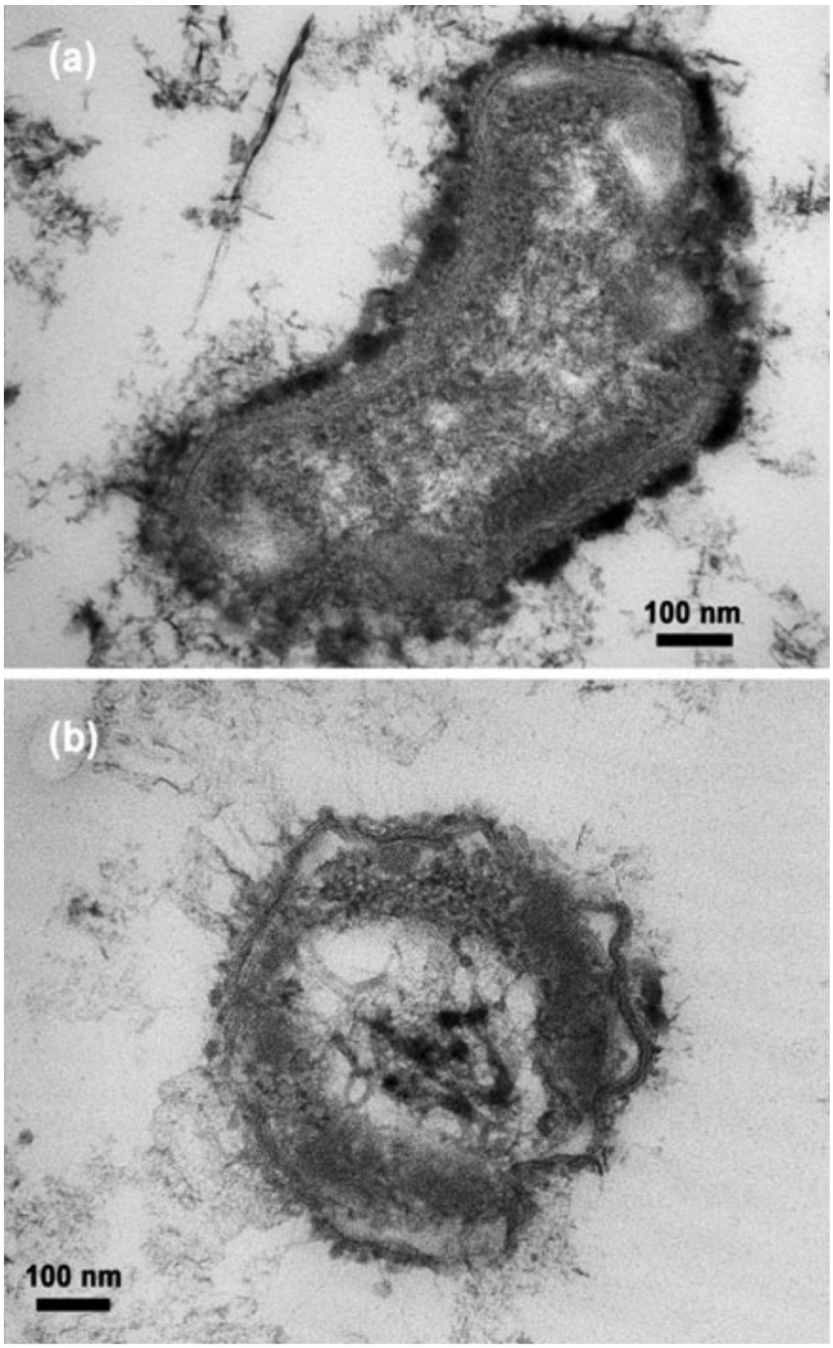

Figure 2. 2 TEM imaging of the S. marcescens, (a) control and (b) exposed bacteria from G. Pyrgiotakis et al. [64], 2012, (Page 9). [With permission from Springer Nature]

2.5 Assessment of egg quality

Egg quality is a multifaceted concept that encompasses all aspects related to the yolk, shell, and albumen of the egg, and can be categorized into external and internal quality. In scientific

studies, external quality traits, which include egg weight, shape, shell deformation (destructive or non-destructive), shell weight, shell thickness, and shell ratio are primarily associated with the appearance of the egg and the strength of the shell. These factors are crucial for consumer appeal and for preventing bacteria from entering the egg. In contrast, internal quality is determined by the size of the air cell, albumen quality, and yolk quality, which include parameters such as the length, width, height, and weight of the albumen and yolk, as well as the Haugh unit, albumen index, yolk index, and yolk albumen ratio [65],[66],[67]. Therefore, to evaluate any potential effects of the decontamination method, it is necessary to assess the properties of both EWNS-treated and untreated eggs, to determine whether the decontamination process has affected their external or internal quality parameters.

Eggshell strength is a crucial factor that determines the external quality of an egg and is generally influenced by the thickness and proportion of the shell. It plays a significant role in determining the viability of eggs during transportation and storage. It is essential for the shell to be thick enough to endure reasonable handling without breaking. Thus, eggs with fragile shells not only result in economic losses but also increase the risk of shell cracking, which can lead to bacterial contamination [66],[68].

One way to determine the freshness of eggs is by examining their specific gravity and weight loss rate. These two factors are good indicators of egg freshness. In general, eggs that have a higher specific gravity and a lower weight loss rate are considered to be fresher, as they indicate that the egg has a smaller air cell and a more tightly sealed shell, respectively. However, the integrity of the cuticle membranes that cover the eggshell is also important for freshness and the prevention of bacterial penetration. Eggs with lower specific gravity and higher weight-loss rates are not only of lower quality but also have a greater likelihood of being penetrated by *Salmonella* spp. due to their weaker, thinner, and more porous shells [9],[15],[69].

When evaluating the quality of an egg, one key factor is the quality of its albumen. This can be measured in a variety of ways, such as through its pH level, height, or by calculating the Haugh unit. pH levels are important indicator of egg freshness, both in the albumen and in the whole egg. When eggs are freshly laid, the albumen pH is typically between 7.6 and 8.5. However, as eggs are stored over time, the pH levels of both the albumen and whole egg tend to increase.

This is due to two factors: gaseous exchanges with the surrounding air through the pores on the eggshell, and the migration of water and minerals between the albumen and yolk through the vitelline membrane. These processes are influenced by various environmental conditions, such as temperature and humidity levels. The increase in pH levels can be an indicator of decreased freshness [15],[68].

The Haugh unit is a measure that takes into account both the height of the albumen and the weight of the egg. Essentially, it is an index that adjusts the height of the albumen based on the size of the egg. A higher Haugh unit (HU) value indicates a higher quality egg. In the United States, the grading of eggs is largely based on the HU value. Eggs can be graded as AA, A, B, or C, depending on their HU score. Grade AA eggs have an HU value between 100-72, Grade A eggs have an HU value between 71-60, Grade B eggs have an HU value between 59-30, and Grade C eggs have an HU value below 29. The higher the HU value, the better the quality of the egg, and the higher the grade it will receive [65],[70],[71].

Furthermore, the yolk of an egg also undergoes changes as it ages. The freshness of eggs can be assessed by examining the yolk and albumen indexes, which are influenced by the migration of water vapor through the cuticle membrane as the eggs age [15],[22]. The deformation in the shape of the yolk is primarily due to the weakening of the vitelline membrane. To assess changes in yolk shape, two indicators are commonly used: the Yolk Index (YI) and the Yolk Coefficient (YC). The Yolk Index is calculated as the ratio of the yolk height to width, while the Yolk Coefficient is expressed as the ratio of the yolk weight to height [68].

Chapter 3 – A comprehensive study of microbial decontamination of egg surface by engineered water nanostructures produced by an electrospray

The content of this chapter will be submitted to a peer reviewed journal of publication.

Contribution of the MSc student

Experiments were planned and performed by Shiva Aminian with the guidance provided by Drs. Lifeng Zhang, Shelley Kirychuk and Karen Schwean-Lardner. Drs. Lifeng Zhang, Shelley Kirychuk and Karen Schwean-Lardner supervised and provided consultation during the entire experimental period as well as thesis preparation. All the writing of the submitted manuscript was done by Shiva Aminian with Drs. Lifeng Zhang, Shelly Kirychuk and Karen Schwean-Lardner providing editorial guidance regarding the style

Contribution of this chapter to the overall study

In this chapter, an electro-nano-spray system was developed and employed to generate EWNS for decontamination of eggshell surface under various operating conditions to determine the most optimal operating condition. *E. coli* W3110 was selected as a representative strain of contamination. Firstly the effect of treatment time, ranging from 2.5 to 15 minutes was investigated. Then, the operating parameters including electric field strength (4 - 9 kV/cm) and liquid flow rate (1 - 3 μL /min/needle) were optimized to find the highest efficacy of the disinfection method. Afterwards, *Salmonella enterica serovar Enteritidis* (ATCC 4931) was tested with the optimized condition.

3.1 Abstract

Eggs and egg products are widely consumed as food globally; however, they can also serve as carriers of diseases in the food industry. Since product safety is the most important aspect of the food industry, the egg industry needs to consider approaches to microbiologically decontamination of eggs. Electrospray is a process of atomizing liquid that uses electrostatic forces to break it down into highly charged fine droplets. In this study, a newly designed prototype for generating the engineered water nanostructures (EWNS) was developed on the lab scale to inactivate *Escherichia coli (E. coli)* that was inoculated onto egg surfaces. The generator had 16 needle injectors connected to a high-voltage power supply and a grounded counter electrode to create the electric field. When the needles released water droplets, the electric field created between the needles and the counter electrode caused the droplets to break into nano-sized droplets. In this study, the effect of different parameters such as exposure time, electric field strength, and the liquid flow rate was investigated to find the optimized operating conditions with the highest rate of *E. coli* inactivation. At 5 min as the targeted treatment time, the highest inactivation efficiency of 1.64 log (97.6 %) was achieved under 9 kV/cm and 1 μL/min/needle. The optimized operating condition was also tested to inactivate *Salmonella* on the inoculated egg surface and the inactivation efficiency of 80.4 % with 0.71 log was obtained. The findings indicate that the EWNS technology has the potential to replace conventional methods of decontaminating egg surfaces without the use of chemicals.

3.2 Introduction

Eggs are a rich source of protein, minerals, and essential vitamins, and they are widely consumed worldwide [2],[3],[15]. However, they can also carry harmful microorganisms, posing a serious health risk to consumers. The risk of contamination can occur during various stages of egg production, processing, packaging, and consumption which could lead to serious health concerns worldwide [8],[17]. Eggs and egg-containing foods are at high risk of being contaminated by *Salmonella* and *E. coli*, which are well-known foodborne pathogens [24]. Although contamination problems are less frequent today compared to the 1980s and 1990s, some eggs are still contaminated with *Salmonella* [72]. The symptoms caused by *Salmonella* infection include fever, stomach cramps, and diarrhea, which can lead to severe illness and even death [11]. In the US, *Salmonella* infections result in about 1.35 million cases, 26,500 hospitalizations, and 420 deaths annually [72]. The constant danger of microbiologically contaminated food poses a

significant threat to public health globally [33]. In Canada, this issue results in the illness of four million people every year, according to a report on food-related illnesses, hospitalizations, and deaths in 2016 [73]. While eggs are a valuable source of nutrition, the contamination of their shells with harmful pathogens can be a severe health concern. Although heat and cooking can help reduce bacterial contamination in eggs, certain microorganisms like *E. coli* that produce heat-stable toxins can still be a health risk to humans [16],[17]. Therefore, to ensure that eggs are safe to consume, the egg industry must take steps to control microbial contamination.

Eggshell decontamination methods are classified as either thermal or non-thermal. One common practice for decontaminating eggshells is washing them with hot water and chemicals like chlorine (sodium hypochlorite) solutions [19]. While egg washing can reduce bacterial load, it does not completely eliminate the risk of contamination [74]. Egg washing is prohibited in the European Union due to various drawbacks such as altering protein structure and producing harmful waste [2],[21]. Additionally, washing eggs with chemicals can damage the cuticle, which acts as a natural barrier to bacterial access, and causes environmental concerns [24],[25]. There are other alternative techniques to decontaminate shell eggs, including hot air treatment, microwaves, as well as non-thermal methods like electrolyzed oxidative water, ozone, ultraviolet light technology, pulsed light technology, and plasma. Although these methods are effective in inactivating microbes, their limitations include dependency on dosage/level, sensitivity to pH, and high operation costs [3],[43],[45].

Therefore, the ideal approach for eggshell decontamination would effectively disinfect egg surfaces while preserving the quality of the egg's constituents, minimizing any sensory changes, having low public health impacts, and being environmentally friendly [10].

More recently, a new type of antimicrobial platform has been developed using nanotechnology-based Engineered Water Nanostructures (EWNS). This platform has been proven to be a successful, eco-friendly, chemical-free, and environmentally friendly method of disinfecting bacteria [27],[28]. The unique physicochemical properties of EWNS play a significant role in inactivating bacteria, and its efficacy has been tested on various surfaces and pathogens, including airborne bacteria [27],[28],[29], bacteria on the surface (stainless steel coupons) [29],[30] and hand hygiene-related pathogens [31]. Furthermore, the microbial inactivation

potential of EWNS was evaluated with food-related microorganisms on the surface of tomatoes [32],[33] , blackberries [26] and spinach [34].

Currently, there has been no testing of the antimicrobial effectiveness of EWNS on eggshell contamination. However, this technology could offer a new solution for disinfecting eggs in the industry.

The current study was aimed to investigate the inactivation efficacy of EWNS droplets by varying operating parameters such as EWNS exposure time, electric field strength (voltage levels, distances between needle tips and counter electrode), and flow rate. The effectiveness of these parameters in inactivating a specific strain of *E. coli* was assessed as a model for contamination. The study then looked into how well the optimal operating condition could inactivate *Salmonella*.

3.3 Materials and methods

3.3.1 Mechanisms for the generation of engineered water nanostructures (EWNS)

In general, the production of EWNS can be attributed to a combined process of electrospray and ionization [27],[28]. As shown in Figure 3.1 [62], the technology relies on a strong electric field generated between a metal capillary containing water and a counter electrode, which aerosolizes the liquid and causes it to break into unstable, highly charged droplets that eventually break down into smaller droplets at the nanoscale level [27],[28],[33] .

