EVALUATION OF PH NEUTRAL ELECTROCHEMICALLY ACTIVATED SOLUTION (ECAS): AS A SANITISER FOR LIVESTOCK HUSBANDRY AND FOOD SAFETY APPLICATIONS

Sangay Tenzin

A thesis submitted in fulfilment of the requirements of the degree of Doctor of Philosophy

School of Animal and Veterinary Sciences
The University of Adelaide
June 2020

I dedicate this Doctoral Thesis to the most precious people in my life:

My wife –Kunzang Wangmo

AND

three lovely children –

Tandin Pema Lhaden, Ngawang Jigme Choden & Ngawang Sangay Rigphel

Table of Contents

Table of Contents ii
Abstract iii
Thesis Declaration v
Acknowledgements vi
Chapter 1: Introduction and Literature Review 7
1.1 General Introduction 7
1.2 Literature Review 11
1.3 Aims and objectives 45
Chapter 2: Decontamination of aerosolised bacteria from a pig farm environment using a pH neutral electrochemically activated solution (Ecas4 anolyte) 46
2.1 Statement of Authorship 47
2.2 Original Article 49
Chapter 3: Reduction of *Salmonella* Enteritidis and total bacterial counts on eggs surface by a pH-neutral electrolyzed oxidizing water fog 65
3.1 Statement of authorship 66
3.2 Original article 68
Chapter 4: Effect of high concentration pH neutral electrochemically activated solution (ECAS) fogging on the cuticle layer of a chicken egg 91
4.1 Statement of Authorship 92
4.2 Original article 94
Chapter 5: A pH-neutral electrolyzed oxidizing water significantly reduces microbial contamination of fresh spinach leaves 107
5.1 Statement of Authorship 108
5.2 Original article 110
Chapter 6: Effects of an eco-friendly sanitizing wash on spinach leaf bacterial community structure and diversity 133
6.1 Statement of authorship 134
6.2 Original article 136
Chapter 7: General Discussions 155
7.1 Introduction 155
7.2 Major Findings 156
7.3 Implications and future directions 163
7.4 Conclusion 164
Bibliography 166

Abstract

The agriculture production, animal farming, and food processing industries heavily depend on biocides to either reduce microbial load or inactivate microorganisms. These chemical biocides are associated with issues such as bacterial co-resistance to antibiotics, production of toxic residues, and reduced consumer acceptability. Electrolysed oxidising (EO) water, also termed electrochemically activated solution (ECAS) is an eco-friendly broad-spectrum biocidal agent. Among acidic, slightly acidic, and neutral pH forms of ECAS, the neutral type is preferred for its non-corrosiveness while still ensuring antimicrobial activity. In veterinary husbandry and shelled egg decontamination, ECAS is currently used in the form of a solution or spray wash. Therefore, in this thesis, the disinfection efficacy of an ECAS aerosol fogging regimen to decontaminate a pig farm environment and shell eggs was evaluated. Additionally, the effectiveness and impact of ECAS as a wash was assessed and compared to commonly used chemical agents for the sanitisation of ready-to-eat spinach leaves, including evaluation of organoleptic properties, shelf life, and nutritional attributes. In turn, this offered the possibility of exploring the effects of ECAS on spinach leaf microbiota composition, in comparison to peroxyacetic acid, a widely used, environmentally friendly sanitiser.

The pig farm fogging experiment was conducted using optimised parameters for farm air sample collection and qPCR techniques that discern between live and dead bacteria. ECAS was fogged at 0.75 mg of free available chlorine (FAC) per cubic meter of air for 3 min every 30 mins, for 5 hours. A time-dependent total bacteria reduction was observed, with the population reduced by 78%, 97%, 99.4%, 99.8%, and 99.998% every hour until the fifth hour. For the shelled egg sanitisation and cuticle integrity experiment, ECAS at 150 mg/L FAC spray washing for 45 s and a fogging for 2 min achieved a complete inactivation of total bacteria and *Salmonella* Enteritidis; a similar reduction was also observed for sodium hypochlorite spray washes (45 s). Most importantly, however, ECAS spray and aerosol fog washing did not significantly affect the cuticle of the eggshell.

In the spinach leaf sanitisation experiment, the effectiveness of ECAS (50 & 85 mg/L of FAC) and peroxyacetic acid (PAA) based sanitiser (50 mg/L of PAA) in

reducing the total microbial load and the purposely inoculated non-pathogenic bacteria, and their effect on sensory attributes in comparison to that of tap water washing was assessed. All types of sanitising washes significantly reduced ($p < 0.05$) total bacteria and coliforms, keeping sensory characteristics above the acceptance level even after ten days of storage at 4ºC. The inactivation of purposely inoculated bacteria varied with the bacterial species and the type of sanitiser. Overall, ECAS (85 mg/L of FAC) showed greater efficacy in reducing *Escherichia coli*, and *Listeria innocua* than PAA. Furthermore, the PAA inactivation trends for these species suggested the induction of a viable but not-culturable state. All three types of sanitising wash significantly reduced *S.* Enteritidis, and the reduction for all three sampling days was higher for PAA. Microbiome profile using 16s rRNA amplicon sequencing showed that all treatment types did not affect the composition and structure of the autochthonous bacterial community, but storage time significantly reduced ($p < 0.05$) the heterogeneity of the identifiable bacterial species.

Compared to other eco-friendly biocidal agents, ECAS could find increasing application in horticulture production, livestock farming and food safety due to its high antimicrobial efficacy and low production costs, as well as due to increasingly restrictive regulations on the use of chemical disinfectants and the change in consumer perception. In addition, the use of ECAS would reduce the use of chemical disinfectants and sanitisers, which may co-select for bacterial antimicrobial resistance and cause occupational safety and environmental issues through the generation of toxic residues.

Thesis Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

I acknowledge that the copyright of published works contained within this thesis resides with the copyright holder(s) of those works.

I give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Sangay Tenzin

March 2020

Acknowledgements

This thesis would not have been possible without the support of many people and institutions. First and foremost, I would like to thank my supervisor Professor Darren Trott for his unyielding support, encouragement, great feedback and excellent networking opportunities. I also would like to thank my other supervisor Dr Permal Deo for his constant guidance, assistance and dedicated involvement. I am also grateful to Dr Sergio Ferro for his support. My thank goes Dr Abiodun David Ogunniyi and Dr Manouchehr Khazandi.

I am very grateful to Endeavour Awards Programme for funding my studies and thanks to my Case Managers from Scope Global.

My sincere appreciation goes to Amanda Ruggero, Lora Bowes and Hong Nguyen (University of South Australia) for extending their professional and technical support during my work at the University of South Australia, City East Campus. I also would like to thank Derrick Prowese, Lyangra farm for his assistance and facilitating my pig farm decontamination work.

My four years of stay in Adelaide would not have been smooth without the support of my family and friends.

I am thankful to my father, parent in-laws and other relatives and friends for their encouragement and for bearing with our absence this long. My deep gratitude to my two in-laws Kinzang Deki and Pema Dorji for taking care of my young son during my absence. Finally, I would like to thank my dear wife Kunzang Wangmo, my daughters Tandin Pema Lhaden, Ngawang Jigme Choden and my son Ngawang Sangay Rigphel for their patience, encouragement, support and love during this time.

Chapter 1: Introduction and Literature Review

1.1 GENERAL INTRODUCTION

The primary production industries, such as agriculture and livestock farming systems, have become more intensive to enhance production because of the ever-increasing demand for affordable food due to the rising population. The intensification of primary production inadvertently led to the large-scale application of biocidal agents in livestock, agriculture, and food industries (Seier-Petersen, Ussery, Aarestrup, & Agersø, 2013). In livestock farming, disinfectants are used primarily to inactivate microorganisms to prevent and control infectious diseases spread to animals and humans. In food processing, biocidal agents are used to enhancing food safety through the elimination of pathogenic bacteria and reduction of food spoilage bacteria to a safe level. Only limited varieties of disinfectant and sanitisation chemicals are used in these industries for cleaning and disinfection processes, as some disinfectant usage is regulated to eliminate risks for human health, minimise hazards to non-target flora and fauna, and control residues affecting environmental safety.

Usually, two-step processes are used in animal farm cleaning and disinfection, shelled egg washing, fruits and vegetable sanitisation. In the first step, the farm environment, eggs, or agriculture products are cleaned either with water or alkaline detergent solutions to remove organic debris that reduces the antibacterial capability of disinfectants. Then in the next step, various disinfectants are used as a solution to reduce bacterial and *Enterobacteriaceae* load and to eliminate specific pathogens that are of risk to animals, farmworkers, and the general public. Disinfectants such as glutaraldehyde, quaternary ammonium compounds (QACs), sodium hypochlorite (NaoCl) and iodophors are commonly used for the disinfection of equipment and barn the environment in livestock farming (Gosling, 2018; Gosling, Mawhinney, Vaughan, Davies, & Smith, 2017). In eggshell sanitisation, QACs and oxidising biocidal agents are the common effective biocidal agents used (Al-Ajeeli, 2013) in reducing general aerobic bacteria, zoonotic pathogens, and *Enterobacteriaceae*. Whereas, for ready-to-eat (RTE) leafy vegetable sanitisation, chlorine-based and peracetic acid (PAA) based

disinfectants are used to sanitise spoilage and food-borne bacterial pathogens, such as *Listeria, Salmonella* and *Escherichia coli* (Premier, 2013). In some instances, though rare, disinfectants in the form of aerosolised mist or fog are applied to prevent transmission of respiratory and food-borne pathogens, reduction of aerobic bacterial load and disinfection of hard to reach areas and equipment.

The antibacterial disinfectants exert antibacterial activity by targeting bacterial cell wall and outer membranes (Hydrogen peroxide - H_2O_2; NaOCl, QACs, PAA) (Maillard, 2002), cytoplasm (QACs, H_2O_2) (Maillard, 2002), respiratory functions (iodophors) (Maris, 1995), enzymes (H_2O_2, NaOCl, PAA) (Maris, 1995; McDonnell & Russell, 1999) and genetic materials (H_2O_2, NaOCl) (McDonnell & Russell, 1999). Since most disinfectants have multiple concurrent modes of action and are used at higher volume and higher concentrations, the occurrence of bacterial resistance is rare. However, the use of disinfectants at suboptimal concentrations is the crucial factor that allows bacteria to develop cross-resistant to antimicrobials (SCENIHR, 2009). The mechanism of bacterial resistance to disinfectants are non-specific and categorised broadly into intrinsic and acquired (Poole, 2002; Russell, 1999). For instance, inherent resistance of Gram-negative bacteria, due to the presence of the outer membrane, makes them less susceptible than Gram-positive bacteria (White & McDermott, 2001) and intrinsic biofilm formation by some bacteria also reduces susceptibility (McDonnell & Russell, 1999). Generally, acquired resistance in bacteria occurs through the acquisition of plasmid-mediated resistance genes associated with the efflux system (White & McDermott, 2001) and changes in outer membrane ultrastructure (Poole, 2002). In case of QACs, bacterial strains isolated from foods (Condell et al., 2012; Mereghetti, Quentin, Marquet-Van Der Mee, & Audurier, 2000), humans (Sidhu, Sorum, & Holck, 2002) and animals (Amass et al., 2000; Beier, Bischoff, & Poole, 2004) acquire resistant determinants such as plasmid-mediated efflux systems (Paulsen et al., 1993; Rouch, Cram, DiBerardino, Littlejohn, & Skurray, 1990) and changes in fatty acids composition in lipopolysaccharide layer (Guerin-Mechin, Dubois-Brissonnet, Heyd, & Leveau, 1999). The resistance mechanism to oxidising agents such as NaOCl, PAA and H_2O_2 is through reduction of cellular permeability (Morente et al., 2013), biofilm formation (Wingender & Flemming, 2011; Wong, Townsend, Fenwick, Trengove, & O'Handley, 2010) and unknown mechanism (Martin, Wesgate, Denyer, McDonnell, & Maillard, 2015).