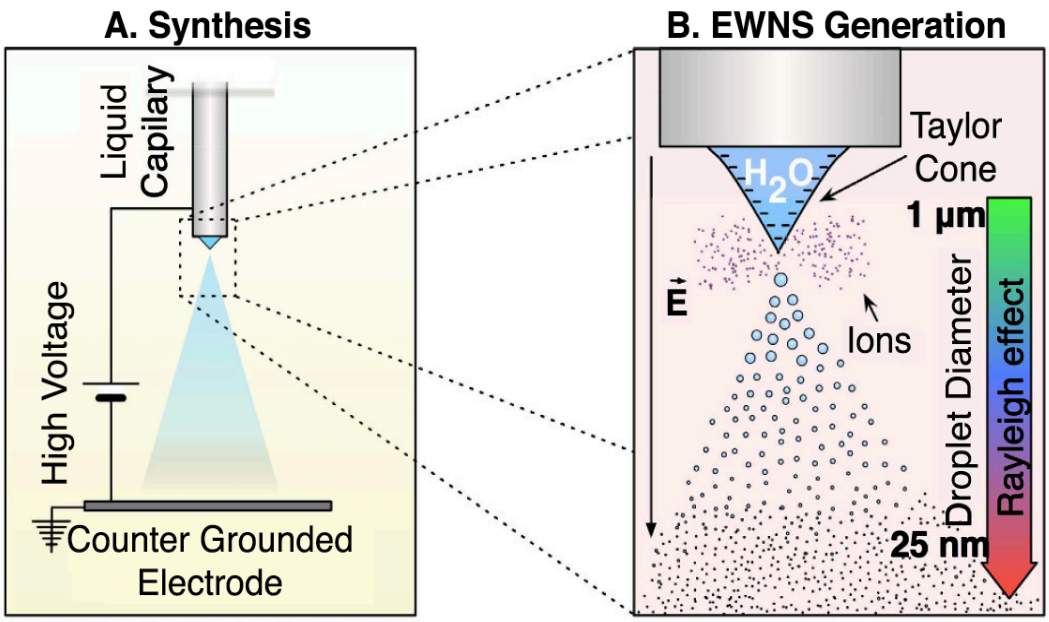

Figure 3. 1 Schematic instruments of an electrospray set-up. from G. Pyrgiotakis et al. [62], 2016 (Page 2). [Open access article]

At the same time, some water and air molecules split and lose electrons due to the high electric field, resulting in the formation of reactive oxygen species (ROS) such as hydroxyl and superoxide radicals. These ROS, which have a very short lifespan, are embedded in the droplets and are responsible for the biocidal properties of EWNS [27],[31],[26]. The unique structure of EWNS consists of an electron-rich water shell containing ROS, making them highly mobile due to their small size (average diameter of 25 nm), and high surface energy. This means that they can stay airborne for a long time, potentially colliding with pathogens suspended in the air or present on surfaces, resulting in microbial inactivation [28],[32],[53].

3.3.2 Experimental setup

The schematic of the electro-nano-spray system is shown in Figure 3.2.

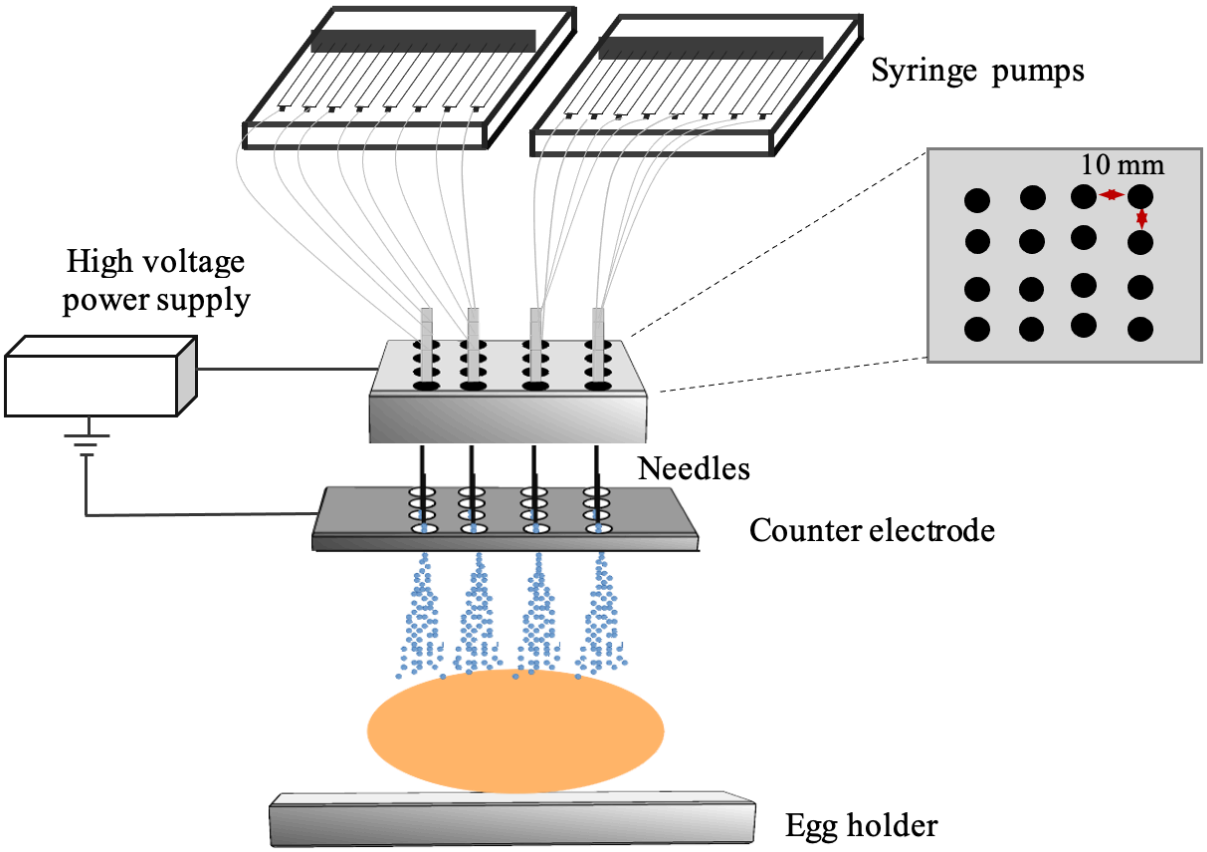

Figure 3. 2 Schematic of the electro-nano-spray system for eggshell surface decontamination

The system consists of 16 emitters/injectors sufficient to cover one-half side of an egg with the EWNS. The injectors used were 30-gauge metal hub blunt point needles (Hamilton, Reno, NV, USA), coupled with plastic syringes to supply water. Each needle was connected to a syringe through a soft plastic tube. Two 8-channel syringe pumps (NE-1800; New Era Pump Systems, Inc., Farmingdale, NY, USA) were used to control the water flow rate through each needle. The needles were fixed on an aluminum plate, which was connected to a high-voltage power supply (XP GLASSMAN FJ30R4, High Bridge, NJ, USA).

The distance between each needle was 10 mm. The aluminum plate served as the charging electrode to ionize the water passing through the needles. Another aluminum plate was used as the counter electrode below the charging electrode and the needles. This electrode was electrically grounded. The distance between the needles and counter electrode was adjustable through two slots on the lateral supporting plates. Figure 3.3 shows a photo of the actual EWNS setup.

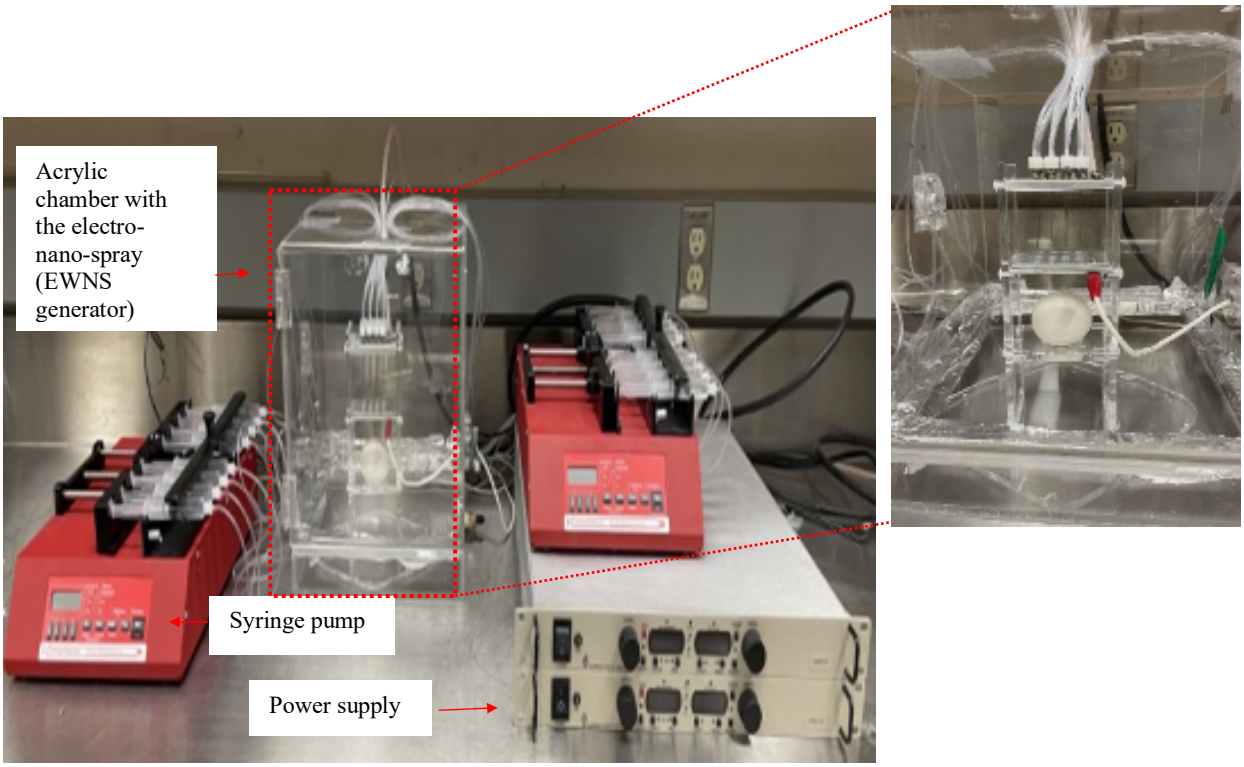

Figure 3. 3 Photo of actual EWNS setup

3.3.3 Microbiological Procedures

3.3.3.1 Preparation of bacteria inoculum

To verify the effectiveness of the EWNS technique on the surface decontamination of eggs, a single strain of *E. coli* (*Escherichia coli* W3110 pTAP337, ATCC, USA) and *Salmonella enterica serovar Enteritidis* (ATCC 4931) were used as representative pathogens.

Each kind of bacteria was incubated in the liquid Lysogeny broth (LB) medium (Becton Dickinson, Mississauga, ON, Canada) and was grown overnight at 37°C for 18-22 hours in an incubator (IMH60, Heratherm, USA). Then, the bacterial concentration of the stock solution was measured based on the optical density using a UV-Vis photo-spectrometer (7000 Aquamate Vis, Thermo Scientific, Canada) at a wavelength of 600 nm and re-adjusted to cfu/mL through Eq. 3.1:

$$\text{Concentratoin}\left(\frac{\text{cfu}}{\text{mL}}\right) = \text{Optical density} \times 10^9 \quad (3.\ 1)$$

The test inoculums were concentrated to final concentrations of $\sim 8.62 \pm 0.02 \times 10^8$ and 5.58×10^8 for *E. coli* and *Salmonella*, respectively.

3.3.3.2 Surface inoculation of eggs

For each experiment, three fresh, large and unwashed eggs (56 to 63 grams) were dipped into 70% ethanol for one minute, brushed with soap solution, and rinsed with warm water. Then, they were washed again with sterile warm water and dried for 30 minutes in a biosafety cabinet. The dried eggs were placed in the bacterial stock solution for 20 minutes under orbital shaking at 100 rpm to inoculate the eggshell surface. Then, the surface inoculated eggs were dried again for 40 minutes.

3.3.3.3 Recovery of bacteria from egg surface

The EWNS-treated eggs were dried for 40 minutes in the biosafety cabinet at room temperature before recovering the bacteria. In order to recover the bacteria from the egg surface, the eggs were transferred into a sterile sampling bag (Nasco Whirl-pack, Thermo Fisher Scientific) containing 50ml Lysogeny broth (LB) medium. The eggs were hand-rubbed through the bag for 3 minutes to remove the bacteria from the eggshell surface. The suspended bacteria was diluted 10 times and 100-µL of the dilutions were transferred to LB agar plates using spread plate method. Plating for each dilution was done in triplicate.

3.3.3.4 Characterize the inactivation efficiency

The inoculated plates were incubated at 37 °C for 16-18 hours (overnight) prior to counting. After approximately 18 hours, the colonies on the plates were counted.

Each operating parameter was done in triplicate. The inactivation efficacy of the EWNS produced by the electrospray was evaluated through Eq. 3.2 and 3.3 for each treatment:

$$\text{Bacteria log reduction } = \text{Abs}\left[\text{Log}_{10}\left(\frac{\text{CFU}_t}{\text{CFU}_c}\right)\right] \quad (3.2)$$

$$\text{Bacteria inactivation efficiency} = \left(1 - \left(\frac{\text{CFU}_t}{\text{CFU}_c}\right)\right) \times 100 \quad (3.3)$$

where CFU_t and CFU_c are the number of CFU recovered from treated and control eggs, respectively.

It is important to note that the surface of the control eggs was also inoculated with the same procedure except exposure to EWNS, and the concentration of bacteria on the egg surface was set as the bacterial concentration at t = 0. Three eggs were used as the control for each experiment.

3.3.4 EWNS Exposure Approaches

One egg which had been surface inoculated and dried was transferred from the biosafety cabinet to the treatment chamber, placed directly under the tip of the needles, and exposed to EWNS (half-side at a time) for the pre-set treatment time, then the other half-side of the egg was exposed for the equal time.

All different operating conditions employed in this study are listed in Table 3.1 Each operating condition was repeated in triplicates to ensure the accuracy of the results. Error bars represent the standard deviation of the measured values. All experiments were conducted at a constant temperature of 20 °C. After each experiment, the treated egg was removed from the chamber and moved back to the biosafety cabinet for bacterial recovery to determine the bacteria inactivation efficacy of the EWNS technology.

Table 3. 1 Operating conditions employed in the *E. coli* inactivation experiments

Operating condition	Electric field strength (kV/cm)	Flow rate (μL/min/needle)	Treatment time (min)
1	5.0 (1.0 cm, -5.0 kV)	1	2.5
2	5.0 (1.0 cm, -5.0 kV)	1	5.0
3	5.0 (1.0 cm, -5.0 kV)	1	7.5
4	5.0 (1.0 cm, -5.0 kV)	1	10.0
5	5.0 (1.0 cm, -5.0 kV)	1	15.0
6	6.0 (1.0 cm, -6.0 kV)	1	2.5
7	6.0 (1.0 cm, -6.0 kV)	1	5.0
8	6.0 (1.0 cm, -6.0 kV)	1	7.5
9	6.0 (1.0 cm, -6.0 kV)	1	10.0
10	6.0 (1.0 cm, -6.0 kV)	1	15.0
11	4.0 (2.0 cm, -8.0 kV)	2	5.0
12	4.5 (2.0 cm, -9.0 kV)	2	5.0
13	5.0 (2.0 cm, -10.0 kV)	2	5.0
14	5.0 (1.0 cm, -5.0 kV)	2	5.0
15	6.0 (1.0 cm, -6.0 kV)	2	5.0
16	6.5 (1.0 cm, -6.5 kV)	2	5.0
17	8.0 (0.5 cm, -4.0 kV)	2	5.0
18	9.0 (0.5 cm, -4.5 kV)	2	5.0
19	4.0 (2.0 cm, -8.0 kV)	3	5.0
20	4.5 (2.0 cm, -9.0 kV)	3	5.0
21	5.0 (2.0 cm, -10.0 kV)	3	5.0
22	4.0 (2.0 cm, -8.0 kV)	1	5.0
23	4.5 (2.0 cm, -9.0 kV)	1	5.0
24	5.0 (2.0 cm, -10.0 kV)	1	5.0
25	5.0 (1.0 cm, -5.0 kV)	3	5.0
26	6.0 (1.0 cm, -6.0 kV)	3	5.0

27	6.5 (1.0 cm, -6.5 kV)	3	5.0
28	6.5 (1.0 cm, -6.5 kV)	1	5.0
29	8.0 (0.5 cm, -4.0 kV)	3	5.0
30	9.0 (0.5 cm, -4.5 kV)	3	5.0
31	8.0 (0.5 cm, -4.0 kV)	1	5.0
32	9.0 (0.5 cm, -4.5 kV)	1	5.0
33	9.0 (0.5 cm, -4.5 kV)	0	5.0
34	9.0 (0.5 cm, +4.5 kV)	1	5.0

3.3.5 Statistical analysis

For each bacteria inactivation, three replicates were conducted. The data represent the average value of three replicates. The standard deviation of the three trials was used to calculate the error. Finally, a statistical analysis of two-way ANOVA using SPSS software was conducted to evaluate the effect of water flow rate and electric field strength and possible interactive effects among them on *E. coli* inactivation.

3.4 Results and discussion

3.4.1 Evaluate the inactivation efficacy of EWNS on egg surface

Electrospray characteristics can be manipulated by altering the operating parameters. To find the most optimal operating condition of EWNS technology in decontamination of egg surface, the effect of different operating parameters was evaluated by establishing a sensitivity test. Experiments were conducted by the one-factor-at-a-time method, where one operating parameter was changed at a time while keeping the other operating parameters constant.

3.4.1.1 Effect of treatment time on *E. coli* inactivation

Figure 3.4 illustrates the influence of the exposure time to EWNS on *E. coli* inactivation efficiency and *E. coli* log reduction at 5 kV/cm and 6 kV/cm, respectively. The results show at 5 kV/cm the log reduction and bacteria inactivation efficiency increased from 0.2 and 36.2% to 1.00 and 90.1%, respectively, with increasing the treatment time from 2.5 minutes to 15.0 minutes. The same trend was also observed at 6 kV/cm. The log reduction and bacteria inactivation efficiency

were 0.28 and 42.8% at 2.5 minutes of exposure time which increased to 1.82 and 98.4% under 15 minutes of treatment. This is attributed to more ROS generated in a longer treatment time, which are the main antimicrobial species produced during the process. Vaze et al. [26] and Si. [30] also obtained similar results that with a longer exposure time, the *E. coli* experienced more protein and DNA damage and consequently more inactivation of bacteria on the eggshell surface.

It is worth noting that the reductions in logarithmic values obtained from the current study were similar to those reported by Vaze et al. [26], who achieved a 1.8 log reduction in *E. coli* on stainless-steel coupons using the EWNS technique for 45 minutes. Ragni et al. [75] found that treating eggshells with non-thermal plasma for 60 to 90 minutes at 35% relative humidity resulted in a maximum reduction of 2.2-2.5 logarithmic units of *S. Enteritidis*, regardless of the medium used. In addition, Al-Ajeeli et al. [5] found that washing eggs with water containing disinfectants such as chlorine, quaternary ammonium compounds, and peracetic acid resulted in a reduction of 0.3 to 1.2 log.

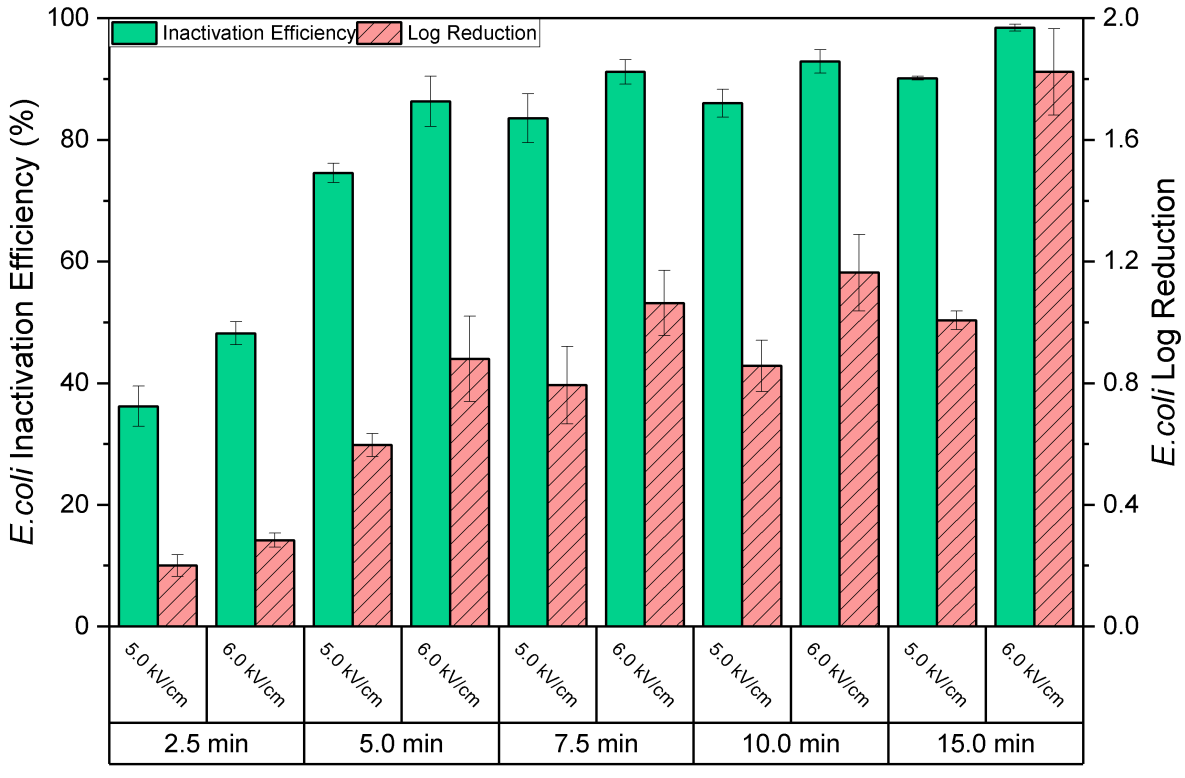

Figure 3. 4 Effect of treatment time on *E. coli* inactivation. This employed operating conditions: 1 to 10 (From Table 3.1). (F = 1 µL/min/needle, D = 1cm, V = -5.0 kV & -6.0 kV)

Since treatment time is an important factor in commercializing the new technology, a treatment duration of 5 minutes is considered be a practical choice for achieveing a balance between efficiency and time optimization. Therefore, a duration of 5.0 minutes was chosen as the exposure time for the remaining tests, as it has the potential to attain a satisfactory level of efficiency while also conserving significant time and resources throughout the experiment.

3.4.1.2 Effect of electric field strength on *E. coli* inactivation

Electric field strength (E) is expressed as the ratio of the applied voltage divided by the distance between the tip of needles and the counter electrode (Eq. 3.4):

$$E = {}^{V}/_{D} \qquad (3.4)$$

where E is the electric field strength (kV/cm), V and D are the applied voltage (kV) and distance between electrodes (cm), respectively. Figure 3.5 shows the effect of the electric field strength on *E. coli* inactivation efficiency and bacterial log reduction. It was obtained by altering the applied voltage at 3 different distances of 0.5, 1.0, and 2.0 cm and a fixed water flow rate of 2 µL/min/needle.

It is important to note that, in each fixed distance between emitters and counter electrode, at lower applied voltages (lower E) a dripping mode occurred [76] while higher levels of voltage created aggressive shaking of the needle and/or arching [30]. Thus, effective electric fields to generate EWNS were achievable in specific ranges of applied voltage. The effective electric field strength was varied from 4.0 to 5.0 kV/cm at 2 cm of distance between electrodes, between 5.0 to 6.5 kV/cm at a distance of 1 cm, and between 8.0 and 9.0 kV/cm at 0.5 cm.

It is obvious from Figure 3.5 that at a water flow rate of 2 µL/min/needle, as the strength of the electric field was increased from 4.0 to 9.0 kV/cm, the *E. coli* numbers on the egg surface decreased. In other words, the *E. coli* on eggshells decreased 59.7% when subjected to 4.0 kV/cm of EWNS, corresponding to 0.39 log reduction. On the other hand, bacterial contamination was reduced by 90.9% (1.04 log) when subjected to 9 kV/cm of EWNS because higher amounts of ROS were generated at the higher strengths of the electric field, which were the main antimicrobial species produced during the process. Based on the results from Ji et al. [77] at a fixed electrode

distance, with increasing the applied voltage, the electron density increases linearly, which resulted in the formation of more ROS radicals.

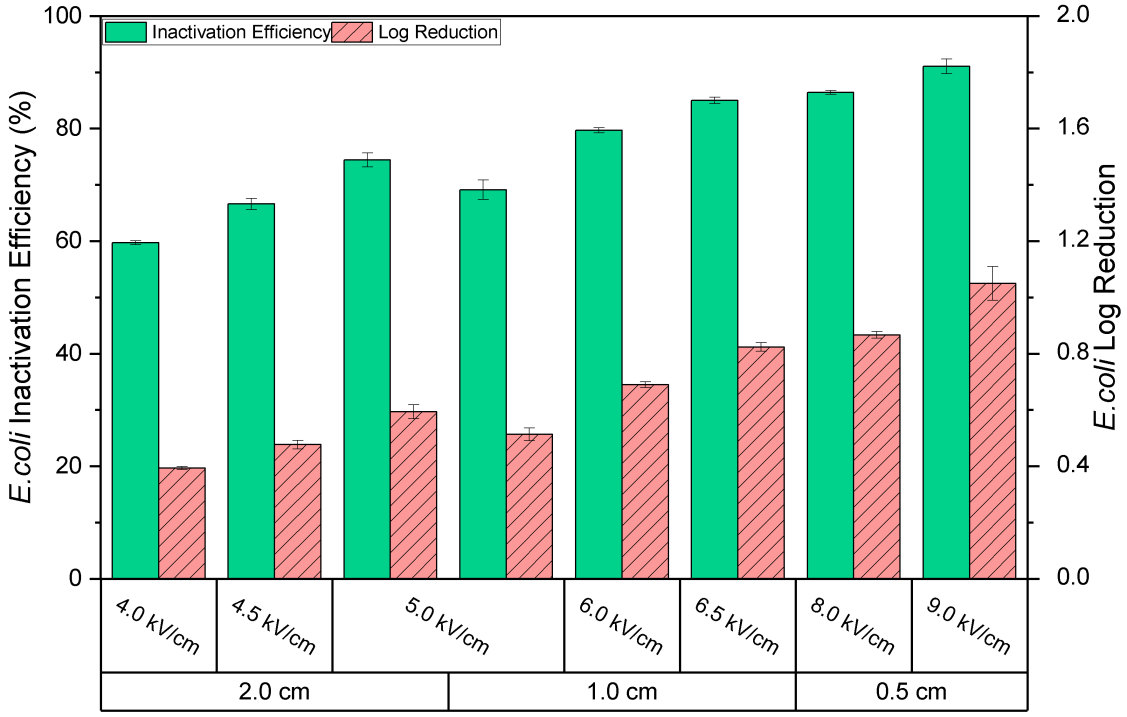

Figure 3. 5 Effect of electric field strength on *E. coli* inactivation. This employed operating conditions: 11 to 18 (From Table 3.1). (F = 2 µL/min/needle, T = 5.0 minutes)

The findings agreed with the observation that was reported by Ying-Qiu Li et al. [78], which showed that inactivating *E. coli* using pulsed electric fields (PEF) was achieved with a 5.2 log reduction in soymilk when PEF was applied at 40 kV/cm compared to less than 2.5 log reduction at 20 kV/cm. Additionally, Jordan Si [30] found that an increase in the applied voltage from -6.6 to -7.6 kV over a 3 cm distance between the capillary and inoculated coupon (resulting in an increase in electric field strength from 2.2 to 2.53 kV/cm) using the EWNS technique, led to an improvement in *E. coli* inactivation efficiency from 2.43 log to 2.95 log after 25 minutes of treatment time.