In addition to co-selection of antibiotic resistance, other disadvantages of these chemical disinfectants are the generation of toxic residues such as chloroforms, tri-halomethanes and chloramines by chlorine-based agents (Amy et al., 2000), industry hosing surface and equipment corrosion, work health and safety (WHS) issues, loss of activity in the presence of organic loads and associated ecological problems related to its disposal. The use of PAA based disinfectant is restrictive because of higher costs (Premier, 2013), inability to accurately monitor PAA concentrations and oxidation-reduction potentials (ORP), and shortening of shelf life of fresh produce when used at the very high concentrations (Bachelli, Amaral, & Benedetti, 2013).

Due to the above-specified limitations of currently used disinfectants, new biocidal agents that have broad-spectrum antimicrobial capability, do not cause environmental toxicity and health hazards, do not select for co-resistance and cross-resistance to antimicrobials, and that appeal to consumer palatability are continuously being sought for application in the sanitisation process in primary industries. Electrochemically activated solution (ECAS), also termed electrolysed oxidising (EO) water, is a general-purpose broad-spectrum biocidal agent produced through the use of inexpensive raw materials. ECAS has potential applications for cleaning and disinfection process in livestock production and post-harvest sanitisation of agriculture produce, as it is an eco-friendly disinfectant that could appeal to environmentally conscious consumers and is non-hazardous easy to use with no reported bacterial resistance.

Broadly, based on the pH, ECAS is classified into acidic, slightly acidic, neutral, and alkaline forms. Acidic ($pH < 5.0$) forms have proven disinfection efficacy against the plethora of bacteria causing animal health and food safety concerns, but their use is limited to in vitro experimental kill efficacy (Meakin, Bowman, Lewis, & Dancer, 2012) and sanitisation of vegetables (Hao et al., 2011) because of inherant corrosiveness to processing equipment (Tanaka et al., 1999; Wang et al., 2019) and high content of elemental chlorine (Cl_2). Slightly acidic forms (pH 5.0-6.5) are known to have higher disinfection potential than acidic forms (Hao, Li, Wan, & Liu, 2015) because of the presence of higher levels of hypochlorous acid (HOCl), which is the main antibacterial oxidising agents present in ECAS (Cao, Zhu, Shi, Wang, & Li, 2009). The application of these anolytes also has caused mild corrosion of equipment and contact surfaces (Ayebah & Hung, 2005). Moreover, acidic forms are known to

be less stable during storage because of rapid chemical relaxation at acidic pH, reverting the ‘metastable’ solution to the brine solution.

A new generation of ECAS, a pH neutral form, contains HOCl as the predominant active antibacterial moiety (Cheng, Dev, Bialka, & Demirci, 2012; Guentzel, Lam, Callan, Emmons, & Dunham, 2008) and only a trace quantity of dissolved chlorine (Cl_2) constituting 0.7 % (Liao, Chen, & Xiao, 2007) which renders it less corrosive and provides a relatively prolonged shelf life (Rahman, Ding, & Oh, 2010). The pH neutral EO anolyte in the form of a wash solution is used for medical applications (Lata et al., 2016; Sergio Ferro, 2017) as well as in the livestock and food safety industries (Khazandi et al., 2017; Martínez-Hernández et al., 2015; Rivera-Garcia et al., 2019). Notably, in livestock sectors, it is used as a spray and wash solution for sanitisation of farmhouses, equipment, air environment and as a drinking water supplement (Bügener, Kump, Casteel, & Klein, 2014; Hao, Li, Wang, Zhang, & Cao, 2013; Hao, Li, Zhang, et al., 2013; Zheng et al., 2014). Similarly, it has a proven bactericidal activity when applied as an immersion or spray wash in reduction and elimination of total bacteria and foodborne pathogens in the shelled egg (Rivera-Garcia et al., 2019; Surdu, Vătuiu, Jurcoane, Olteanu, & Vătuiu, 2017). Therefore, in this study the disinfection effectiveness of ECAS as an aerosol fog to decontaminate the animal farm environment and disinfect the shelled egg surface of total bacteria and pathogens was assessed.

In the case of RTE vegetable sanitization, the effectiveness of neutral ECAS washing of spinach leaves to reduce total bacterial load and eliminate foodborne pathogens such as *E. coli, Salmonella enterica* serovar Typhimurium*, S. aureus, L. monocytogenes,* and *E. faecalis* depends on free available chlorine concentration (FAC) (Izumi, Kiba, & Hashimoto, 1999; Guentzel et al., 2008). The wash duration and FAC recorded for effective sanitization for the above experiments were 3 min (20 mg/L) and 10 minutes (50, 100 and 120 mg/L) for total aerobic bacteria and foodborne pathogens, respectively. As, PAA-based sanitizer is widely used for RTE washing, because of its effectiveness at short duration (45 to 60 s at 50 mg/L PAA), so effectiveness of neutral ECAS at 50 and 85 mg/L FAC washing of spinach leaves for 60 s was compared to 50 mg/L PAA in reducing total bacteria and surrogate pathogens. In addition, as different disinfectants at varying concentrations of active biocidal agents affect the shelf life, nutritional content and microbiota differently, the effect of

the above washings on organoleptic attributes, vitamin C and bacterial microbiome composition was also evaluated.

The structure of this thesis is publication format with five experimental chapters. In chapter 1, the literature review presents the use of ECAS and other disinfectants in livestock farming and food safety applications, specifically on the use of pH-neutral ECAS in disinfecting pig farm environment, shelled eggs and minimally processed vegetables. In chapter 2, the effectiveness of ECAS aerosol fogging in decontamination of a pig farm environment was assessed. The aerosol fog was generated using an ultrasonic humidifier and air samples before and after fogging were collected using a robust cyclonic air sampling technique. In chapter 3, the effectiveness of ECAS fogging at reducing both aerobic bacteria and experimentally inoculated *Salmonella* spp. on shelled eggs was assessed. In chapter 4, the effectiveness of high concentration ECAS fogging on disinfection of the eggshell cuticle layer was assessed. In chapter 5, the effectiveness of ECAS washing at reducing aerobic bacteria and experimentally inoculated foodborne pathogens on fresh spinach leaves was assessed in comparison to PAA-based sanitizer. In Chapter 6, the effect of ECAS on the bacterial microbiome structure of spinach leaves at two-time points after wash treatment was assessed.

1.2 LITERATURE REVIEW

This review first discusses electrochemically activated solution (ECAS), highlighting the various types, generation techniques, antimicrobial and chemical properties, and limitations of its application. A brief overview of other biocidal agents used in livestock farming and food safety management is introduced to contextualise the importance of cleaning and disinfection. The essential constituents of this review are the current status of the application of pH-neutral ECAS in various forms in the sanitisation of the farm environment, equipment and agriculture produce of total bacteria and specific pathogens to ensure animal wellbeing and human health. As the neutral form of this disinfectant is not widely used, reference is made to other types of ECASs and chemical disinfectants and their effect on bacterial pathogens, wherever necessary, to connect with the research aims and objectives described in this thesis.

1.2.1 Electrochemically activated solution

ECAS is widely referred to as electrolysed water, electrolysed oxidising (EO) water or electro-activated water (EW) in the scientific literature. The ECAS is synthesised by passing dilute (0.1%) sodium chloride (NaCl) solutions through an electrolytic cell containing anode and cathode chambers separated by a membrane, where direct current triggers electrochemical activity at the material electrode interface (Hricova, Stephan, & Zweifel, 2008). In the anodal chamber, negatively charged ions transform into an activated "metastable" state containing elemental oxygen (O_2) and chlorine (Cl_2), and free available chlorine (FAC) compounds. Principal constituents of the FAC (oxidising moieties) are hypochlorous acid (HClO), hypochlorite ions (OCl-), hydrochloric acid or chlorine oxide (ClO). This resultant solution in the anodal chamber is acidic anolyte (ECAS) with low pH (pH < 5.0) (Hsu, 2003; Prilutsky & Bakhir, 1997). At the cathodal chamber, a high pH (pH 10.0 - 11.5) catholyte termed electrolysed reduced water containing elemental hydrogen (H2), and sodium hydroxide (NaOH) is produced concurrently. Slightly acidic and neutral ECAS are also produced by redirecting electrolysed reduced water from the cathodal chamber into the anodal chamber, where electrolysis of dilute NaCl solution occurs. Umimoto (2013, 2015) categorizes ECAS anolyte depending on its pH as strongly acidic anolyte (pH: 2.2-2.7.0), slightly acidic anolyte (pH: 5.0 -6.5), weakly to slightly acidic electrolyzed water (pH 4.0 to 6.5) and neutral anolyte (pH: 6.5-7.5). Generally, the most preferred ECAS anolyte is of near-neutral pH with high oxidation-reduction potential (ORP) (> 800 mV) and contains a higher concentration of FAC (particularly HClO) compounds which are responsible for bactericidal activity. The prevalence of FAC in the resulting anolyte (ECAS) is dependent on characteristics of the electrochemical cell and its operating parameters such as flow rate and salt concentration (Hsu, 2005; McPherson, 1993; Stoner, Cahen, Sachyani, & Gileadi, 1982). The flow and salt concentrations also determine the pH as well as ORP of the anolyte. FAC proportion and abundance and pH of the anolyte are correlated (Hsu, 2005; Prilutsky & Bakhir, 1997). Empirical evidence from experiments shows that ORP has higher significance (Cloete, Thantsha, Maluleke, & Kirkpatrick, 2009) than the elemental chlorine (Cl_2) content in the determination of disinfection potential of ECAS anolyte (Park, Hung, & Chung, 2004). Moreover, slightly acidic and neutral

ECAS primarily has HOCl as an antimicrobial species and has higher bactericidal activity than OCl- and Cl_2 (Kim, Yen-Con Hung, & Robert E. Brackett, 2000b). Slightly acidic or neutral anolyte is preferred over-acidic anolyte as it contains a relatively higher concentration of HOCl and acidic ECAS causes mild corrosion to the disinfected surfaces.

pH neutral ECAS used in this project was obtained from a patented novel anolyte generation technology that has a reactor with four chambers (Bohnstedt, 2006) consisting of two cathode compartments and two anode compartments. This ECAS is produced from a dilute brine solution of 0.4% to 0.5% of NaCl at neutral pH, and contains about 350–400 mg/L of FAC (Migliarina & Ferro, 2014). Figure 1 presents a simple schematic diagram of slightly acidic and neutral ECAS production.