It is noteworthy that two different bacterial inactivation efficiencies were obtained despite the application of the same electric field strength of 5 kV/cm. In the shorter space between two

electrodes, the lower voltage was able to produce high electric field strength but *E. coli* inactivation efficiency was lower. It is due to the electrical current between two electrodes which means a lower applied voltage between the needles and the counter electrode results in lower current and less electric charges which leads to producing less ROS (54 mA for 10 kV at 2 cm distance compared to 19 mA for 5 kV at 1 cm).

3.4.1.3 Effect of water flow rate on *E. coli* inactivation

The liquid flow rate is another operating parameter that plays a crucial role in the performance of EWNS in the decontamination of shell egg surface. Figures 3.6, 3.7 and 3.8 depict the effect of different flow rates at three different needle-to-counter electrode distances of 2, 1 and 0.5 cm, respectively. A general declining trend of bacterial inactivation efficiency with increasing the water flow rate was observed.

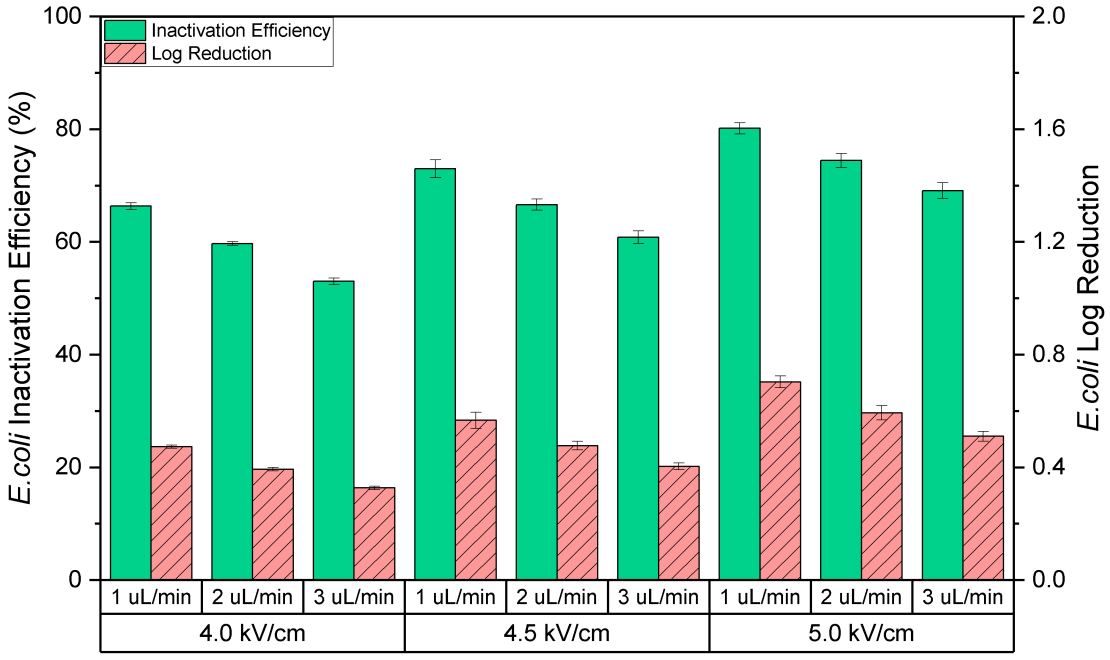

Figure 3. 6 Effect of water flow rate per needle on *E. coli* inactivation. This employed operating conditions: 11-13 & 19-24 (From Table 3.1). (D = 2 cm, T = 5.0 minutes)

As shown in Figure 3.6 at 4.0 kV/cm strength of the electric field, *E. coli* inactivation efficiency and log reduction decreased from 66.3% and 0.47 to 53.0% and 0.33 by increasing the water flow rate from 1 to 3 µL/min/needle (total water flow rate from 16 to 48 µL/min. Similar trends were observed for both 4.5 and 5.0 kV/cm which means the highest flow rates resulted in significantly low ($p < 0.05$) bacterial inactivation in comparison with the lower ones.

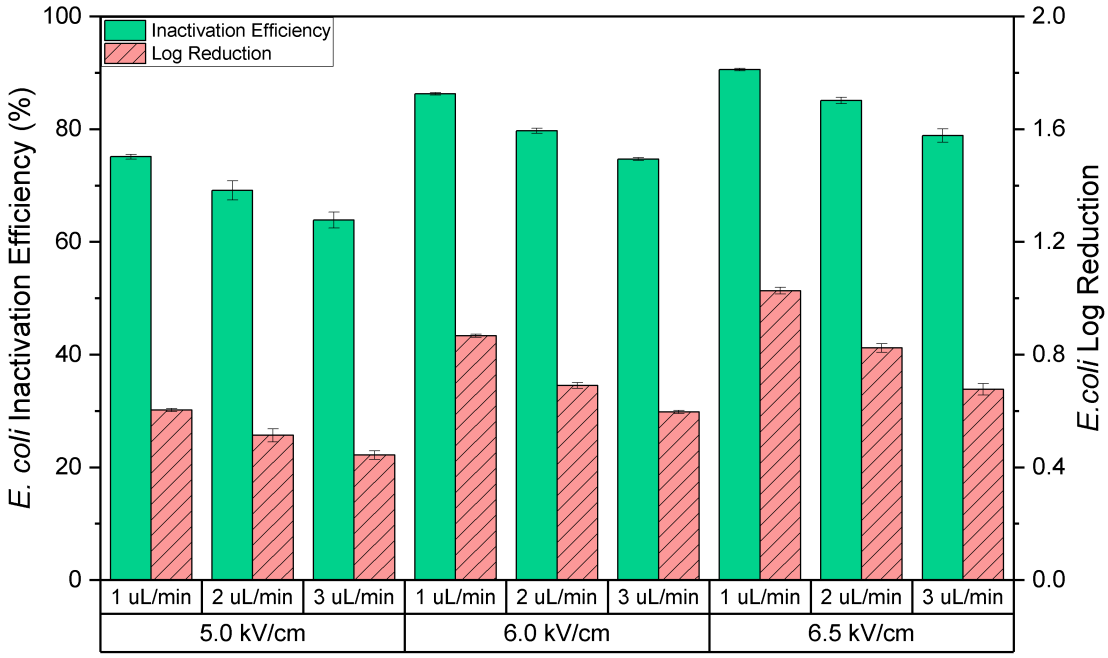

Figure 3. 7 Effect of water flow rate per needle on *E. coli* inactivation. This employed operating conditions: 2,7, 14-16 & 25-28 (From Table 3.1). (D = 1cm, T = 5.0 minutes)

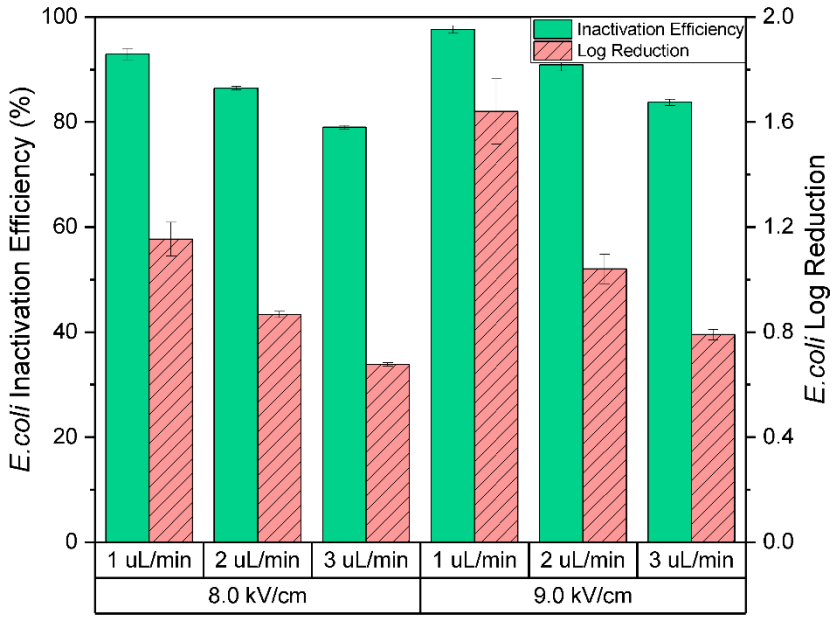

Figure 3. 8 Effect of water flow rate per needle on *E. coli* inactivation. This employed operating conditions: 17,18 & 29-32 (From Table 3.1). (D = 0.5cm, T = 5.0 minutes)

The results obtained at 1 and 0.5 cm distance of electrodes (Figure 3.7 and 3.8) were consistent with those found at 2 cm (Figure 3.6). A declining trend of bacterial inactivation efficiency with increasing the water flow rate was observed. As seen in Figure 3.7, in the highest electric field strength (6.5 kV/cm), when the liquid flow rate increased from 1 to 2 and 3 µL/min, the corresponding *E. coli* inactivation was dropped from 90.3% (1.01 log) to 85.1% (0.83 log) and 78.9% (0.68 log), respectively. Similarly, as Figure 3.8 depicts, the lower the flow rates, the higher the bacterial inactivation. For instance, at both electric field strengths of 8.0 and 9.0 kV/cm, the bacterial inactivation at 1 µL/min/needle flow rate were 93.0% (1.16 log) and 97.6% (1.64 log), respectively, which were better than the higher flow rates.

This can be explained by the theory that the quantity of electric charges (Q) in the electrospray system is proportional to the current (I) and contact time (t) between the fluid and counter electrode as shown in Eq. 3.5 [79].

$$Q = It \quad (3.5)$$

Lower flow rates result in longer contact time between the metal capillary and the liquid. Consequently, more electric charges and energy are accumulated leading to the higher formation of ROS and higher inactivation of *E. coli* [1],[79]. This is also in agreement with studies by Si [30] and Lin et al. [15] which showed bacterial inactivation reduced with elevated flow rates of water and argon, respectively. Ouyang et al. [1] also found that in Flow-Through Pulsed UV Light Treatment System, the reduction of *E. coli K12NSR* in Liquid Egg White was decreased from 1.43 to 0.21 log CFU/mL by increasing the flow rate from 40 to 80 ml/min with 2 passes.

3.4.1.4 Comparison with empty needles

Figure 3.9 shows that when empty needles were used in the electrospray system, the *E. coli* inactivation percentage was 65.5%, corresponding with a 0.46 log reduction. This was significantly lower compared to 97.6% inactivation efficiency (1.64 log reduction) which was obtained under operating condition 32 in Table 3.1 ($p < 0.05$). Although the bacterial inactivation principle of empty needles and EWNS is the same and the air around the metal capillaries is ionized to ROS, the higher inactivation rate in EWNS with water is mainly due to the water nanodroplets. The nanoscale water droplets enhance the production of ROS and also facilitate transport of the generated ROS to the inoculated egg surface, while in the electrospray system with empty needles, there is no medium to help diffuse the ROS to the inoculated surface of the eggs. In addition, the surface charge of water droplets prevents ROS from neutralizing with other air molecules which increases their lifetime from milliseconds to minutes [64],[79].

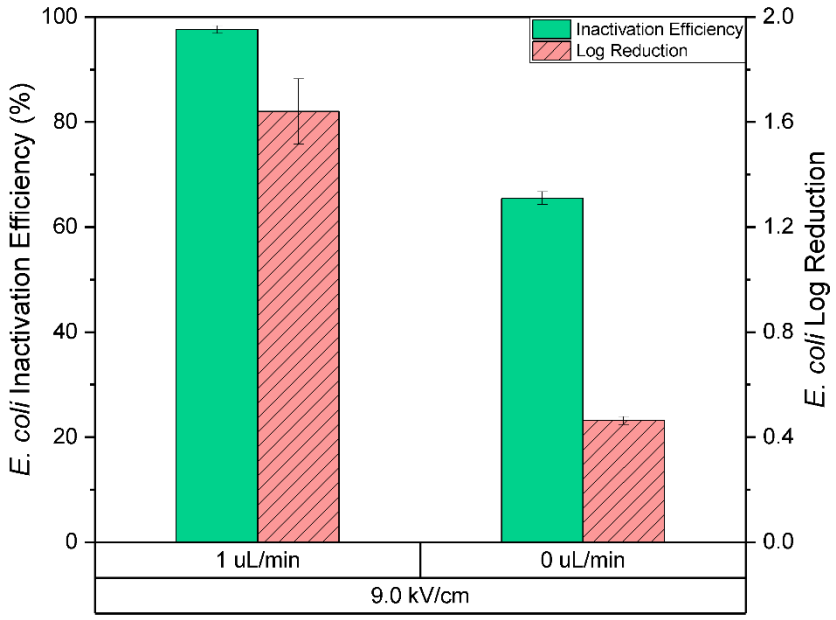

Figure 3. 9 Comparison of EWNS with empty needles on *E. coli* inactivation. This employed operating conditions: 32 & 33 (From Table 3.1). (E= 9.0 kV/cm (D = 0.5 cm & V = -4.5 kV), T = 5.0 min)

3.4.1.5 Comparison with positive applied voltage (same electric field strength)

Figure 3.10 shows the *E. coli* inactivation of egg surface under positive and negative applied voltage with an equal electric field strength and other same operating conditions. The efficiency of the treatment with negative voltage was significantly higher ($p < 0.05$) than that from the positive voltage (89.1% with 0.96 log). More production of reactive oxygen species (ROS) can occur under negative voltage levels, attributed to the higher mobility of negative charges, resulting in greater ROS generation compared to positive voltage [30],[80].