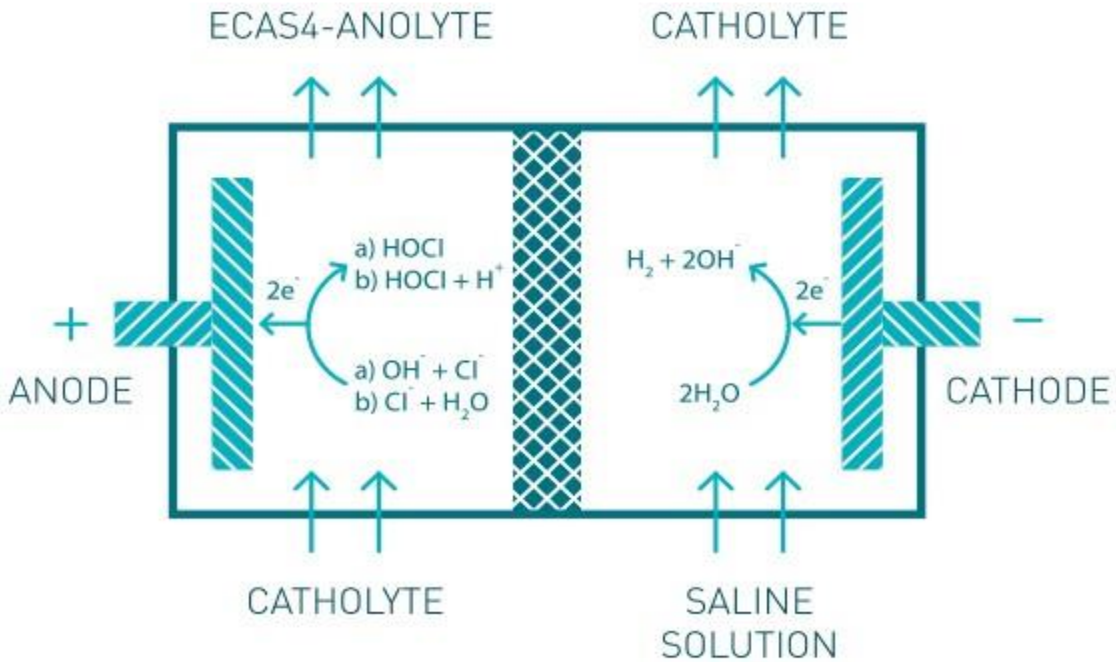


Figure 1. Schematic representation of slightly acidic and neutral ECAS anolyte production. Figure reproduce from www.ecas4.com.au

1.2.1.1 Antimicrobial mode of action of neutral ECAS on bacteria

pH neutral ECAS antimicrobial constituents are composed of HOCl, ClO, HO_2 (perhydroxyl radical), and O_2 and are less corrosive compared to other sanitisers (Ayebah and Hung, 2005). Neutral ECAS is highly effective in inactivating bacteria because it contains a proportionately higher concentration of HOCl than acidic ECAS

anolyte (Marritto & Gravani, 2006). It is also more stable than the acidic form because of a significant reduction of chlorine loss at pH 6.0 – 9.0 (Len et al., 2002).

Broadly, ECAS causes antimicrobial action by damage to the bacterial cell membrane, intracellular proteins and oxidation of critical metabolic systems (Barrette Jr, Hannum, Wheeler, & Hurst, 1989; Feliciano, Lee, & Pascall, 2012; Tang et al., 2011; Zeng et al., 2010). FAC compounds and other reactive oxygens moieties are known antimicrobial compounds present in ECAS. The most predominant FAC oxidising agent found in neutral ECAS is HOCl. It exerts antibacterial action by penetrating and damaging the bacterial cell walls and membranes, inhibiting enzymatic activity and damaging bacteria deoxyribonucleic acid (DNA) by production of hydroxyl radical (Barrette Jr et al., 1989; Folkes, Candeias, & Wardman, 1995; Fukuzaki, 2006; Hurst, Barrett Jr, Michel, & Rosen, 1991). Hypochlorite ion (OCl^-) oxidises the cell membrane from outside because of its inability to penetrate the plasma membrane (Fukuzaki, 2006). Other oxidative species in ECAS also contribute to the antimicrobial activity (Jeong, Kim, & Yoon, 2009; Jeong, Kim, Cho, Choi, & Yoon, 2007). The degradation of proteins and nucleic acids in *Escherichia coli* after 5 minutes of exposure to ECAS has been confirmed (Zinkevich, Beech, Tapper, & Bogdarina, 2000). Additionally, degradation of protein, by oxidative stress induced by the highly reactive non-specific oxidant has been confirmed for both *Pseudomonas aeruginosa* and *E. coli* (Cloete et al., 2009).

Hao et al. (2017) further investigated the difference in efficacy of acidic (pH 2.48 ± 0.06) and slightly acidic (pH 5.95 ± 0.10) anolyte on bactericidal effect and the mode of action on *E. coli, Staphylococcus aureus,* and *Bacillus subtilis*. Scanning electron microscopy images showed damage to the bacterial cell membrane by ECAS on both Gram-negative (Figure 2.1) and Gram-positive organisms (Figure 2.2) such as *E. coli* and *S. aureus,* respectively. Figure 2.1 and 2.2 showed the leakage and exposure of intracellular contents to oxidising agents in the solution resulting in bacterial death. Interestingly, degradation of RNA was caused by slightly acidic anolyte but not with the acidic anolyte indicating RNA is one of the targets of antibacterial activity (Figure 2.3) (J. Hao, Wu, Li, & Liu, 2017), which might explain the differential efficacy of anolytes with varying pH (Hao et al., 2017). However, the mechanism of action of degradation caused by slightly acidic ECAS on RNA is yet to be understood, and one

plausible explanation could be the presence of a higher concentration of oxidising moieties than in the acidic anolyte (Zinkevich et al., 2000).

Figure 2.1 Scanning electron microscopy picture of *E. coli* treated with slightly and strongly acidic electrolysed water. No treatments control (top image); strongly acidic electrolysed water (AEW) treatment (bottom right picture), and slightly acidic electrolysed water (SAEW) treatment (bottom left picture). Picture source; Hao and group, 2017.

This image has been removed due to copyright.

Figure 2.2 Photos of the scanning electron microscope of *S. aureus* treated by slightly and strongly acidic electrolysed water. No treatments as control (top); AEW treatments (bottom right) and SAEW treatment (bottom right). Picture source; Hao and group 2017.

LIBRARY NOTE:

This image has been removed due to copyright.

Figure 2.3 Electrophoretogram of RNA of three tested strains, including *E. coli, S. aureus,* and *B. subtilis* treated by slightly and strongly acidic electrolysed water. In the photogram, Lane 1–2 indicate the results of *B. subtilis* treated with slightly acidic electrolysed water (SAEW) treatments and strongly acidic electrolysed water (AEW) treatments. Lanes 3–4 for *E. coli* and lanes 5–6 *S. aureus* treated with slightly acidic

electrolysed water (SAEW) treatments and strongly acidic electrolysed water (AEW) treatments. Picture source; Hao and group 2017.

1.2.1.2 Toxicity of ECAS

The safety of a disinfectant is paramount especially when applied to live organisms or tissues that might cause unwanted toxic effects. No adverse oral toxicity was observed in rats fed with ECAS in drinking water (Morita, Nishida, & Ito, 2011). Moreover, no toxic effects were observed in mucous membranes, and no sensitivities reported when acute oral toxicity tests (LD_{50}) were conducted (Marais, 2002). An in vitro mutagenicity study reported ECAS to have no genotoxicity (Gutiérrez, 2006; Tsuji et al., 1999) and no detrimental effects on dermal fibroblasts (Martínez-de Jesüs, Remes-troche, Armstrong, & Beneit-montesinos, 2007) indicating ECAS as a safe, biocompatible disinfectant.

ECASs' reactive properties with the inanimate material surfaces and environmental toxicity are essential characteristics for consideration as a disinfectant for farmhouses and equipment. ECAS has been demonstrated to have limited corrosive effects on material surfaces and is less corrosive than 0.1% sodium hypochlorite solution (Ayebah & Hung, 2005; Tanaka et al., 1999). Moreover, since it reverts to brine solution during chemical relaxation, and it is quenched effectively by organic materials, it is one of the most environmentally safe disinfectants.

1.2.1.3 Limitations of ECAS4

The most prominent limitation of ECAS is a significant reduction in its antimicrobial potential in the presence of high organic matter load (Marais, 2002; Oomori, Oka, Inuta, & Arata, 2000; Park et al., 2004; Selkon, Babbt, & Morris, 1999). Organic materials transform FAC into combined available chlorine (N-chloro compounds) which have significantly lower bactericidal activity than that of the FAC (Oomori et al., 2000). Moreover, the removal of FAC through oxidation-reduction reaction in the presence of organic materials also causes loss of its antimicrobial potency (Oomori et al. 2000). The other drawback with the strongly acidic ECAS is it is less stable for storage and causes corrosion of equipment surfaces (Abadias, Usall, Oliveira, Alegre, & Viñas, 2008; Cao et al., 2009; Guentzel et al., 2008). However, to effectively overcome the inhibition of antibacterial activity with organic materials, firstly, ECAS could be used at a higher concentration of FAC at increased exposure

time. The other option would be to apply it in the form of micron-sized droplets or aerosolised fog form with frequent continual delivery because aerosolisation of ECAS increases the surface area coverage for disinfection. Another important issue associated with ECAS is the HOCl, that reverts to NaCl and water as it is in a 'metastable state'. Although, data on bactericidal effect of neutral ECAS within the published literature is limited, a study showed that acidic ECAS retain its bactericidal activity for over a year (Robinson, Thorn & Reynolds, 2013).

1.2.2 Application of ECAS in livestock farming and food industries

In livestock farming disinfectants are used as part of farm biosecurity management strategy to reduce environmental microorganisms affecting animal and farmworkers' health and eliminate the reservoir of disease-causing pathogens. Similarly, in table egg production, disinfectants are used for the reduction of general microbial load, and elimination of infectious microbial agents and food-borne pathogens from the eggshell surface. In the case of ready-to-eat vegetables and fruits, disinfectants are used in reducing the microbial load to prolong shelf life through reduction of spoilage bacteria and ensuring food safety through the elimination of pathogenic bacteria. Common disinfectants used for cleaning and disinfection processes are benzalkonium chloride, formaldehyde, sodium hypochlorite, H_2O_2, PAA and glutaraldehyde (Amass et al., 2000; G. Sundheim, S. Langsrud, E. Heir, & A. Holck, 1998). The concerns with use of these chemical disinfectants are; toxic effect on animals, workers and environment (Blackwell, Kang, Thomas, & Infante, 1981; Pinkerton, Hein, & Stayner, 2004; Til et al., 1989); corrosiveness to farm equipment (Nicklas, Böhm, & Richter, 1981); and selection of disinfectant-resistant organisms and bacterial cross-resistance to antibiotics (Russell, 2000; Sidhu et al., 2002; Slifierz et al., 2015). Despite having above mentioned detrimental effects, disinfectants are a vital biosecurity measure that reduces or eliminates the risk of transmission of infectious diseases and human pathogens. Alternative disinfectants that do not have adverse effects mentioned above are assessed for antibacterial efficacy for application in the cleaning and disinfection regimen in these industries. ECAS is one such disinfectant with a broad-spectrum antimicrobial efficacy, produced from low cost readily available raw material (brine solution) with no reported toxicity. Moreover, it is an environmentally safe disinfectant that can be generated in situ for disinfection applications.

Acidic ECAS has disinfection activity equivalent to 80% ethanol and superior to 0.1% chlorhexidine or 0.02% povidone-iodine (Tanaka et al., 1999). However, there is a limitation for applications of an acidic form of ECAS due to the corrosion of processing equipment and the phytotoxic effect on plants (Abadias et al., 2008). Slightly acidic or neutral pH ECAS having higher antimicrobial effectiveness against a broad range of bacterial pathogens (Deza, Araujo, & Garrido, 2005; Kim et al., 2000b), has been widely trialled as a decontaminant in livestock farming and wash sanitiser in food industries. In veterinary husbandry, ECAS has proven disinfection efficacy i) on milking implements in dairy farming (Kalit, Kos, Kalit, & Kos, 2015; Kawai et al., 2017), ii) in swine barns and poultry houses (Hao, Li, Wang, et al., 2013; Hao, Li, Zhang, et al., 2013) iii) for carcase and surface cleaning in meat processing industry (Bach et al., 2006; Rasschaert et al., 2013) and iv) for shelled eggs sanitisation for hatching and consumption (Fasenko, O'Dea Christopher, & McMullen, 2009; Ni, Cao, Zheng, Chen, & Li, 2014; Rivera-Garcia et al., 2019). In food processing, ECAS was found to be an efficacious disinfectant for food processing tools (Deza et al., 2005; Handojo, Lee, Hipp, & Pascall, 2009) and wash disinfectants for fruits (Graça, Abadias, Salazar, & Nunes, 2011) and vegetables (Rahman, Ding, et al., 2010; Rahman, Jin, & Oh, 2010).

1.2.2.1 Application of ECAS in pig barn decontamination

Cleaning and disinfection of the barn environment are an integral part of disease management in swine production as farm environment and equipment can harbour pathogens, and aerosolised pathogens are readily transmitted between stock in close contact. Concrete environment surfaces are disinfected by first washing off organic debris using either water or alkaline soap solution and then decontaminating with disinfectant. Air environment is disinfected by spraying chemical disinfectant or using an aerosol generator that generates mist or fog from the disinfectant solution. The use of disinfectants in decontaminating the air environment is further restricted in comparison to that of the concrete surface disinfection because disinfectants release chemicals that pose respiratory health risks both to animals and workers. For example, formaldehyde and NaOCl release carcinogenic chemicals (Amy et al., 2000) and elemental chlorine, respectively posing health risks to both animals and farmworkers.

1.2.2.1.1 Airborne bacteria and respiratory diseases in pig farms

Worldwide significant financial losses are implicated to veterinary services, poor feed to body conversion ratio, carcase disposal and management expenses in swine production. Airborne bacteria in the pig farm environment affect the health and welfare of animals and workers, and productivity (Kim & Ko, 2019; Popescu, Borda, Diugan, & Oros, 2014). Building types, animal numbers, ventilation type and climatic conditions (seasons) influence the bacterial load in the pig farm environment (Chang, Chung, Huang, & Su, 2001; Gustafsson, 1997; Kim & Ko, 2019; Popescu et al., 2014). The total recoverable bacterial load in the pig farm air was recorded to be between 3.4 to 6.4 Log_{10} CFU/m^3 (Bilić, Habrun, Barač, & Humski, 2000; Kim & Ko, 2019; Kristiansen, Saunders, Hansen, Nielsen, & Nielsen, 2012; Popescu et al., 2014). Culture-based and culture-independent molecular approaches are used to study the airborne bacterial community. Both Gram-positive and Gram-negative bacteria are isolated from indoor pig farm air environment (Roque et al., 2016; Vestergaard et al., 2018). The bacteria composition included some pathogens such as *A. pleuropneumoniae*, *Streptococcus suis* and *B. cereus* (Bonifait, Veillette, Létourneau, Grenier, & Duchaine, 2014; Loera-Muro et al., 2013; Roque et al., 2016). Bioaerosols of pig farm also harbour many viral respiratory pathogens such as coronavirus (CoV) (Borkenhagen et al., 2018).

Respiratory diseases are a potent inhibitor of economic returns from intensive swine production. The degree of production losses varies with various causative agents involved in respiratory infection of pigs. Swine respiratory infection is caused by bacterial and viral etiological agents individually or in tandem and referred to as porcine respiratory disease complex (PRDC) (Brockmeier, Halbur, & Thacker, 2002). The clinical manifestations of PRDC depend upon the farm production management system and the infective organism causing the disease (Jackson, Stone, & Tyler, 2015). Based on overt disease clinical symptoms and epidemiology, PRDC is classified broadly into an acute severe self-limiting disease and a chronic pneumonic or/and pleuro-pneumonic category. The self-limiting form is caused by viral pathogens such as swine influenza virus, classical swine fever virus, pseudorabies virus, porcine circovirus and porcine reproductive and respiratory syndrome virus. The common cause of chronic forms is primary and secondary bacterial pathogens such as *Mycoplasma hyopneumoniae, Pasteurella multocida, Streptococcus suis,*

Haemophilus parasuis, Bordetella bronchiseptica, Actinobacillus pleuropneumoniae, Actinobacillus suis, and *Salmonella* Choleraesuis.

A. pleuropneumoniae is a fastidious Gram-negative coccobacillus that causes *A. pleuropneuominae* pleuropneumonia (APP). It had two biovars, NAD-dependent biovar 1 with fourteen serovars and NAD-independent biovar 2 with four serovars (Blackall, Klaasen, Van Den Bosch, Kuhnert, & Frey, 2002; Bossé et al., 2018; Fodor, Varga, Molnar, & Hajtos, 1989; Nielsen, 1986a, 1986b; Nielsen et al., 1997; Sárközi, Makrai, & Fodor, 2015). Different serovar dominates different geographical locations and common serovars found in Australia are 1, 5, 6, 7, 8, 9 and 15 (Turni, Singh, Schembri, & Blackall, 2014). *A. pleuropneuominae* serovars 1, 5 and 9 are most virulent followed by *A. pleuropneuominae* 15 (McKenzie, 2010). *A. pleuropneuominae* 6, 7 and 8 has mild virulence potential and still causes serious pleuropneumonia in farms with high stock density and poor air quality (McKenzie, 2010). Epidemics observed are often in farms with naïve animals that are previously not exposed, or farms with circulating infection with carrier animals stressed with key precipitating factors such as high stock density, poor ventilation and other respiratory pathogens.

Globally APP is one of the leading causes of PRDC (Bochev, 2007; Thacker & Thanawongnuwech, 2002). *A. pleuropneuominae* infections present as acute, chronic and subclinical manifestations and APP diagnoses are confirmed either through culture and PCR detection of *ApxIV* toxin gene or serodiagnosis of ApxIV toxin using ELISA (Dreyfus et al., 2004). Generally, *A. pleuropneuominae* is controlled by instituting management protocols such as batch farrowing and All-In All-Out (AIAO) strategies that result in a noticeable reduction in mortalities and ill thrift independent of prevalent serovars. APP infection is also prevented or controlled through in-feed antibiotic inclusions and serovar specific vaccines. The antibiotic treatments result in resistance to common antibiotics by *A. pleuropneuominae* (McKenzie, 2014).

Recently McKenzie (2010) used a live vaccine to prevent APP and had some success in delayed APP occurrence by about two weeks. McKenzie (2014) also used quaternary ammonium compound (QAC) to nebulise / fog *A. pleuropneuominae* endemic farm environments with success. However, the environmental toxicity and resistance development and co-selection of resistance against other anti-infectives caused by this compound is a setback for extensive scale application. An *A.*

pleuropneumoniae phenotype resistant to antimicrobials including erythromycin, ampicillin, penicillin, tetracycline and tilmicosin has been reported (Turni, 2014).

Concerning the association between management and respiratory diseases in pig, McKenzie (2014) reported that increasing pigs from 400 to 3200 increases the risk of respiratory infections by sixty-four-fold. Specifically, APP outbreaks with high mortality and morbidity rates with persistent ill thrift occur in a production system consisting of 1000 multi-age pigs per airspace, compounded by the presence of *A. pleuropneumoniae, Mycoplasma hyopneumoniae* and porcine circovirus type 2 (PCV2) (McKenzie, 2014). So, the prevalence of the respiratory disease in swine farms is related to the respiratory health of neighbouring piggeries, farm air quality and herd density of pigs (Alawneh et al., 2018; Gardner, Willeberg, & Mousing, 2002). Since APP is a significant endemic economic disease in intensive pig farming in Australia, therefore, cost-effective prevention and control measure that is safe and does not contribute to antimicrobial resistance is urgently needed for this disease.

1.2.2.2 ECAS in the pig farm

Recently, use of slightly acidic and neutral ECAS in disinfection of the livestock premises has gained traction because of its negligible corrosiveness to the equipment, and the fact that it is non-hazardous to animals and farm workers (Bügener et al., 2014; Hao, Li, Wang, et al., 2013; Hao, Li, Zhang, et al., 2013; Majd et al., 2015; Nan et al., 2010; Ni, Cao, Zheng, Zhang, & Li, 2015; Zheng, Li, Cao, Zhang, & Yang, 2012; Zheng et al., 2014). This is in comparison to acidic ECAS, that corrodes farm equipment surfaces (Guentzel et al., 2008) and has reduced bactericidal activity due to loss of elemental Cl_2 (Cui, Shang, Shi, Xin, & Cao, 2009).

Pig and poultry farms mostly use ECAS as a solution or spray wash for sanitisation of animal houses, disinfection of farm air environment and equipment (Hao, Li, Wang, et al., 2013; Hao, Li, Zhang, et al., 2013; Zheng et al., 2014). In the dairy industry, it is used for disinfection of equipment and cattle teats infected with mastitis bacteria (Kawai et al., 2017; Nagahata et al., 2011; Nan et al., 2010). The spray disinfection of hard to reach areas and farm air environment in combination with solution washing of animal house surfaces are also a common strategy. Though uncommon, ECAS is also used in drinking water as a supplement in poultry production (Bügener et al., 2014), spray sanitisation of the poultry farm air environment to reduce

airborne bacteria, ammonia and particular matter is also documented (Majd et al., 2015; Zheng et al., 2012; Zheng et al., 2014). However, so far, there is no known report of ECAS aerosol fog disinfection of animal farm environment to reduce aerobic bacteria and respiratory pathogens in livestock industries.

Typically, the initial anti-microbial activity of the biocidal agent is assessed in vitro against microorganisms of interest before testing for its efficacy in the field situation. ECASs in vitro antimicrobial activity against pathogens of animal origin has shown it to be an effective anti-infective. *Enterococcus faecium, Mycobacterium avium, Proteus mirabilis, P. aeruginosa, S. aureus and Candida albicans* from animals were susceptible to acidic ECAS of 40 mg/ L FAC within 30 mins of exposure (Fenner, Bürge, Kayser, & Wittenbrink, 2006). The slightly acidic form of ECAS inactivated these bacteria (*Salmonella, S. aureus,* and coliforms) isolates from piggery environments at a minimum free available chlorine concentration of 80 mg/L within 4 min of exposure time. The FAC concentration differences required to inactivate bacteria between the two experiments could be attributed to the use of different media to perform the experiment that may or may not contain organic material that quenches active antimicrobial components of ECAS. The other reason for these discrepancies could be the use of a different form of ECAS anolyte (acidic and slightly acidic) containing different proportions of active chlorine contents such as HOCl, ClO^- and oxidative moieties. However, no data on the efficacy of neutral ECAS anolyte against the bacterial strain of pig origin is currently available.