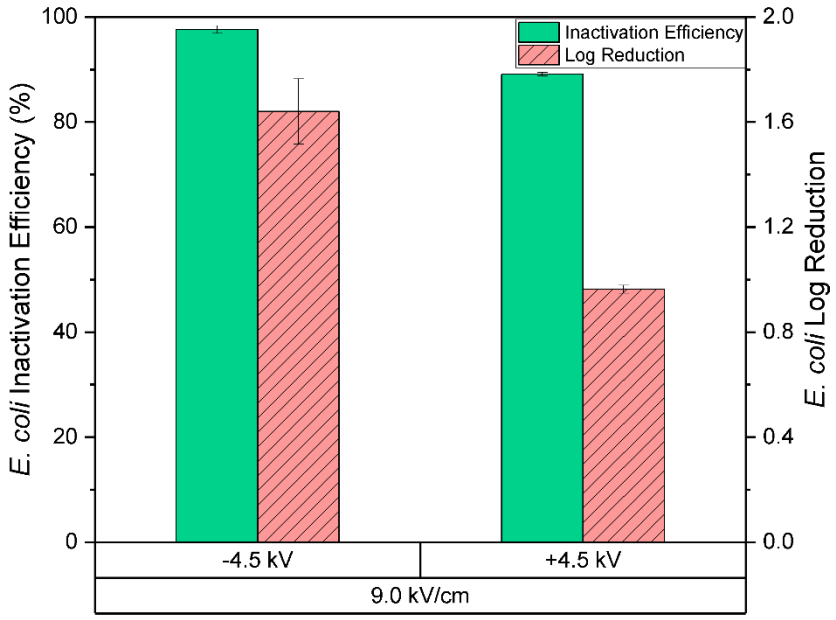

Figure 3. 10 Comparison of positive applied voltage with negative on *E. coli* inactivation. This employed operating conditions: 32 & 34 (From Table 3.1). (D = 0.5 cm, F = 1 µL/min/needle, T = 5.0 minutes)

3.4.2 Evaluate the inactivation efficacy of EWNS technology in treatment *Salmonella* on egg surface

As shown in Figure 3.11, a log reduction of 0.71 corresponding with inactivation percentage of 80.4 was obtained for the inactivation of *Salmonella* on the egg surface under optimized treatment conditions of 1 µL/min/needle, 9.0 kV/cm electric field strength and treatment time of 5.0 minutes which were lower than those for *E. coli* inactivation at the same condition which was 97.9% with 1.69 log reduction.

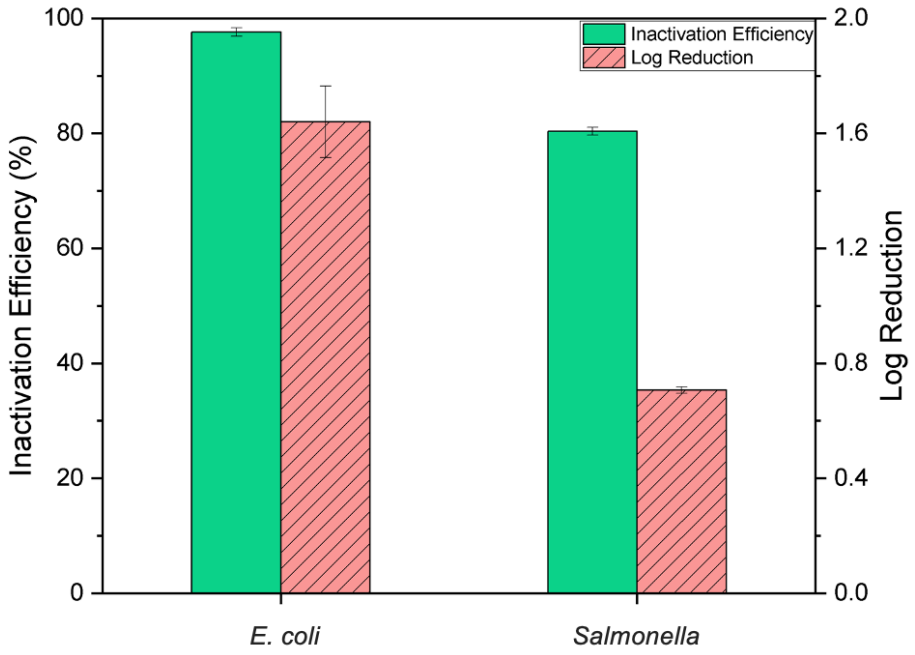

Figure 3. 11 Effect of EWNS on inactivation of *Salmonella* on egg surface compared to *E. coli*. This employed operating conditions: 32 (From Table 3.1). (F = 1 µL/min/needle, E = 9.0 kV/cm, T = 5.0 minutes)

3.4.3 Statistical analysis

3.4.3.1 Two-way ANOVA analysis

The results of two-way ANOVA statistical tests are presented in Table 3.2 for optimizing of operating condition at 5 minutes of the EWNS exposure time. It can be observed from the table that there was no statistically significant (p-value = 0.926) two-way interaction between flow rate and electric field strength. However, the simple main effects due to both flow rate and electric field strength were statistically significant ($p < 0.05$).

Table 3. 2 Two-way ANOVA results for optimizing at 5 minutes of treatment time

Tests of Between-Subjects Effects

Dependent variable: *E. coli* inactivation Efficiency

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	9001.275[a]	20	450.064	139.360	<0.001
Intercept	404127.779	1	404127.779	125135.950	<0.001
FlowRate	1765.493	2	882.747	273.338	<0.001
ElectricFieldStrength	7160.344	6	1193.391	369.527	<0.001
FlowRate*ElectricFieldStrength	18.009	12	1.501	0.465	0.926
Error	164.705	51	3.230		
Total	430818.730	72			
Corrected Total	9165.980	71			

a. R Squared = 0.982

3.4.4 limitations of the electro-nano-spray technique

Electro-nano-spray has certain limitations in generating engineering water nanostructures. Some of these limitations include:

1) It is generally a relatively slow technique compared to other decontamination methods such as cold plasma.
2) Electro-nano-spray typically requires high voltages (a few kilovolts) to generate the charged droplets and ions. Maintaining a stable voltage and avoiding arcing or electrical discharge can be challenging, particularly when dealing with complex samples or high flow rates.
3) Generating stable and controlled EWNS through electro-nano-spray can be challenging. Factors such as the applied voltage, flow rate, and the properties of the water sample can affect the size, uniformity, and stability of the generated EWNS.
4) Electro-nano-spray systems can be prone to clogging or blockages, particularly when working with water samples. Water may contain impurities, particulate matter, or biological molecules that can accumulate and cause blockages in the spray emitter or capillary. This can hinder the consistent and continuous generation of water nanostructures.

3.5 Conclusions

In this work, the effects of EWNS exposure time, water flow rate, and electric field strength (combination of applied voltage and distance between needle tips and counter electrode) on the inactivation of *E. coli* on egg surface were investigated. The main findings can be summarized as follows:

1) It was found that longer exposure time (15 minutes) resulted in higher *E. coli* inactivation rates. After selecting 5.0 minutes as exposure time for the rest of the tests, the highest *E. coli* inactivation efficiency was obtained at the lowest liquid flow rate (1 μL/min/needle) and highest electric field strength (9 kV/cm).
2) Microbial inactivation efficiency was higher when EWNS was used than when empty needles are used.
3) Microbial inactivation efficiency was higher under negative voltage in comparison with positive.

4) The antimicrobial efficiency obtained at the most effective operating conditions for *Salmonella* was lower than the results for inactivation of *E. coli*.

Chapter 4 – Investigation of the impact of EWNS technology on egg quality

The content of this chapter will be submitted to a peer reviewed journal of publication.

Contribution of the MSc student

Experiments were planned and performed by Shiva Aminian with the guidance provided by Drs. Lifeng Zhang, Shelley Kirychuk and Karen Schwean-Lardner. Drs. Lifeng Zhang, Shelley Kirychuk and Karen Schwean-Lardner supervised and provided consultation during the entire experimental period as well as thesis preparation. All the writing of the submitted manuscript was done by Shiva Aminian with Drs. Lifeng Zhang, Shelly Kirychuk and Karen Schwean-Lardner providing editorial guidance regarding the style.

Contribution of this chapter to the overall study

In this chapter, the impacts of the EWNS method on the quality of treated eggs were evaluated, and compared the results to unwashed (control) and washed eggs. In order to investigate the impacts of the EWNS on the physical properties of eggs, in Part 1 a total of 60 eggs were used and categorized into 3 different groups of treated, washed, and unwashed (control) eggs. The quality analyses were done 1 week after treatment and washing the eggs. In Part 2, the impact of the EWNS treatment on the physical properties of eggs during a longer storage period was evaluated. Three eggs from each group were taken for physical quality analyses at 0, 7, 14, and 21 days of storage period after treatment (a total of 12 eggs per group). In addition, changes in moisture content and albumen proteins of treated eggs were analyzed and compared with washed and unwashed eggs (3 eggs per group).

4.1 Abstract

The table-egg industry worldwide predominantly relies on chicken eggs, which are highly valued as a nutritious food source and widely used as an ingredient in various food products. However, eggs have the potential to act as carriers for microorganisms and spread diseases within the food chain, and worldwide foodborne illnesses, linked to the consumption of raw egg products, are a global public health concern. Since food contamination remains a persistent threat and product safety is a crucial aspect of the food industry, the poultry industry must explore effective approaches and develop innovative technologies for preserving food. Thus, the ideal technique should guarantee complete disinfection of the egg exterior, while preserving the quality of the egg's constituents, causing minimal changes to its sensory characteristics, and promoting public health and environmental sustainability.

After determining the most optimal operating conditions for EWNS technology in this research, the physical and chemical properties of eggs were examined to assess any potential negative impacts of the decontamination process. Eggshell thickness, eggshell specific gravity, albumen pH, yolk pH, yolk index, Haugh unit, moisture content, and protein content of albumen were assessed and compared between treated eggs, washed eggs, and untreated eggs (control). The data analysis showed that there were no notable variations in the quality features of EWNS treated eggs compared to unwashed and washed eggs.

4.2 Introduction

Egg quality is a complex concept that encompasses external and internal parameters, with both being crucial for consumers. External quality is primarily concerned with egg weight, shape, shell deformation, shell weight, shell thickness, and shell ratio. These factors affect the appearance of the egg and the strength of the shell, which are essential for consumer appeal and preventing bacterial contamination. Internal quality, on the other hand, is determined by the size of the air cell, albumen quality, and yolk quality, which include the Haugh unit, albumen index, yolk index, and yolk albumen ratio [65],[66],[67].

Eggshell strength is a crucial factor in determining external quality, influenced by the thickness and proportion of the shell, playing a significant role in the viability of eggs during

transportation and storage [68]. Specific gravity and weight loss rate are good indicators of egg freshness, with higher specific gravity and lower weight loss rate indicating freshness. The integrity of cuticle membranes that cover the eggshell is also essential for freshness and for preventing bacterial penetration. Eggs with lower specific gravity and higher weight-loss rates have a greater likelihood of being penetrated by bacteria due to their weaker, thinner, and more porous shells [9],[15],[69]. Furthermore, measuring the quality of albumen is an important aspect of determining egg quality, and it can be evaluated by examining pH levels, height, or the Haugh unit. A higher Huagh unit value indicates a superior quality egg, and eggs are assigned grades AA, A, B, or C based on their Huagh unit score [65],[71]. The freshness of eggs can be determined by examining the yolk and albumen indexes, which are influenced by the migration of water vapor through the cuticle membrane as eggs age. Yolk shape deformation is primarily caused by the weakening of the vitelline membrane, and the yolk index (YI) and the yolk coefficient (YC) are frequently used to gauge changes in yolk shape [22],[15],[68].

One can assess egg freshness by examining the pH levels of the entire egg and the albumen. Normally, when an egg is newly laid, the albumen pH varies from 7.6 to 8.5. But, as the egg gets older, the pH levels may rise because of gaseous exchanges taking place through the pores on the eggshell and the transfer of minerals and water between the yolk and albumen. An increase in pH levels might indicate decreasing freshness [22],[68].

The main objective of this study was to investigate how the EWNS technique affects the physical and chemical properties of eggs. The study was divided into two parts. In the first part, 60 eggs were used and divided into three groups: treated, washed, and unwashed (control). Quality analysis was performed after one week of treatment and washing of the eggs. The eggs were stored at a temperature of 4 °C. Additionally, the chemical characteristics of the treated eggs were compared to the unwashed and washed eggs using SDS-PAGE analysis (three eggs per group). In the second part, the impact of the EWNS treatment on the physical properties of eggs was evaluated over a longer storage period. Physical quality analysis was done on three eggs from each group at 0, 7, 14, and 21 days of storage period after treatment (a total of 12 eggs per group). The eggs were stored at a temperature of 4 °C during this period.

4.3 Materials and methods

4.3.1 Study Design

The study consisted of two parts. In the first part, a total of 60 eggs were utilized, and they were divided into three groups: treated, washed, and unwashed (control).

After subjecting the eggs to the EWNS treatment as treated eggs and washing with warm water and soap as washed eggs, a comprehensive analysis of their quality (eggshell specific gravity, eggshell thickness, albumen pH, yolk pH, yolk index, and Haugh unit) was conducted following one week of storage at a temperature of 4 °C. The chemical properties of the treated eggs were compared to those of the unwashed and washed eggs using SDS-PAGE analysis, with three eggs analyzed per group. Moreover, the moisture content of all groups were evaluated (3 eggs per group). The eggs were stored at a temperature of 4 °C for one week after treatment and washing.

In the second part, the influence of the EWNS treatment on the physical attributes of eggs was assessed over an extended storage period. A total of 36 eggs were selected, which were divided into three groups. Physical quality analysis was conducted on three eggs from each group at multiple time points: 0, 7, 14, and 21 days after treatment. The eggs were stored at a temperature of 4 °C throughout this period.

4.3.2 Physical quality of eggs

The impact of EWNS treatment on the egg quality was evaluated by measuring physical properties (eggshell specific gravity, eggshell thickness, albumen pH, yolk pH, yolk index, and Haugh unit). Eggs were divided into 3 different groups, unwashed (control), washed, and EWNS-treated eggs. Twenty eggs were tested per group. The eggs were stored at 4 °C after treatment and washing for one week.

4.3.2.1 Eggshell thickness

After breaking each egg and removing the shell membrane, the thickness of the eggshell (μm) was measured with the use of a digital caliper (Mastercraft, Mastercraft tools).