Swine barns are regularly cleaned and disinfected because of pathogenic bacterial strains present on surfaces and equipment contaminated with faeces, and aerosols contaminated by bacteria from shedding animals and bedding materials. Hao et al. (2013) studied the effect of slightly acidic electrolysed water (SAEW) in decontaminating piggery farm surfaces, equipment and air, and reported a significant reduction in microbial load on the surfaces of the shed treated with SAEW flushing, statistically comparable to the efficacy of trichloro isocyanurate acid and better than povidone-iodine flushing. In spraying the barn air environment, Hao's group (2013) observed that reduction of airborne bacteria with SAEW (59%) was higher than those of trichloro isocyanurate (49%) and povidone-iodine (26%). The disinfection efficiency of all the three disinfectants, however, was not significantly different from the control water spraying. The disinfection non-significance in the above experiment

could be attributed to the interval of use of disinfectant, that is once every four days. So, experimental design with increased frequency of disinfection that exposes airborne bacteria to neutral ECAS aerosols for a sustained period, similar to McKenzie (2014) fog disinfection using QAC, could reduce total bacteria and respiratory pathogens significantly in the barn air environment.

1.2.3 ECAS in food safety applications

In food safety management, disinfectants reduce microorganisms and eliminate pathogens such as bacteria, fungi, viruses or parasites to prevent spoilage of food and occurrence of foodborne illnesses. Wide varieties of biocidal disinfectants are used to control the growth or to inactivate microorganisms on the food or on materials that come into contact with the food. New alternative disinfectants are sort after for food safety applications, as the use of chemical biocidal agents is associated with environmental pollution, occupational hazards and co- and cross-resistance to antimicrobials. ECAS represents an effective alternative eco-friendly treatment for food sanitisation since 2002 in Japan and the United States of America in 2013 (Venturini, 2013).

Meat, eggs, fish, vegetables and fruits are food industries that use ECAS to reduce or eliminate spoilage and foodborne pathogenic microorganisms. Food related bacteria susceptible to antibacterial activity of ECAS are *S.* Enteritidis and *L. monocytogenes* (Cao et al., 2009; Ni et al., 2014; Ozer & Demirci, 2006; Park, Hung, Lin, & Brackett, 2005; Venkitanarayanan, Ezeike, Hung, & Doyle, 1999; Yang, Swem, & Li, 2003), *E. coli* O157:H7 (Ni et al., 2014; Ozer & Demirci, 2006; Venkitanarayanan et al., 1999; Yang et al., 2003), *S. aureus* (Ni et al., 2014), *Salmonella* Typhimurium (Yang et al., 2003), *Listeria innocua* (Abadias et al., 2008), *Bacillus cereus* (Kim, Yen-Con Hung, & Robert E Brackett, 2000a)¸ *Campylobacter jejuni* (Ovissipour et al., 2015)*, E. faecalis* (Guentzel et al., 2008)*, P. aeruginosa* (Deza et al., 2005), *Yersinia enterocolitica* (Han, Hung, Bratcher, et al., 2018; Han, Hung, & Wang, 2018) and *Vibrio parahaemolyticus* (Ovissipour et al., 2015; Ren & Su, 2006).

1.2.3.1 ECAS in eggshell sanitisation

The predominant sector that uses ECAS for sanitisation in livestock farming is poultry. In poultry production, ECAS is used as a drinking water supplement (Bügener et al., 2014), for sanitisation of carcases (Fabrizio, Sharma, Demirci, & Cutter, 2002;

Rasschaert et al., 2013), birdhouses (Hao, Li, Wang, et al., 2013; Ni et al., 2015) and shelled egg surfaces (Achiwa & Nishio, 2003; Bialka, Demirci, Knabel, Patterson, & Puri, 2004; Ni et al., 2014; Rivera-Garcia et al., 2019). It is also used for spray sanitisation of the poultry farm air environment to reduce airborne bacteria, ammonia and particulate matter (Hao, Li, Wang, et al., 2013; Majd et al., 2015; Zheng et al., 2012; Zheng et al., 2014). Many researchers assessed ECAS in the sanitisation of table eggs for food safety application (Achiwa & Nishio, 2003; Bialka et al., 2004; Rivera-Garcia et al., 2019; Zang et al., 2019) and hatching eggs for quality production of chicks (Fasenko et al., 2009). Achiwa and Nishio (2003), Russell (2003), Bialka and coworkers (2004), Park's group (2004) and Fasenko and company (2009) used acidic ECAS for sanitisation of eggshell surfaces. Whereas, Zang and colleagues (2019) and Bing and associates (2019) employed slightly acidic form and Surdu and associates (2017) and Rivera-Garcia and group (2019) the neutral type. The majority of these researchers followed two-step processes of disinfection. In the initial washing step, dirt and debris are washed off the eggshell with water or alkaline detergent, followed by ECAS disinfection.

Some studies compared ECAS efficacy to chemical disinfectants, others between acidic and slightly acid forms and some to no-treatment controls. Achiwa and associates (2003) compared sodium hypochlorite washing with ECAS washing of egg cleaning and disinfection unit. The eggs for ECAS washing were pre-washed with alkaline ECAS before acidic ECAS (20-30 mg/L FAC) sanitisation. Overall, they concluded that use of ECAS improved sanitisation control of eggshells, egg washer and egg washing facilities and allowed for automation of sanitisation process that contributed significantly in sanitisation efficiency. In other studies, ECAS (70-80) dip washing of eggs performed better in comparison to that of chlorine-based (100 mg/L FAC) commercial sanitiser in inactivating *S.* Enteritidis and *E. coli* K12 from the eggshell surface (Bialka et al. 2004). But acidic ECAS affected the cuticle layer on the eggshell surface similar to that of chlorine-based sanitiser. Park and colleagues (2005) found the pre-washing of eggs with alkaline ECAS followed by acidic ECAS (41 mg/L FAC) anolyte for 1 min reduced *S.* Enteritidis and *L. monocytogenes* by 4.4 and 3.7 log CFU/shell egg, respectively. This wash efficiency was similar to the of chlorinated (200 mg/L FAC) water wash for 1 min in reducing *S.* Enteritidis and *L. monocytogenes* (Park et al., 2005).

ECAS also can be applied as a fine particle mist or aerosols as it contains only a trace amount of dissolved elemental chlorine that could be used without being a health hazard. Acidic ECAS (50 mg/L FAC) spray washing of hatching eggs reduced total aerobic bacteria significantly, without affecting hatchability and chick quality (Fasenko et al. 2009). Moreover, Russell (2003) reported electrostatic spraying of acidic ECAS (8 mg/L FAC) effectively sanitised hatching eggs inoculated with pathogens such as *S.* Typhimurium, *S. aureus*, *L. monocytogenes*, and *E. coli*.

Commonly used eggshell sanitisers are chlorine-based oxidising agents such as sodium hypochlorite and quaternary ammonium compounds. Selection of these chemical disinfectants is based on their potency in reducing indicator bacteria and inactivating pathogens on the eggshell surface. Moreover, these chemicals are also cheaper and readily available. Zang and associates (2019) compared disinfection efficacy of slightly acidic ECAS and acidic ECAS and sodium hypochlorite on shelled egg washing. They reported similar disinfection efficacy of all the three sanitiser solutions at a maximum of 26 mg/L FAC concentration on eggs inoculated with *S.* Enteritidis, and *E. coli.* But slightly acidic ECAS performed better than the other two sanitisers in preserving egg quality parameters such as yolk index, weight loss, albumen pH and yolk pH attributable to reduced corrosion caused on the eggshell surface. Bing and associates (2019) experimented on the simultaneous sanitisation of eggshell inoculated with *S.* Enteritidis by slightly acidic ECAS (30 mg/ L) plus ultra-violet (UV) light and found higher inactivation efficiency than standalone use of these disinfection protocols.

Only two previous reports are presented for eggshell sanitisation using neutral ECAS. In 2017, Surdu and co-authors reported eggs immersed (15 mins) in neutral ECAS containing 8 mg/L and 12 mg/L of FAC had 50% and 62% of total bacterial load reduction. The non-significant reduction of bacteria observed in the above study could be because of the use of dilute neutral ECAS solution containing less than 12 mg/L of FAC. In 2019, Rivera-Garcia and colleagues used neutral ECAS to spray wash eggs inoculated with *L. monocytogenes* and compared it with untreated (salt solution washed) and citric acid solution (2%) washed controls for disinfection efficacy and effect on cuticle integrity. In this research, neutral ECAS showed higher disinfection efficiency without affecting the cuticle integrity.

Since acidic and slightly acidic ECASs are corrosive on the equipment, neutral ECAS could be a potential alternative to chemical-based biocidal agents in shelled egg washing. Moreover, the neutral form could be used as an aerosol fog in disinfecting eggshell surfaces without posing occupational hazard risk due to the release of chlorine as it contains only a trace amount of elemental Cl_2 (Liao et al., 2007). Since neutral ECAS spray or solution wash reduced native bacteria and inactivated *L. monocytogenes* from the shelled eggs, neutral ECAS would inactive other significant egg-related foodborne pathogens such as *S.* Enteritidis, *S.* Typhimurium, *E. coli* 0157:H7, *Shigella* and other opportunistic bacteria such as *S. aureus*, *P. aeruginosa*, *Streptococcus* species (spp.) and *Klebsiella* spp.

Salmonella is a leading cause of foodborne diseases throughout the world. The proportion of salmonellosis of food origin in Australia is about 40,000, out of the annual estimate of 4.1 million cases of foodborne gastroenteritis and causes more serious disease often requiring hospitalisation (Kirk, Ford, Glass, & Hall, 2014). *Salmonella* causing foodborne illness has been increasing over time for the past 20 years, and this rate of salmonellosis is highest in Australia when compared to similar economically affluent countries (NNDSS, 2015). The estimates present that foodborne illness due to *Salmonella* spp. have caused 35% of hospitalisations and 28% of mortality cases due to foodborne illness, and the hospitalisation and death cases resulting from Salmonellosis was highest in comparison to foodborne diseases of different origin (Kirk et al., 2014). Among the salmonellosis cases of foodborne source, raw eggs and egg products have the highest frequency (Painter et al., 2013; Braden, 2006; Moffatt et al., 2016). Besides public health hazard, for instance, foodborne illnesses are estimated to have cost the Australian economy, AUD$ 125 billion per annum (Kirk et al., 2014). As egg industries in Australia are booming due to rise in per capita egg consumption to about 245 eggs, per person per year, egg production has risen to 516 million dozen eggs annually (AEL, 2019). Contamination of egg by *Salmonella* spp., however, remains a key challenge despite the implementation of various decontamination procedures. Eggs can harbour *Salmonella* on the outer surface of the eggshell and internally in the egg yolk. Transmission of *Salmonella* on eggshell occurs either through *Salmonella*-positive hens during the formation of the egg (transovarian route), the oviductal route and through contaminated external environmental sources like faeces and soil. Many serotypes,

such as Infantis, Virchow, Newport, Hadar, Stanley, Bredney, Livingstone and Deby are recovered from eggs (Martelli and Davies, 2012). Though serovars such as *S.* Enteritidis, *S.* Typhimurium and *S.* Infantis are a concern for the egg and poultry industry, the first two serovars are also a significant concern for public health. *S.* Enteritidis is one of the most frequently isolated serotypes associated with eggs and egg products worldwide (Hendriksen et al., 2011). Therefore, to address the *Salmonella* food poisoning issue, an array of methods for sanitisation of eggs to remove pathogenic bacteria are employed.