4.3.2.2 Eggshell specific gravity

Determining specific gravity of eggs was accomplished by flotation of eggs in different salt solutions with specific gravity ranging from 1.060 to 1.100 in 0.005-unit intervals. Firstly, the egg was placed into the first (lowest) specific gravity solution for about 15 to 20 seconds. If the egg floated (break the surface and remain there), the specific gravity was considered equal to the solution (1.060). Otherwise, the egg was raised out of the bucket and left to drip for 10–15 seconds. Then, it was placed in the next higher specific gravity solution. This procedure was continued until the egg was placed in an appropriate amount of specific gravity [5].

4.3.2.3 Albumen pH and yolk pH

The pH value of albumen and yolk was determined by a pH meter (Hanna-HI9125, Hanna Instruments, Inc., Laval, QC, Canada) by submerging the tip of the pH meter in the yolk and albumen separately.

4.3.2.4 Yolk Index

The yolk index (YI) was calculated from Eq. 4.1:

$$\mathrm{YI} = (\mathrm{h}/\mathrm{d}) \times 100 \qquad (4.\ 1)$$

Where h and d are the height and diameter of the yolk (mm), respectively. Both of the yolk height and diameter are measured with a digital caliper [22].

4.3.2.5 Haugh Unit

The Haugh unit (HU) value is given by Eq. 4.2:

$$\mathrm{HU} = 100 \log 10\ (\mathrm{h} - 1.7\mathrm{w}^{0.37} + 7.6) \qquad (4.\ 2)$$

where h is the height (mm) of the thick albumen that immediately surrounds the yolk and is measured with an albumen height gauge, and w is the whole egg weight (grams) [22].

4.3.2.6 Moisture Content

The albumen and yolk of eggs were separated. Then 5 – 10 gr samples were weighed and placed in a clean and dried weighing bottle (m_3). The total mass of the weighing bottle and sample was m_1.

The samples were placed in an oven at 105 °C for 24 hours to dry, then cooled for 30 minutes and weighed (m_2). Finally, the moisture content in the sample was calculated according to Eq. 4.3 [81].

$$\text{Moisture content (\%)} = \left(\frac{m_1 - m_2}{m_1 - m_3}\right) \times 100 \quad (4.3)$$

4.3.3 Chemical properties of eggs

4.3.3.1 SDS-PAGE

SDS-PAGE analysis was conducted to compare the chemical properties of the treated eggs with both the unwashed and washed eggs. Unwashed (control), washed, and EWNS-treated albumen samples were subjected to SDS-PAGE analysis. Firstly, freeze-dried albumen samples were diluted to a concentration of 2 mg/mL using PBS buffer. Then, 80 µL of diluted sample solution was mixed with 20 µL of Laemmli Sample Buffer (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada), incubated in boiling water for 10 min and 10 µL/lane was loaded on a gel. In order to compare the molecular weights, a pre-stained protein molecular weight standard was used. Then SDS-PAGE was employed using 4–20% Mini-PROTEAN® TGX™ Precast Protein Gel (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) at 150 V voltage at room temperature until the blue marker reached the bottom of the gel (almost 40 minutes). After electrophoresis, the gel was stained with Bio-Safe™ Coomassie Stain (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) at room temperature for 2 hours, then was washed with deionized water to have a clear background [82],[83]. The intensity of stained protein bands obtained by SDS-PAGE was analyzed using Image Lab 6.1 software.

4.3.4 Statistical analysis

The data represent the average value of each group of eggs. The standard deviation was used to calculate the error. The differences between means values of the egg quality parameters

among three different groups of eggs and at different storage times were investigated using one-way ANOVA.

4.4 Results and discussion

4.4.1 Evaluate the impact of EWNS technology on egg quality

4.4.1.1 Evaluate the egg quality one week after treatment

As shown in Table 4.1, the impact of EWNS treatment on the egg quality was evaluated by measuring physical properties (eggshell specific gravity, eggshell thickness, albumen pH, yolk pH, yolk index, and Haugh unit). Statistical analyses of the results showed that there was no substantial difference in qualitative characteristics among unwashed, washed, and treated eggs after one week.

Table 4. 1 Evaluation the impact of the EWNS treatment on egg quality (20 eggs per group)

Group	Eggshell Specific Gravity	Eggshell Thickness (mm)	Albumen pH	Yolk pH	Yolk Index	Haugh Unit
Unwashed	1.083 ± 0.004	0.38 ± 0.01	8.53 ± 0.10	6.31 ± 0.11	0.35 ± 0.04	81.46 ± 4.73
Washed	1.083 ± 0.003	0.39 ± 0.01	8.54 ± 0.15	6.31 ± 0.21	0.34 ± 0.03	80.97 ± 4.02
Treated	1.083 ± 0.003	0.39 ± 0.02	8.52 ± 0.10	6.32 ± 0.16	0.35 ± 0.03	81.22 ± 3.88

4.4.1.2 Evaluate the egg quality in a longer storage period

To evaluate the impact of the EWNS treatment on the physical properties of eggs during a longer storage period, three eggs from each group were taken for physical quality analysis at 0, 7, 14, and 21 days of storage period after treatment. The eggs were stored at 4 °C. Results of egg quality characteristics are presented in Table 4.2.

Table 4. 2 Evaluation the impact of the EWNS treatment on egg quality in longer storage period (3 eggs per group)

Day	Group	Eggshell Specific Gravity	Albumen pH	Yolk pH	Yolk Index	Haugh Unit
0	Unwashed	1.090 ± 0.000	8.45 ± 0.07	6.05 ± 0.02	0.39 ± 0.02	85.89 ± 0.62
	Washed	1.092 ± 0.003	8.45 ± 0.05	6.06 ± 0.06	0.39 ± 0.01	85.01 ± 0.82
	Treated	1.090 ± 0.000	8.45 ± 0.05	6.05 ± 0.04	0.39 ± 0.01	85.71 ± 0.70
7	Unwashed	1.082 ± 0.003	8.56 ± 0.06	6.27 ± 0.05	0.34 ± 0.00	80.08 ± 3.68
	Washed	1.082 ± 0.003	8.59 ± 0.04	6.33 ± 0.11	0.34 ± 0.00	79.78 ± 2.36
	Treated	1.085 ± 0.000	8.59 ± 0.03	6.32 ± 0.07	0.34 ± 0.01	79.92 ± 3.48
14	Unwashed	1.077 ± 0.003	8.76 ± 0.04	6.61 ± 0.03	0.32 ± 0.01	75.45 ± 0.76
	Washed	1.077 ± 0.003	8.75 ± 0.08	6.64 ± 0.08	0.32 ± 0.01	73.97 ± 1.08
	Treated	1.077 ± 0.003	8.76 ± 0.05	6.59 ± 0.04	0.32 ± 0.00	74.79 ± 1.20
21	Unwashed	1.068 ± 0.003	8.88 ± 0.03	6.76 ± 0.08	0.28 ± 0.01	69.93 ± 0.99
	Washed	1.068 ± 0.006	8.90 ± 0.07	6.79 ± 0.07	0.28 ± 0.02	69.29 ± 1.31
	Treated	1.072 ± 0.003	8.89 ± 0.03	6.78 ± 0.03	0.28 ± 0.01	69.82 ± 1.21

Based on results showed in Table 4.2 and statistical analyses, although the quality of all egg groups reduced after each week during the storage period, no considerable change ($p < 0.05$) was observed in any of the analyzed parameters among control, washed, and treated eggs.

Figure 4.1 shows the effect of storage time (weeks) on the specific gravity of unwashed (control), washed, and treated eggs. The specific gravity of all egg groups declined significantly as the storage time increased, indicating that eggs floated in a salt solution with lower specific gravity compared to the previous week. This might occur due to the increase in the size of the air cell with enhancing storage time [84].

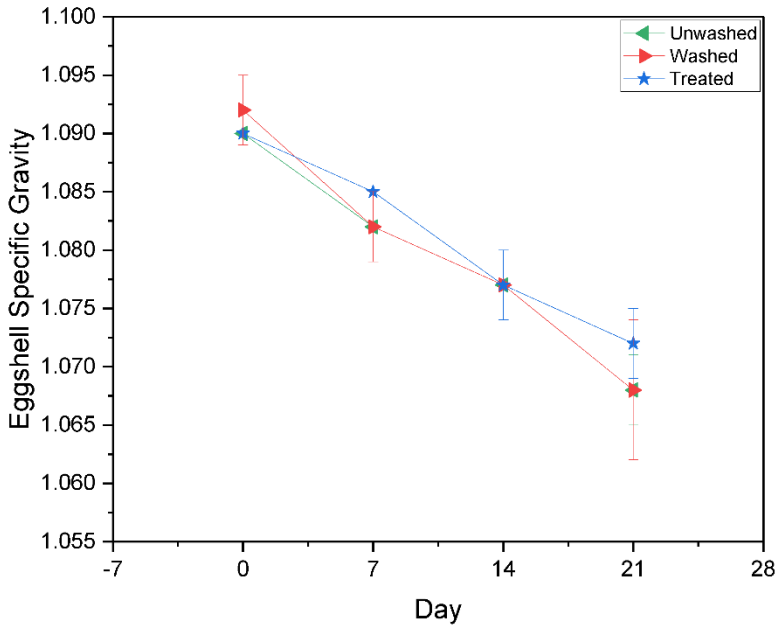

Figure 4. 1 Changes in specific gravity of eggs during storage (3 eggs per group)

As shown in Figure 4.2 and Figure 4.3, the pH levels of both albumen and yolk in unwashed, washed, and decontaminated eggs enhances every week, but the change only became significant after two weeks of storage ($p < 0.05$). This can be due to evaporation and loss of carbon dioxide from eggs by diffusion leading to an increase in pH [85].

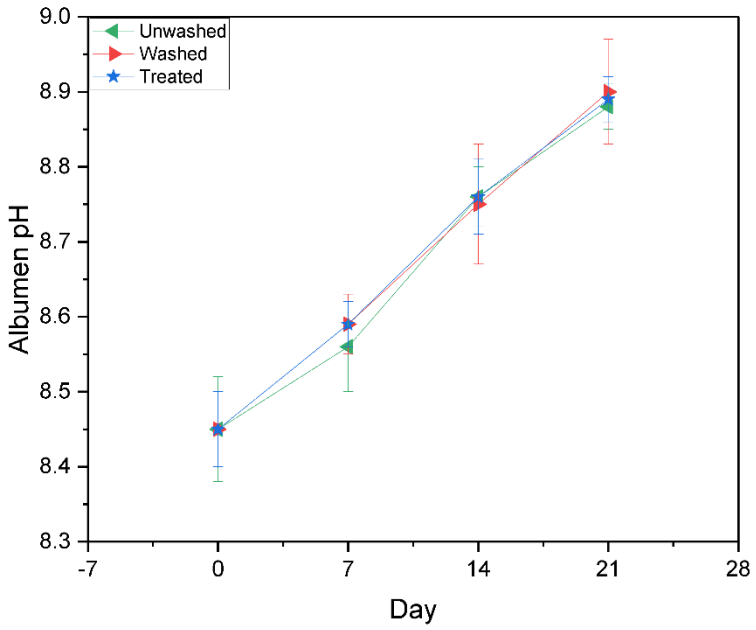

Figure 4. 2 Changes in albumen pH during storage (3 eggs per group)

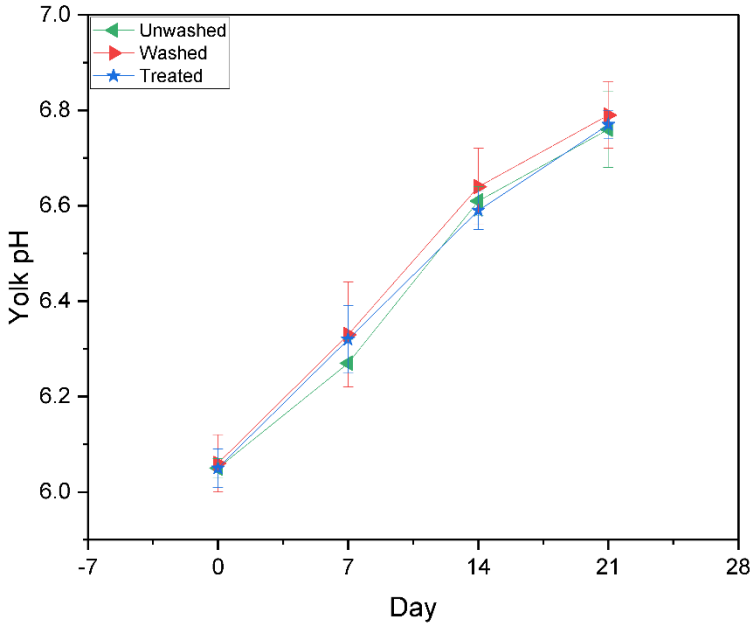

Figure 4. 3 Changes in yolk pH during storage (3 eggs per group)

Figure 4.4 presents the effect of storage time on the yolk index. A significant decline was noticed in the yolk index in all types of eggs at all 7 days intervals ($p < 0.05$), and the yolks became flatter and less round. This means that the ratio of the yolk's height to its diameter, and therefore the yolk index, decreased. This could be attributed to the weakening of vitelline membrane strength during the storage period which results in the migration of water from albumen to yolk and consequently a reduction in the yolk index due to the flattening of yolk [85].

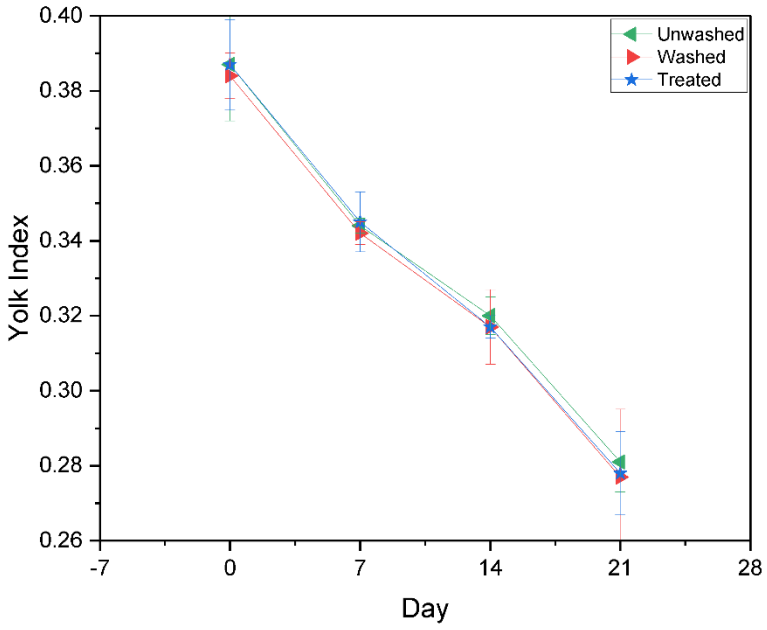

Figure 4. 4 Changes in yolk index during storage (3 eggs per group)

The effect of storage time on the Haugh unit is depicted in Figure 4.5. The Haugh unit showed a notable reduction as storage time increased for all groups of eggs each week ($p < 0.05$) which means that the thickness of the albumen decreased, resulting from albumen protein degradation. The result is then a reduction in albumen height and a lower value for the Haugh unit [65],[86].