Protocols employed for reduction or elimination of *Salmonella* cells can broadly be classified into thermal and non-thermal disinfection. Thermal disinfection, such as egg pasteurisation, is a highly effective method, but negatively affects egg proteins and rheological properties (Rodriguez Romo, 2004). The most common non-thermal sanitisation for eggshell disinfection is the use of alkaline detergent wash solution at a temperature above 40 °C, with high pH (11.0) for pre-washing of dirt and faecal materials followed by sanitisation using chlorine-based compounds at the concentration between 50 to 200 mg/L (USDA, 2001). Chlorine-based oxidising sanitisers are used because of easy availability, relatively low cost and high efficacy in adequately removing bacteria during washing. But the accumulation of organic load from dirt, manure and broken eggs reduces pH and chlorine concentrations which affect the effectiveness of the sanitiser solution and make it a potential source of contamination. Moreover, because of the environmental impacts caused by chlorine by-products and problems with wastewater disposal, its use is not without issues such as bacterial resistance development to its persistence usage (Ridgway & Olson, 1982). So, egg industries are continuously exploring for alternative egg wash protocol to safely and effectively decontaminate *Salmonella* and other pathogens. Neutral ECAS spray and fog washing of eggs would disinfect shelled eggs of native bacteria and pathogens.

1.2.3.2 The effect of the ECAS use on the cuticle integrity of eggshell

Avian eggshell can be distinguished into six layers morphologically (Nys, Hincke, Arias, Garcia-Ruiz, & Solomon, 1999; Solomon, 1991). The layers consist of innermost uncalcified membrane layers (inner and outer shell membranes of 22 and 48 µm thickness, respectively), inner calcified mammillary knob layer of 100-110 µm, fourth palisade layer of ~300 µm, thin vertical crystal layer of 3-8 µm and the outmost

cuticle layer (0.5-12.8 µm) (Cain & Heyn, 1964; Gilbert, 1971; Parsons, 1982; Simons, 1971). Eggshell layers are formed in the acellular environment of the uterine fluid sequentially from the innermost membrane layer to the outermost crystal layer (Nys et al., 1999; Nys, Zawadzki, Gautron, & Mills, 1991). The cuticle is *in utero* deposition on the egg surface consisting of an inner thin zone of hydroxyapatite crystals and outer superficial non-calcified water-insoluble organic pigments (Dennis et al., 1996; Nys, Gautron, Garcia-Ruiz, & Hincke, 2004; Nys et al., 1991; Parsons, 1982). Cuticle is synthesised in the non-ciliated secretory cells of the uterus (Solomon, 1991) 1.5 to 2.0 hours prior to oviposition (Hincke et al., 2000) and it covers the crystal layer by bridging the outer pore openings or by extending down into the pore canals (Board, 1982; Cooke & Balch, 1970; Williams, Dillard, & Hall, 1968).

The eggshell cuticle is mainly composed of 90% glycoprotein, 4% polysaccharide, 3 % lipids and inorganic phosphorus (Becking, 1975; Dennis et al., 1996; Mikšík, Charvátová, Eckhardt, & Deyl, 2003; Wang & Slavik, 1998; Wedral, Vadehra, & Baker, 1974). Of about 850-870 types of proteins, the majority is water-insoluble (Wedral et al., 1974) and Du (2013) reported cuticle protein has a high content of amino acids such as cysteine, glycine, glutamic acid, lysine and tyrosine. The amino acid sequence of the cuticle is different from that of the shell membrane and shell matrix (Baker & Balch, 1962). Additionally, the cuticle also contains traces of magnesium, potassium, copper, zinc, calcium, phosphorus, carbon and oxygen (Board & Love, 1980; Kusuda, Iwasawa, Doi, Ohya, & Yoshizaki, 2011; Wedral et al., 1974)

The cuticle is the first protective protein layer on the eggshell, and cuticle thickness in hens are moderately inheritable (Dunn et al., 2019). Other factors, such as hen housing and hen age, are parameters that affect the cuticle properties. In terms of hen housing, eggs from caged flocks have better cuticle coverage than these from free-range flocks (Samiullah et al., 2013). Spark and Board (1984), Messens and co-workers (2005), and Leleu and associates (2011) reported an inverse co-relationship between the cuticle coverage and hen age, whereas some researchers reported non-significant effects of hen age on cuticle coverage (Ball, Logan, & Hill, 1975; Roberts & Chousalkar, 2013). On the quantitative measurement of cuticle composition, eggs from older hens were reported to have significantly depleted polysaccharides and lipid

components of the cuticle (Rodríguez-Navarro, Domínguez-Gasca, Muñoz, & Ortega-Huertas, 2013) which could be attributed to the physiological status of the hen.

De Reu and co-authors (2006) found cuticle coverage to be the most crucial eggshell structure that prevents bacterial trans-shell penetration. Polysaccharides and lipids are antibacterial proteins and hydrophobic structure of cuticle that prevent bacteria penetration and colonisation (Board & Halls, 1973; D'Alba, Jones, Badawy, Eliason, & Shawkey, 2014; De Reu et al., 2006; Rose-Martel, Du, & Hincke, 2012; Wellman-Labadie, Picman, & Hincke, 2008). But in newly laid eggs, immature and moist cuticle make eggs easily susceptible to bacterial penetration (Miyamoto et al., 1998; Sparks & Board, 1984) which is further aggravated by positive temperature difference that facilitates easy bacterial penetration (Bruce & Drysdale, 1994). Although mature cuticle is effective in preventing bacteria penetration, as it is deposited during egg-laying, about 3.5% percentage of eggs were found without cuticle and 8% of eggs without cuticle on the apex or blunt end of the egg (Board & Halls, 1973).

Salmonellosis of egg origin is a significant contributor to food poisoning (OzFoodNet, 2007). The rate of *Salmonella* penetration in eggs increases significantly with the age of laying hens (12.9% to 25.0 %) (Bruce & Drysdale, 1994; Nascimento, Cranstoun, & Solomon, 1992). The increase in *Salmonella* penetration could be because of reduction of cuticle coverage on eggs from old hens (Nascimento et al., 1992) and significantly depleted polysaccharides and lipid components of the cuticle (Rodríguez-Navarro et al., 2013) necessary for resistance to bacterial penetration and colonisation. Moreover, abrasions on the eggshell are also known to increase eggshell penetration by bacteria (Board, Loseby, & Miles, 1979) and specifically *Salmonella* spp. (Gole et al., 2014).

Preventive measures such as the production of eggs from *Salmonella* free flocks, wash and sanitisation of shelled eggs and coating of eggs with mineral oil are employed for safe and hygienic production of eggs to decrease the incidence of food poisoning. Many countries, including Australia, sanitize shell eggs using chemical sanitisers to reduce eggshell contamination (Hutchinson et al., 2004). Chemical sanitisers employed are QACs, sodium carbonate and sodium hypochlorite (Al-Ajeeli, 2013). Some egg washing chemicals cause cuticle layer damage (Wang & Slavik, 1998) that facilitate the trans-shell transmission of bacteria, including *Salmonella*

(Gole et al., 2014). The effect on the cuticle varies with the type of sanitiser (Wang and Slavik 1998), and corrosion of cuticle leads to higher bacterial penetration (Gola et al., 2014). Moreover, QAC wash leaves residues on the eggshell surface (Wang and Slavik, 1998) and can cause co-selection of antibiotic resistance (Fernandez Marquez, Burgos, Pulido, Gálvez, & López, 2017). NaOCl washing also is known to leave residual chlorine that adversely affects consumer acceptability (Wang & Slavik, 1998). An alternative to these chemicals that do not produce environmental waste and residues, that do not affect eggshell cuticle and do not select genes for antibiotic resistance are currently being sorted for application in table egg and hatching egg production.

Many researchers also assessed the effect of ECASs on the cuticle of the eggshell. Bialka and associates (2004) found acidic electrolysed water as dip solution had a significant impact to the cuticle layer when compared to that of untreated eggs and the effect observed above was reported to be similar to that of chlorine-based sanitiser. Zang and colleagues (2019) observed slightly acidic ECAS washing performed better in preserving egg quality parameters such as yolk index, weight loss, albumen pH and yolk pH in comparison to the acidic ECAS and sodium hypochlorite washes. These findings are attributable to reduced corrosion by slightly acidic ECAS on the eggshell surface, particularly the cuticle. In the case of neutral ECAS, Rivera-Garcia et al. (2019) found that neutral ECAS spray washing to have not affected the cuticle integrity. Hence, the sanitization of shell eggs with a higher concentration of neutral ECAS fog would not significantly corrode the cuticle layer.

1.2.3.3 ECAS for washing fresh leafy-green vegetable - Spinach leaves

The increase in consumption of raw agriculture produce such as minimally processed vegetables is driven by changes in human lifestyle of eating vegetables that require reduced cooking time, understanding the benefits of nutritional contents and public health sectors campaigns promoting plants as an extremely healthy food. The increase in consumption of ready-to-eat (RTE) vegetables increases the risk of illnesses arising from foodborne pathogens. Worldwide each year 600 million cases of foodborne illnesses and 420, 000 deaths are caused by 31 foodborne agents, including pathogens (WHO, 2015). Centre for Disease Control and Prevention reported that half of the foodborne illnesses out of 48 million in the United States occurred through consumption of fruits, vegetables and nuts (CDC, 2016). European Food Safety

Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) reported an increase in nonanimal origin foodborne outbreaks in Europe from 5.2 % in 2006 to 20% in 2010 with a slight reduction to 13.1% in 2016 (ECDC, 2007, 2012, 2017). In Australia, fresh produce identified as a source of foodborne diseases accounted for 8 % of cases in 2006 (OzFoodNet, 2007) which increased to 9% in 2012 (OzFoodNet, 2018).

A meta-analysis of literature identified RTE vegetables (lettuce, spinach, cilantro, watercress) causing the highest number of outbreaks (Machado-Moreira, Richards, Brennan, Abram, & Burgess, 2019). Food-borne illnesses associated with fresh vegetables are due to the introduction of various bacterial pathogens through exposure to contamination sources at production (cultivation and harvest), during processing (sorting and washing), at distribution chains, at point of sale in the market and in kitchens. Moreover, minimally processed vegetables (MPV) requires cutting and slicing for packaging and become more susceptible to bacterial and parasitic contamination as they release nutrients that facilitate growth (Harris et al., 2003).

Sources of contamination of leafy-green vegetables identified at the farm level are irrigation water (Taban & Halkman, 2011), contaminated manures (Alegbeleye, Singleton, & Sant'Ana, 2018) and contaminated soil (Bernstein, Sela, & Neder-Lavon, 2007). Microorganisms such as bacteria, virus, and parasites have caused RTE produce–related disease outbreaks. Surveys on MPV microbial loads has recorded the presence of bacteria such as *Salmonella* spp., *E. coli*, *Listeria* spp. and spoilage bacteria, besides yeasts and moulds (Abadias et al., 2008; Ilic, Odomeru, & Lejeune, 2008; Mritunjay & Kumar, 2017; Tournas, 2005; Valentin-Bon, Jacobson, Monday, & Feng, 2008).

Prevalence studies of bacterial contamination of leafy vegetables reported higher aerobic plate counts (APC) from spinach leaves and the mean counts observed was > 5.0 log CFU/g (Mritunjay & Kumar, 2017; Tango, Choi, Chung, & Oh, 2014; Valentin-Bon et al., 2008). APC is an indicator of overall microbial quality of the food rather than food safety (Oliveira et al., 2010), whereas coliform counts measure the bacterial contamination of faecal origin. Mritunjay and Kumar (2017) observed higher count of coliforms in spinach among the vegetables with mean counts higher than log 5.0 CFU/g, Valentin-Bon's group (2008) reported wide-ranging mean counts (<47 to >4.0 log MPN/g) with Tango and group (2014) recoding lower counts (mean count <

2.4 log CFU/g). The presence of *E. coli* (Ilic et al., 2008; Mritunjay & Kumar, 2017; Valentin-Bon et al., 2008), *E. coli* O157:H7 (Mritunjay & Kumar, 2017), *Salmonella* spp. (Ilic et al., 2008; Mritunjay & Kumar, 2017) *Listeria* spp. (Mritunjay & Kumar, 2017; Tango et al., 2014), including *L. monocytogenes* (Ilic et al., 2008; Mritunjay & Kumar, 2017) has been confirmed in spinach leaves. The high counts of indicator bacteria and the presence of pathogens in spinach leaves can be attributed partly to its physiology, i.e., rough and hydrophobic leaf surface favouring bacterial adhesion (Zhang, Oh, Cisneros-Zevallos, & Akbulut, 2013), making spinach leaves the favourable vehicle for foodborne pathogens. So, WHO (2008) exclusively listed spinach as a potential foodborne hazard. Therefore, to ensure food safety, product quality and to prolong the shelf life of spinach leaves, sanitising treatments are used for the reduction of general bacteria and pathogens.

The microbiological safety of RTE fruits and vegetables relies on chemical disinfectant disinfection. Chlorine-based disinfectant, NaOCl remains the most widely used wash solution in the vegetable production industries because of its ease of availability, low cost and ease of application (Goodburn & Wallace, 2013; Premier, 2013). The issue with this wash solution is its disinfection efficacy where bacterial log reductions are reported below 2 logs (Delaquis, Stewart, Toivonen, & Moyls, 1999; Lang, Harris, & Beuchat, 2004; Zhang & Farber, 1996) and formation of carcinogenic by-products such as chloroforms, tri-halomethanes and chloramines. Other popular sanitisers include broad-spectrum bactericidal PAA based sanitizer that contains PAA and H_2O_2 which breaks down into natural compounds such as acetic acid, water and oxygen. PAA has exceptional disinfection efficacy at low temperature, and it is used at a concentration in between 50 to 150 mg/ L. Some drawbacks of its usage are higher cost, inability to accurately monitor PAA concentrations and ORP, as well as affecting the shelf life of fresh produce when used at very high concentrations by damaging the leaf tips.

Other alternative options explored for sanitisation of RTE vegetables used organic acid formulations, gamma irradiation, stabilised chlorine dioxide, the combination of sanitisers and process steps known as hurdle technology, biological control agents such as bacteriocins and bacteriophages and ECAS water. Use of organic acids in sanitisation is limited because of its kill efficacy (Allende, Selma, López-Gálvez, Villaescusa, & Gil, 2008; López-Gálvez, Allende, Selma, & Gil, 2009),

high cost and inability to measure its active components onsite. Gamma irradiation has excellent inactivation efficacy against many foodborne pathogens and adoption of the technique is affected by negative consumer perception (Hsu, Simonne, Jitareerat, & Marshall Jr, 2010; Junqueira-Gonçalves et al., 2011), because of the incomplete understanding of health issue associated with consumption of irradiated foods (Niemira & Fan, 2006). Chlorine dioxide though effective, required longer treatment times (Han, Linton, Nielsen, & Nelson, 2001), making it impractical for industry application. The issue in applying hurdle technology sanitisations is its efficacy plus the exorbitant cost of investment. Biological control agents inactivate only a particular strain of bacteria, whereas vegetable sanitisation requires broad-spectrum antimicrobial agents. Moreover, some bacteriocins showed health hazards in the mouse model (Daniel et al., 2006). So, use of bacteriophages and bacteriocins in commercial level disinfection is currently not feasible.

Exhaustive review on the efficacy ECAS on fruits and vegetables (Abadias, Usall, Anguera, Solsona, & Viñas, 2008; Huang et al., 2008; Shiroodi & Ovissipour, 2018; Turantaş, Ersus-Bilek, Sömek, & Kuşçu, 2018) suggest electrolysed water to be a valuable alternative biocidal agent. Acidic, near neutral, neutral form and hurdle enhancement of ECAS were assessed for sanitisation of total aerobic bacteria and pathogenic bacteria on spinach leaves. Moreover, their effect on vitamin C, nitrite levels, and sensory (texture and colour) attributes in comparison to common wash sanitiser like sodium hypochlorite and PAA were also analysed.

Total aerobic bacteria reduction on spinach leaves washed with neutral ECAS observed by Izumi (1999) varied with the dosing of free available chlorine concentration. For instance, 50 mg/L FAC neutral ECAS completely inactivated total bacteria on the leaf surface, whereas log 2.3 CFU/g reduction was reported for ECAS at 20 mg/L. The 50 mg/L FAC concentration of neutral ECAS had similar efficacy to that of NaOCl concentration of about 100-150 mg/L. Lin and colleagues (2005) recorded 1.7 log CFU/g of bacterial load reduction on the spinach leaves when washed with acid ECAS at 50 mg/L FAC. Total bacterial reduction observed by Rahman's group (2010) was similar (log 1.9 CFU/g) for both the acidic and slightly acidic forms at 50 mg/L and 5 mg/L of FAC, respectively. Total bacterial load reduction observed by Gomez-Lopez and associates (2013) by slightly ECAS at 2 mg/L FAC was about 1 log CFU/g. Hao and group (2015) reported slightly acidic ECAS (20 mg/L FAC) to

have significantly higher efficacy than the acidic form (80 mg/L FAC) in reducing total bacterial load in spinach leaves. Rahman and colleagues (2010) did not observe differences in total bacteria reductions between slightly acidic (5 mg/L FAC) and acidic ECAS (100 mg/L FAC) treatments. The above findings show that the slightly acid form is much more potent in reducing aerobic bacterial load than the acidic analogue.

Importantly, the efficacy of ECAS also has been evaluated in disinfecting surrogate foodborne pathogens. Park et al. (2008) assessed the efficacy of acidic ECAS (FAC ~ 40 mg/L) disinfection of spinach leaves inoculated (spiked) with *E. coli* O157:H7, *S.* Typhimurium and *L. monocytogenes* and observed linear association of efficacy with exposure time. The susceptibility to acidic ECAS varied with the bacterial species and significant log reduction (> 3.0 log CFU/g) of *E. coli* O157:H7 and *L. monocytogenes* within 1 min of treatment and 30 s for *S.* Typhimurium was reported (Park et al. 2008). Rahman and group (2010) reported 2.4 and 2.8 log CFU/g reduction of *E. coli* O157:H7 and *L. monocytogenes* when treated with slightly acidic ECAS (5 mg/L FAC) and corresponding similar reduction with acidic ECAS (100 mg/L FAC) wash. Similar to earlier findings for total bacterial load reduction, a slightly acidic form is more efficacious than the acidic form for the reduction of surrogate pathogens. The differential efficacy between slightly acid and acid form is because of the presence of higher HOCl concentration in slightly acidic ECAS (Cao et al., 2009). Moreover, in the acidic form, bactericidal efficacy is further reduced due to off-gassing of Cl_2 (Len, Hung, Erickson, & Kim, 2000). Because of higher content of HOCl in neutral ECAS, Guentzel and acquaintances (2008) reported higher (4.0–5.0 $\log_{10}$) CFU/mL reduction of spinach inoculated with *E. coli, S. typhimurium, S. aureus, L. monocytogenes,* and *E. faecalis* at 100 and 200 mg/L FAC, comparing to 2.1 – 4.8 $\log_{10}$ CFU/mL for 20 and 50 mg/ L FAC concentrations.

In the case of ECAS disinfection of different vegetables, Izumi (1999) reported greater disinfection efficacy of neutral ECAS (15, 30, and 50 mg/L of FAC) on spinach leaves than in non-leafy cut vegetables like bell peppers, carrots, radish, and potatoes. Similarly, Lin and associates (2005) reported 1.7 log CFU/g reduction of aerobic bacterial load, higher than the reduction observed in leafy cabbage and chinjon, with acidic ECAS (FAC of 50 mg/L). Guentzel's group (2008) also observed superior efficacy of neutral ECAS (at 100 and 120 mg/L FAC) in inactivating *E. coli, S.*

Typhimurium, *S. aureus, L. monocytogenes* and *E. faecalis* in spinach leaves than on lettuce. Additionally, Park et al. (2008) recorded that a short exposure time (within 1 min) was required to significantly reduce pathogens on spinach leaves in comparison to the lettuce leaves (within 3 min) with acidic ECAS at 40 mg/L FAC. The higher efficacy of ECAS anolyte on spinach in contrast to other vegetables might be because of structural characteristics such as surface area and micro-structure of leaf tissue (Izumi, 1999).

Many researchers compared the differential efficacy of ECAS, NaOCl and PPA washing of spinach leaves, and also assessed its differential effect on sensory attributes and nutritional qualities. The effectiveness recorded by Rahman and co-workers (2010) for total bacteria and pathogen reduction by acidic and slightly acidic types was similar to that of NaOCl at 100 mg/L FAC. Izumi (1999) observed the bactericidal efficacy of neutral ECAS at 50 mg/L of FAC on total bacterial load to be similar to 100-150 mg/L of NaOCl wash on spinach leaves. Gomez-Lopez (2013) highlighted slightly acidic ECAS (2 mg/L) had a lower efficacy on the reduction of total bacteria in comparison to that of peroxyacetic acid (80 mg/L FAC) and similar effectiveness to that of NaOCl at 4 mg/L of FAC. Premier (2013) observed ECAS (unspecified concentration and type) have a higher bactericidal effect on total load in comparison to 100 mg/L PAA and 100 mg/L chlorine-based solution. The majority of these findings show that lower FAC concentration of ECASanolyte is required to achieve a similar level of bacterial inactivation by NaOCl and PAA sanitizers.

Park and associates (2008) observation of no significant effect of acidic ECAS treatment on taste, colour and appearance of the spinach agreed with earlier findings by Izumi (1999) on sensory attributes. Gomez-Lopez (2013) compared the effect of ECAS with PAA and NaOCl and reported no significant changes in the sensory quality of the baby spinach for all the treatments. In terms of nutritional quality, vitamin C content after washing was lower for PAA washed relative to high levels observed in ECAS and NaOCl treatments (Gomez-Lopez et al. 2013). Chlorine-based compounds are usually used for their enhancement of the shelf life of fresh produce, but Premier (2013) reported 15 days of shelf life for ECAS to 12 days of shelf life achieved by that of chlorine-based chemical. Izumi and Kiba (1999) deduced from their experiment that the residual antimicrobial effect of neutral ECAS during storage to be higher than NaOCl washing, hence prolonging the shelf life of the spinach leaves. Besides, not

affecting organoleptic attributes, ECAS also inhibits nitrate reductase activity through reduction of bacterial load and nitrate reductase (Hao et al., 2015). Gomez-Lopez and co-authors (2013) documented ECAS not producing dangerous levels of trihalomethanes (THM) in process wash water, unlike that of NaOCl wash. However, the THM level on the baby spinach leaves for both the treatments was well below the prescribed acceptable level.

The significant factor to be considered in sanitiser use is the induction of a viable but nonculturable (VBNC) bacterial state among foodborne pathogens. In the VBNC state, bacteria retain their viability and virulence and can be resuscitated under favourable growth conditions. The VBNC bacteria are often observed with use of a suboptimal concentration of the active bactericidal component present in disinfectants (Gu et al., 2020; Ogunniyi et al., 2019; Teixeira, Fernandes, Silva, Dias, & Azeredo, 2020; Zhang, Chen, Xia, Li, & Hung, 2018). Among the commonly used disinfectants, use of NaOCl and PAA in washing leafy vegetables like lettuce at concentration below 50 mg/ L were demonstrated to induce the VBNC state to foodborne pathogens such as *E. coli* (Gu et al., 2020; Teixeira et al., 2020), *S. enterica* and *L. monocytogenes* (Gu et al., 2020). More importantly, Gu et al. (2020) showed that PAA treatment of spinach induced VBNC in all the above three surrogate foodborne pathogens.