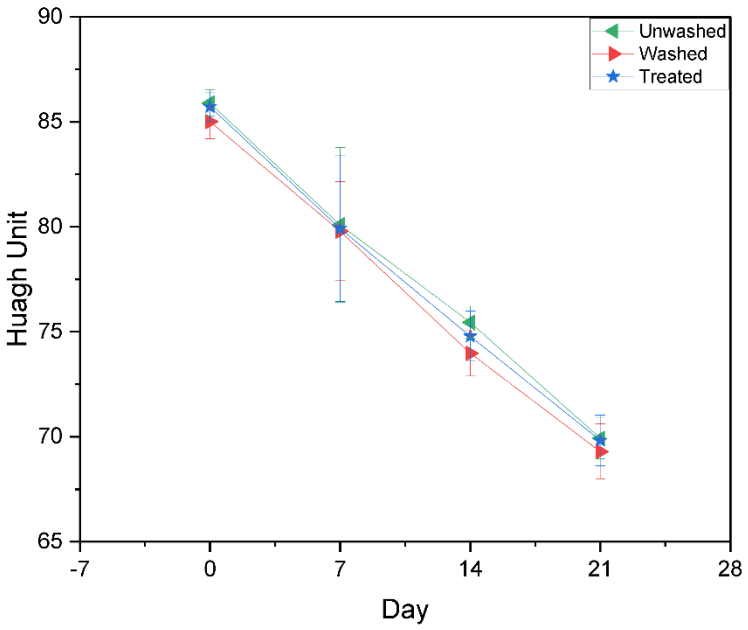

Figure 4. 5 Change in Haugh unit during storage (3 eggs per group)

Therefore, statistical analysis of the results demonstrated that the qualitative characteristics of eggs (eggshell specific gravity, albumen and yolk pH, yolk index, Haugh unit) stored in the fridge (at 4 °C) decreased significantly ($p < 0.05$) with a prolonged storage period of 3 weeks. However, there is no statistical difference among the three group eggs.

4.4.1.3 Moisture Content

Table 4.3 indicates the moisture content of albumen and yolk for 3 different groups of unwashed, washed, and treated eggs. Statistical analyses showed there was no significant difference among all groups and moisture contents were between 88 to 89% and 55 to 57% for albumen and yolk, respectively.

Table 4. 3 Evaluation the impact of the EWNS treatment on moisture content (3 eggs per group)

Group	Moisture content of Albumen	Moisture content of Egg Yolk
Unwashed	88.83 ± 1.44	55.77 ± 1.43
Washed	88.06 ± 1.40	56.68 ± 1.79
Treated	88.89 ± 1.15	56.41 ±1.59

4.4.1.4 SDS-PAGE

Figure 4.6 shows the SDS-PAGE gel image of protein bands of the 3 different groups of unwashed, washed, and EWNS-treated eggs. In each line, egg albumen proteins were sorted from top to the bottom according to their molecular weights and the marker proteins lane (Lane 1) was used as a reference to determine the proteins. As the most abundant protein in albumen is Ovalbumin, it was the largest band on the gel. Lysozyme and Conalbumin (Ovotransferrin) were the other protein bands detected in the albumen [87],[88]. Statistical analysis of the intensity of identified protein bands showed that there was no considerable difference in protein characteristics among the three groups of control, washed, and treated eggs.

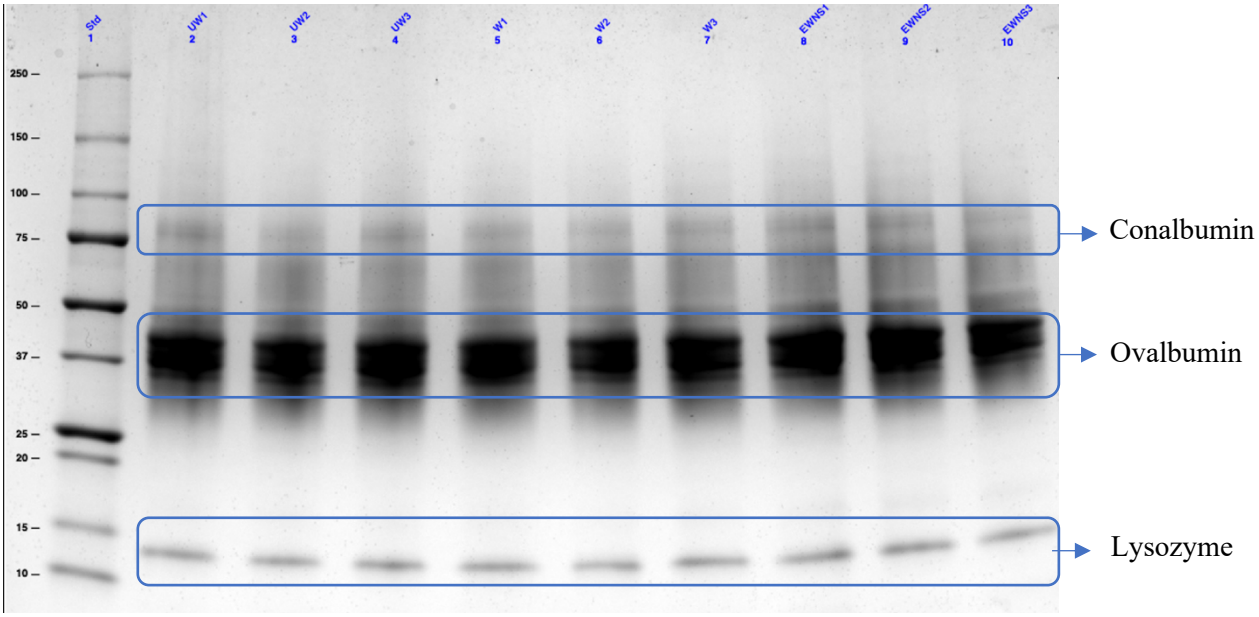

Figure 4. 6 SDS-Page gel image of protein bands of albumen samples

4.5 Conclusions

In this work, the impact of the novel EWNS decontamination method on the physical and chemical properties of treated eggs was evaluated in both short and long storage time and compared with washed, and unwashed (control) eggs. The results can be summarized as follows:

1) After one week, there were no significant differences in quality characteristics between unwashed, washed, and treated eggs, according to statistical analysis.
2) Over a period of 3 weeks in storage at 4°C, the quality characteristics of eggs (eggshell specific gravity, albumen and yolk pH, yolk index, Haugh unit) decreased significantly ($p < 0.05$), but there were no significant differences between the three groups of eggs studied.
3) Moisture contents of albumen and yolk were around 88-89% and 55-57%, respectively, and there were no significant differences between all groups.
4) Statistical analysis of the intensity of egg albumen protein bands showed that there were no significant differences in protein characteristics among the control, washed, and treated eggs.

Chapter 5 – Conclusions and recommendations

5.1 Summary of results

The research aimed to investigate the effectiveness of Engineered Water Nanostructures (EWNS) generated using electro-nano-spray technology for decontaminating egg surfaces of *Escherichia coli* (*E. coli*) and *Salmonella*. Firstly, the effect of exposure time was examined on the inactivation of *E. coli* inoculated on egg surfaces over a range of 2.5 to 15 minutes. The results indicated that increasing the treatment time led to an increase in the log reduction and bacterial inactivation efficiency, with the longest treatment time producing the highest results at 1.82 log and 98.4%. Due to the practical significance of the treatment time in the commercialization of the new technology, a duration of 5 minutes was chosen as the exposure time for the remaining tests. Next, the effect of electric field (E) strength on *E. coli* inactivation was investigated. When the water flow rate was set to 2 μL/min/needle, increasing the strength of the electric field from 4.0 to 9.0 kV/cm led to a decrease in *E. coli* numbers on the egg surface. Specifically, the *E. coli* on eggshells decreased by 59.7% (0.39 log) when subjected to 4.0 kV/cm of EWNS. In contrast, bacterial contamination was reduced by 90.9% (1.04 log) when subjected to 9 kV/cm of EWNS. It also observed a general declining trend of *E. coli* inactivation efficiency with increasing the water flow rate.

Moreover, it was found that the use of empty needles in the electrospray system resulted in a significantly lower *E. coli* inactivation compared to the same situation with EWNS. Additionally, *E. coli* inactivation of egg surfaces under positive applied voltage was significantly lower than that under negative applied voltage with equal electric field strength and other same operating conditions.

After conducting various experiments, it is determined that at 5 minutes as the treatment time, the optimal operating parameters for surface decontamination of eggs via EWNS were 1 μL/min/needle (16 μL/min as total water flow rate) and 9 kV/cm electric field strength. This strength of the electric field was calculated at a 0.5 cm distance between two electrodes and an applied voltage equal to -4.5 kV. At these optimal operating conditions, the highest antimicrobial efficiency for *E. coli* was 97.9% with a 1.69 log reduction, and for *Salmonella*, it was 80.4% with a 0.71 log reduction.

To assess the impact of EWNS technology on egg quality, various physical properties of eggs such as eggshell specific gravity, eggshell thickness, albumen pH, yolk pH, yolk index, and Haugh unit were measured. Statistical analyses showed no significant differences in qualitative characteristics among unwashed, washed, and treated eggs after 1 week (20 eggs per each group). Furthermore, it was investigated that the impact of the EWNS treatment on the physical properties of eggs during a longer storage period and the physical quality at 0, 7, 14, and 21 days of storage period after treatment was analyzed. The results indicated that while the quality of all egg groups reduced after each week during the storage period, no considerable change ($p < 0.05$) was observed in any of the analyzed. The moisture content of the egg albumen and the yolk was analyzed, and statistical analysis revealed that there were no significant differences among all groups, with the moisture content ranging between 88 to 89% for albumen and 55 to 57% for yolk (3 eggs per group). Additionally, the intensity of identified protein bands on the SDS-PAGE gel image was analyzed, and there were no substantial differences in protein characteristics among the control, washed, and treated eggs. In summary, the application of EWNS did not have any negative effects on the physical and chemical properties evaluated for egg quality. These findings suggest that EWNS can be used as an eco-friendly approach for decontaminating eggshells.

5.2 Conclusions

In this research work, the effects of a novel, chemical free, shell egg EWNS exposure time, water flow rate, and electric field strength (combination of applied voltage and distance between needle tips and counter electrode) on the inactivation of *E. coli* on egg surface were investigated. The main findings can be summarized as follows:

1) Eggshell surface can be effectively disinfected using the nano-sized droplets generated through electrospray.
2) At 5.0 minutes as exposure time, the highest efficiency of EWNS for inactivating the *E. coli* on the egg surface (97.6% with 1.64 log) was obtained through the lowest water flow rate (1 μL/min/needle), and the highest electric field strength (9.0 kV/cm).
3) Under the optimal operating condition, the efficiency of the EWNS system on the inactivation of *salmonella* inoculated on the egg surface was lower (80.4% with 0.71 log) in comparison with *E. coli.*
4) According to statistical analysis of the physical and chemical characteristics of eggs, there

were no substantial differences in these properties between control (unwashed) and treated eggs. This indicates that the EWNS treatment poses no adverse effects on the egg quality with respect to the physical and chemical properties evaluated.

The results presented above demonstrate that the EWNS generated by the electro-nano-spray method has the potential to inactivate bacteria on the egg surface. This work develops a novel, effective, non-thermal, and safe decontamination technology for eggshell surfaces.

5.3 Recommendations

This study focused on the effectiveness of EWNS technology for decontaminating egg surfaces. The model bacteria used for testing was *Escherichia coli*, as it is known to be a leading cause of foodborne illness outbreaks. The results showed that the EWNS treatment was successful in reducing bacterial levels on the egg surfaces.

In future studies, it may be worthwhile to investigate the application of this technology on the inside of eggs such as liquid egg products. Additionally, targeted delivery of EWNS-based nano-sanitizers to the surface of interest (egg) could be explored.

It may also be beneficial to combine the EWNS treatment with other decontamination methods, such as cold plasma to inactivate the bacteria on the egg surface.

Finally, scaling up the system for larger-scale trials with a larger number of eggs could be a useful area of research in the future. This would allow for a more comprehensive understanding of the practicality, feasibility, and effectiveness of the EWNS technology in commercial settings. Scaling up the electrospray system for generating EWNS can be achieved by considering the following suggestions:

- Increase the number of emitters/injectors to cover a larger area (higher number of eggs).
- Optimize the needle size and configuration such as using needles with different gauges or multiple needles per emitter to enhance the spray pattern and coverage.

- Utilize other technologies to atomize water into nano-size droplets for higher water flow rates such as ultrasonic nebulizers or microfluidic devices.

- Modify the electrode design to accommodate the increased number of injectors and ensure proper spacing between the needles to maintain consistent spraying.

- Consider automation and control systems: As the system scales up, it may be beneficial to introduce automation and control systems for precise control over flow rates, voltage, and other parameters. This can improve efficiency, reproducibility, and ease of operation.

- Monitor safety considerations to ensure that safety measures are in place and adhere to electrical and operational safety protocols considering the handling of water, potential electrical hazards, and other safety concerns.

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Appendix A – Procedures for preparing *E. coli*

1. Preparation of LB broth (lysogeny broth) (Done in HSc 2D10)

1.1. Materials Needed

i. LB Broth powder (BD Difco, Fisher Scientific, Cat#DF0446-17-3)
ii. Distilled water
iii. Top Loading Balance (Mettler Toledo)
iv. Weigh boats

1.2. Preparation

i. Weigh out 12.5g of LB Broth powder using a top loading balance and place into a 1L Nalgene bottle.
ii. Dissolve the LB broth powder in 500 mL of distilled water.
iii. Unscrew the lid on the bottle so it is loose, cover the lid with tin foil and put a piece of autoclave tape with: media type, date, lab #, and initials/PI's initials. Put the bottles of media in a solid container and place on the cart for autoclaving.

2. Preparation of LB Agar plates (Done in HSc 2D10)

2.1. Materials Needed

i. LB Broth powder (BD Difco, Fisher Scientific, Cat#DF0446-17-3)
ii. Granulated Agar (BD Difco, Fisher Scientific, Cat#DF0145-17-0)
iii. Distilled water
iv. Top Loading Balance (Mettler Toledo)

2.2. Preparation

i. Weigh out 7.5 g of granulated Agar and 12.5 g of LB broth powder using a top loading balance. Place both into a 1L Nalgene bottle.
ii. Dissolve the powder in 500 mL of distilled water.
iii. Unscrew the lid on the bottle so it is loose, cover the lid with tin foil and put a piece of autoclave tape with:
    a. media type
    b. date,

c. lab #,
d. initials/PI's initials.

iv. Put the bottles of media in a solid container and place on the cart for autoclaving.
v. Cool the agar down in a 65 °C water bath.
vi. In the biosafety cabinet, pour agar into petri plates covering approximately ¾ of the plate. Close the lids to the plates and swirl gently to distribute the agar across the petri plate.
vii. Allow the plates to solidify. Store at 4°C if not using right away.

3. Preparation of *E. coli* after receiving from supplier

3.1 Materials Needed

i. ATCC 27325 *Escherichia coli* (Migula) Castellani and Chalmer
ii. LB Broth
iii. Pipettes, 1000 µL volume
iv. Pipette Tips, 1000 µL volume
v. LB Agar plates
vi. 37°C Incubator

3.2 Preparation of E. coli Culture

i. In the biosafety cabinet, open the vial according to the manufacturer's instructions.
ii. Add 0.5 to 1 mL of LB broth to the tube containing the pellet using a pipette.
iii. Mix contents of the tube by pipetting up and down gently.
iv. Aseptically transfer this aliquot to a tube containing LB Broth (5 to 6 mL) and mix well.
v. Using a sterile disposable loop, streak the culture onto LB agar plates.
vi. Incubate the tube and plates at 37 °C for 16-18 hours (overnight).

4. *E. coli* inoculation

4.1 Materials Needed

i. Sterile disposable loops
ii. LB Agar plates
iii. LB broth
iv. Pipettors

v. 37 °C incubator

4.2 Plate to Plate *E. coli* Inoculation

i. Use sterile disposable loop attaches one single colony of *E. coli* from original agar plate.
ii. Close the original petri dish.
iii. Partially lift the lid of the petri dish of the LB Agar plate.
iv. Hold the charged loop parallel with the surface of the agar, smear the inoculum backwards and forwards across a small area of the medium (see streaked area A in photograph).
v. Ture the dish with 90° anticlockwise, with the same loop streak the plate from area A across the surface of the agar in three or four parallel lines (B). Make sure that a small amount of culture is carried over.
vi. Turn the dish through 90° anticlockwise again and streak from B across the surface of agar in three or four parallel lines (C).
vii. Turn the dish through 90° anticlockwise and streak loop across the surface of agar from C into the centre of the plate (D).
viii. Remove and dispose the lope, close the Petri dish.
ix. Incubate plates overnight at 37 °C.
x. Seal and storage at 4 °C for maximum two weeks.

4.3 Plate to liquid culture media *E. coli* inoculation

i. Use sterile disposable loop attaches one single colony of *E. coli* from agar plate.
ii. Close the Petri dish.
iii. Dip the loop inside a 15 ml Falcon tube contains 5 ml of LB broth and mix gently until the loop is clear of the bacteria colony.
iv. Close the Falcon tube and dispose of the loop in the biowaste bin.
v. Incubate the culture medium at 37 °C for 18 to 24 hours (overnight).

(All procedures of inoculation above are performed in the Biosafety cabinet.)

5. Optical density determination

5.1 Materials Needed

i. Small sized Kimwipes
ii. Sterile LB Broth (used as blank)

iii. Cuvettes
iv. UV-Vis Spectrophotometer
v. 1000 μL Pipette Tips
vi. 1000 μL pipettors

5.2 OD readings

i. Take 1 mL of culture and put it into a plastic cuvette.
ii. Recap the Falcon tube with liquid culture medium.
iii. Wipe down the outside of the cuvette with a kimwipe to remove any thing that could interfere with the OD reading.
iv. Use the single wavelength mode of UV-Vis spectrophotometer measures the absorbance of 1 ml incubated LB medium at 600 nm (LB culture as blank).
v. If the OD>1, the sample dilution is needed.

(The OD reading can be performed on the bench)

6. Surface inoculation of egg

vi. Weigh the fresh egg.
vii. Dip the egg into 70 % ethanol for one minute.
viii. Brush the egg with soap and rinse it with warm water.
ix. Wash the egg again with sterile warm water.
x. Put the egg in biosafety cabinet (a sterile laminar air flow) and allow to dry for 30 minutes.
xi. Place the dried egg in the pre-determined concentration of *E. coli* stock solution for 20 minutes under orbital shaking at 100 rpm.
xii. Leave the egg to dry for 40 minutes in the biosafety cabinet prior to treatment.

(All procedures of inoculation above are performed in the Biosafety cabinet.)

7. Characterize the inactivation efficiency

i. Transfer the inoculated and dried egg from the biosafety cabinet to the treatment chamber
ii. Expose the eggs to EWNS (half-side at a time) for the desired treatment/exposure time, then the other half-side off egg will be exposed for the equal time.
iii. Dry the treated egg for 40 minutes in the biosafety cabinet.

iv. After the treatment, to recover the *E. coli* from the egg surface, place each egg in a sterile sampling bag (Nasco Whirl-pack).
v. Add 50ml of LB Broth solution into the sterile bag.
vi. Hand-rub the shell egg through the bag for 3 minutes to detach the bacteria.
vii. Treat the control egg with the same procedure except for exposure to EWNS and set the concentration of bacteria on the egg surface as *E. coli* concentration at t=0.
viii. Take three 100-µL of the mixed solution from the sampling bag for agar plating (three plates for the three 100-µL solutions).
ix. Incubate the plates at 37 °C for 16-18 hours (overnight) prior to CFU counting.
x. The next day count the colonies on the plates. Record this number in your logbook.
xi. If number of colonies are too numerous to count (>300 colonies) on the plates, then
xii. dilution series need to be used.
xiii. Do each test in triplicates.

(All procedures of recovery above are performed in the biosafety cabinet.)

Appendix B – Procedures for preparing *Salmonella*

1. Preparation of LB broth (lysogeny broth) (Done in HSc 2D10)

1.2. Materials Needed

i. LB Broth powder (BD Difco, Fisher Scientific, Cat#DF0446-17-3)
ii. Distilled water
iii. Top Loading Balance (Mettler Toledo)
iv. Weigh boats

1.2. Preparation

i. Weigh 12.5 g of LB Broth powder using a top loading balance and place it into a 1L Nalgene bottle.
ii. Dissolve the LB broth powder in 500 mL of distilled water.
iii. Unscrew the lid on the bottle, so it is loose, cover the lid with tin foil and put a piece of autoclave tape with media type, date, lab #, and initials/PI's initials. Put the bottles of media in a solid container and place them on the cart for autoclaving.

2. Preparation of LB Agar plates (Done in HSc 2D10)

7.1. Materials Needed

i. LB Broth powder (BD Difco, Fisher Scientific, Cat#DF0446-17-3)
ii. Granulated Agar (BD Difco, Fisher Scientific, Cat#DF0145-17-0)
iii. Distilled water
iv. Top Loading Balance (Mettler Toledo)

2.2. Preparation

i. Weigh 7.5 g of granulated Agar and 12.5 g of LB broth powder using a top loading balance. Place both into a 1L Nalgene bottle.
ii. Dissolve the powder in 500 mL of distilled water.
iii. Unscrew the lid on the bottle, so it is loose, cover the lid with tin foil and put a piece of autoclave tape with:
    e. media type
    f. date,

g. lab #,
h. initials/PI's initials.

iv. Put the bottles of media in a solid container and place on the cart for autoclaving.
v. Cool the Agar down in a 65 °C water bath.
vi. In the biosafety cabinet, pour Agar into petri plates covering approximately ¾ of the plate. Close the lids to the plates and swirl gently to distribute the Agar across the Petri plate.
vii. Allow the plates to solidify. Store at 4 °C if not used right away.

3. *Salmonella* Inoculation

3.1 Materials Needed

i. ATCC 4931 *Salmonella enterica serovar Enteritidis*
ii. LB Broth
iii. Pipettes, 1000 µL volume
iv. Pipette Tips, 1000 µL volume
v. 37 °C Incubator

3.2 Preparation of *Salmonella* Culture

i. In the biosafety cabinet, open the frozen sample.
ii. Add 5 µL of frozen culture to a 15 ml Falcon tube containing 10 mL of LB broth.
iii. Mix contents of the tube by pipetting up and down gently.
iv. Incubate the tube and plates at 37 °C for 18-24 hours (overnight).

(All procedures of inoculation above are performed in the Biosafety cabinet.)

4. Optical density determination

4.1 Materials Needed

i. Small sized Kimwipes
ii. Sterile LB Broth (used as blank)
iii. Cuvettes
iv. UV-Vis Spectrophotometer
v. 1000 µL Pipette Tips
vi. 1000 µL pipettors

4.2 OD readings

i. Take 1 mL of culture and put it into a plastic cuvette.
ii. Recap the Falcon tube with a liquid culture medium.
iii. Wipe down the outside of the cuvette with a Kim wipe to remove anything that could interfere with the OD reading.
iv. Use the single wavelength mode of UV-Vis spectrophotometer to measure the absorbance of 1 ml incubated LB medium at 600 nm (LB culture as blank).
v. If the OD > 1, the sample dilution is needed.

(The OD reading can be performed on the bench)

5. Surface inoculation of egg

i. Weigh the fresh egg.
ii. Dip the egg into 70 % ethanol for one minute.
iii. Brush the egg with soap and rinse it with warm water.
iv. Rewash the egg with sterile warm water.
v. Put the egg in a biosafety cabinet (a sterile laminar airflow) and allow it to dry for 30 minutes.
vi. Place the dried egg in the pre-determined concentration of *Salmonella* stock solution for 10 minutes under orbital shaking at 100 rpm.
vii. Leave the egg dry for 30 minutes in the biosafety cabinet prior to treatment.

(All procedures of inoculation above are performed in the Biosafety cabinet.)

6. Characterize the inactivation efficiency

i. Transfer the inoculated and dried egg from the biosafety cabinet to the treatment chamber
ii. Expose the eggs to EWNS (half-side at a time) for the desired treatment/exposure time, then the other half-side-off egg will be exposed for an equal time.
iii. Dry the treated egg for 30 minutes in the biosafety cabinet.
iv. After the treatment, to recover the *Salmonella* from the egg surface, place each egg in a sterile sampling bag (Nasco Whirl-pack).
v. Add 50ml of LB Broth solution into the sterile bag.

vi. Hand-rub the shell egg through the bag for 3 minutes to detach the bacteria.
vii. Treat the control egg with the same procedure except for exposure to EWNS and set the concentration of bacteria on the egg surface as Salmonella concentration at t=0.
viii. Take three 100-µL of the mixed solution from the sampling bag for agar plating (three plates for the three 100-µL solutions).
ix. Incubate the plates at 37 °C for 16-18 hours (overnight) prior to CFU counting.
x. The next day, count the colonies on the plates. Record this number in your logbook.
xi. If the number of colonies is too numerous to count (> 300 colonies) on the plates, then
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xiii. Do each test in triplicates.

(All procedures of recovery above are performed in the biosafety cabinet.)

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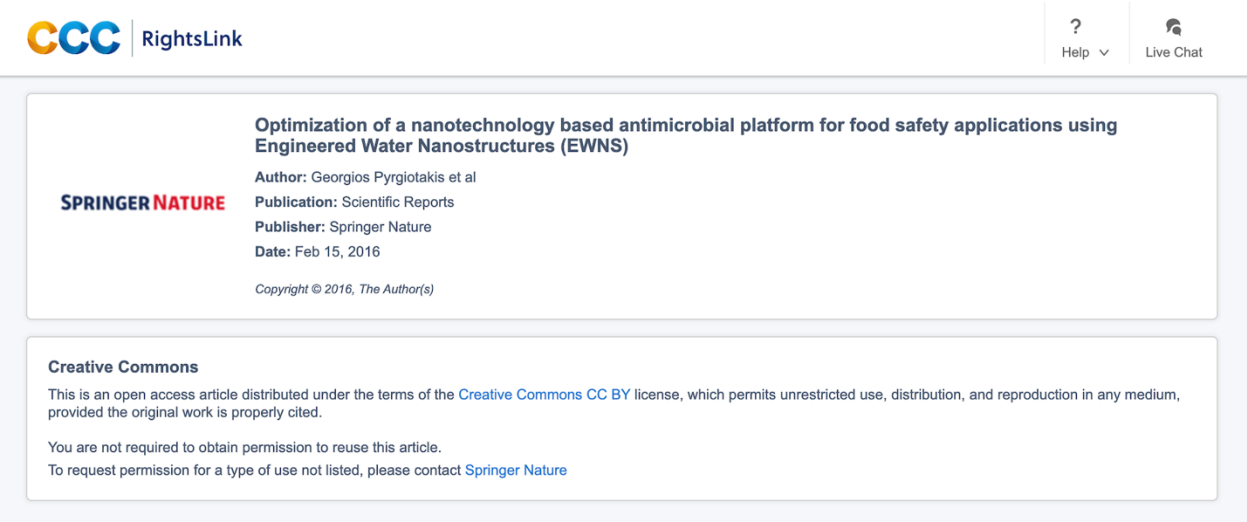

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