ECAS is also known to induce VBNC in foodborne pathogens such as *E. coli* O157:H7, *L. monocytogenes¸ Y. enterocolitica and S.* Enteritidis (Afari, Liu, & Hung, 2019; Han et al., 2018; Zhang et al., 2018). The VBNC formation in ECAS treated bacterial cells depended on multitude of factors such as bacterial species (Afari et al., 2019; Han et al., 2018), ECAS FAC concentration (Afari et al., 2019; Ogunniyi et al., 2019; Zhang et al., 2018), type (Zhang et al., 2018) and organic load (Afari et al., 2019; Ogunniyi et al., 2019). Since ECAS has higher disinfection efficacy than sodium hypochlorite, acidic ECAS at 30 mg/ L (FAC) and slightly acidic ECAS at 40 mg/ L FAC induced VBNC in comparison to NaOCl inducing it at 60 mg/ L of FAC in an *E. coli* suspension (Zhang et al. 2018). Higher inactivation efficacy of ECAS is attributable to acidic pH weakening the bacterial cell membrane integrity that allows for easy penetration of antimicrobial moieties such as HOCl (Park et al., 2004). Neutral ECAS at 20 mg/ L did not induce VBNC in *E. coli* cells with a similar observation made for 50 mg/L of NaOCl treatment (Ogunniyi et al., 2019). Another neutral ECAS VBNC study (Han et al. 2018) on representative foodborne pathogens showed VBNC

induction in *E. coli* O157:H7¸ *Y. enterocolitica and S.* Enteritidis at 18.5 mg/ L of FAC, whereas when FAC was increased to 37 mg/ L no induction of VBNC for these organisms was recorded.

In summary, neutral ECAS 60s wash of spinach leaves at concentrations of 50 and 85 mg/L of FAC will achieve a significant reduction of general bacteria load, *E. coli*, *S.* Enteritidis and *L. innocua,* without affecting the nutritional and other quality parameters. Moreover, neutral ECAS, when used above 50 mg/ L FAC, would not induce VBNC in the bacteria isolates. The generation of comprehensive data on neutral ECAS sanitization would offer alternative fresh produce sanitizer choices to the industries and farmers.

1.2.3.4 Effect of ECAS on microbiome composition of spinach leaves

The world's top three producers of spinach are China, United States and Japan, accounting for 90.6 %, 1.4 % and 1.1 % of production respectively (Noel J Riggs & Scott, 2019). Australia produced about 37, 000 tonnes in 2018 recoding a 20% rise from the previous year (Calughton, 2019) and spinach is grown mainly in three regions of Victoria (Greater Melbourne, Latrobe Gippsland and North West). Spinach (*Spinacia oleracea*) is produced in cold climatic conditions (10 to 20 °C) in a wide variety of soil types requiring nitrogen fertilisation and minimal irrigation, and the mature plant withstands subfreezing temperatures. Spinach leaves are harvested when they have about 5 to 8 fully developed leaves which usually takes about one to three months depending on temperature and season. Spinach leaves harbour microbial populations acquired throughout the farming process. The microbial community on spinach plant surface (phyllosphere) are predominately bacteria adapted to extreme conditions such as exposure to ultraviolet radiation, variable temperature, inadequate nutrients and moisture availability (Beattie & Lindow, 1999; Ercolani, 1991; Jacobs, Carroll, & Sundin, 2005; Lindow & Brandl, 2003). Harvest and processing techniques cause plants to release nutrient-rich extracellular polysaccharides that propagate faster bacterial growth and colonization (European Commission Health and Consumer Protection, 2002).

Conventional culture-based techniques or species-specific polymerase chain reaction (PCR) is used for assessment of bacteria population on the RTE spinach leaves and to detect known public health risk pathogens. The aerobic bacterial load and coliform counts indicate the quality and safety of spinach leaves. Earlier, clone

library molecular techniques such as denaturing gradient gel electrophoresis (DGEE) and terminal restriction length polymorphism (T-RFLP) analysis of bacterial 16S ribosomal RNA (rRNA) genes were employed to understand the phyllosphere bacterial community on spinach leaves (Handschur, Pinar, Gallist, Lubitz, & Haslberger, 2005; Jackson, Randolph, Osborn, & Tyler, 2013; Rudi, Flateland, Hanssen, Bengtsson, & Nissen, 2002). Contemporary next-generation sequencing (NGS) techniques are now widely used for comprehensive analysis of bacterial community composition because of increase in depth of sequence reads and, improved, easier to use bioinformatics analysis pipelines (Lopez-Velasco, 2010; Truchado, Gil, Suslow, & Allende, 2018). This high throughput sequencing and analysis method, besides community structure, provided insights into the association of bacterial phyllosphere diversity to the environmental factors (Leff & Fierer, 2013; Truchado et al., 2018), use of biocidal agents (Leff & Fierer, 2013; Truchado et al., 2018) and pesticides (Gu et al., 2010; Leff & Fierer, 2013). It also provides insights into the dynamics of interaction of the bacterial community composition and RTE leafy vegetables phyllosphere community of the plant in the field, post-harvest, during processing and storage (Gu et al., 2018; Lopez-Velasco, Welbaum, Boyer, Mane, & Ponder, 2011; Soderqvist et al., 2017; Truchado et al., 2018). Phyllosphere bacterial population structure has been observed to change in packaged RTE spinach after treating with sanitiser and during refrigerated storage (Gu et al., 2018; Lopez-Velasco et al., 2011; Soderqvist et al., 2017).

Bacteria are the dominant microbial inhabitants of the phyllosphere of leafy vegetables with bacteria counts observed between < 4.0 to 8.3 $\log_{10}$ CFU/g for bagged RTE spinach leaves (Valentin-Bon et al., 2008). Bacteria species, represented as operational taxonomic units (OTUs), varying between 20 to 2915 OTUs have been reported in spinach leaves (Gu et al., 2020; Jackson et al., 2013; Leff & Fierer, 2013; Lopez-Velasco et al., 2011; Soderqvist et al., 2017; Tatsika, Karamanoli, Karayanni, & Genitsaris, 2019). Phyla ranges between 4 to 14 have been observed in bacteria microbiome profiles of spinach leaves (Gu et al., 2018; Soderqvist et al., 2017) with the vast majority represented by Proteobacteria (Gu et al., 2020; Leff & Fierer, 2013; Lopez-Velasco et al., 2011). The other predominant phyla reported are Bacteroidetes, Firmicutes and Actinobacteria (Gu et al., 2020; Gu et al., 2018; Leff & Fierer, 2013; Lopez-Velasco, Carder, Welbaum, & Ponder, 2013; Soderqvist et al., 2017; Tatsika et

al., 2019; Truchado et al., 2018). At order-level Bacilli, Gammaproteobacteria, Actinobacteria, Alphaproteobacteria, and Betaproteobacteria were the predominant orders reported in spinach leaves (Truchado et al., 2018).

Bacterial families *Enterobacteriales* and *Pseudomonadaceae* are common isolates on spinach leaves observed by both the culture-based and culture-independent techniques (Babic, Roy, Watada, & Wergin, 1996; Lopez-Velasco et al., 2011; Tatsika et al., 2019; Truchado et al., 2018). Pyrosequencing approaches in spinach leaves also recorded *Burkholderiaceae* (Tatsika et al., 2019), *Bacillaceae* (Trichado et al., 2018) and *Spingomonadaceae* (Lopez-Velasco et al., 2011; Gu et al., 2018) families. Family *Enterobacteriales* are mostly represented by genera *Erwinia, Pantoea, Serratia* and *Enterobacter* (Leff & Fierer, 2013; Tatsika et al., 2019). *Pseudomonadaceae* by genera *Pseudomonas* and *Acinetobacter* (Gu et al., 2018), and *Burkholderiaceae* by genus *Janthinobacterium* (Tatsika et al., 2019; Gu et al., 2020). The genus *Massilia* belonging to family *Oxalobacteraceae* (Lopez-Velasco et al., 2013; Truchado et al., 2018) and *Phyllobacterium* of *Phyllobacteriaceae* (Darlison et al., 2019) was also reported. At species level Darlison et al., (2019) found *Curtobacterium plantarum* to be the most prevalent bacteria followed by *Pantoea agglomerans, Pantoea ananatis, Lelliottia amnigena, Pseudomonas koreensis, Pantoea brassicacearum* and *Pseudomonas beatica. Pantoea* species are typically plant commensals, but human and plant pathogens of this species exist (Delétoile et al., 2009; Rezzonico, Smits, Montesinos, Frey, & Duffy, 2009). For example, *Pantoea agglomerans* is a plant pathogen that causes human disease (Cruz, Cazacu, & Allen, 2007). *Pseudomonas* species are ubiquitous bacteria present in food and production premises that compete with other bacteria for nutrition (Hibbing, Fuqua, Parsek, & Peterson, 2010). Some *Pseudomonas spp.* were also recognised as potential plant pathogens and others involved in spoilage of leafy vegetables causing soft rot due to production of pectinolytic enzymes (Adams & Moss, 2005). *Erwinia, Janthinobacterium* and *Massilia* species are widely recognised plant pathogens. Taksika and group (2019) recorded a substantially higher number of relative abundances of OTUs of Rhizobiales and Cytophagales in spinach leaves than in rocket salads. OTUs of phyla Planctomycetes, Verrucomicrobia and TM7 previously not associated with plant phyllosphere were also reported by Lopez-Velasco and associates (2011) and, genera of bacteria belonging to Planctomycetes (Kulichevskaya et al., 2007) and TM7

(Hugenholtz, Tyson, Webb, Wagner, & Blackall, 2001) are adapted to grow under harsh conditions like exposure to UV light.

Lopez-Velasco and colleagues (2011) observed the reduction of richness, diversity and evenness of microbiota of spinach leaves stored at refrigerated conditions after minimum processing. These reductions were caused by a decrease in abundance of OTU composition of all phyla and an increase in the relative abundance of Gammaproteobacteria. Tatsika's group (2019) observed a similar reduction in richness and diversity indices in microbiome composition of rocket salad. Lopez-Velasco and co-authors (2011) recorded a decrease in phyla composition to 5 from 11 after overnight storage at 4 °C. Order Pseudomonadales mostly represented by a known psychrotroph species *Pseudomonas* spp. were the dominant bacterial populations in RTE spinach stored at refrigeration temperature (Lopez-Velasco, Davis, Boyer, Williams, & Ponder, 2010; Rudi et al., 2002; Soderqvist et al., 2017). All these studies also reported that *Pseudomonas* species and *Enterobacteriales* to be the predominant bacterial components stored at refrigeration temperatures. Lopez-Velasco and associates (2011) recorded a pronounced reduction of families *Oxalobacteraceae, Rhizobiales, Acinetobacter* and *Sphingomonadaceae,* and increase in genera *Pseudomonas* spp., *Stenotrophomonas* spp., *Pantoea* spp. and *Escherichia* spp., 15 days post storage at a higher temperature (10 °C). A most recent study on the effect of storage temperature on spinach microbiota composition by Gu and colleagues (2018) conducted using pyrosequencing and 16s rDNA qPCR techniques observed marked difference in composition at day 7 in comparison to that of day 0. The compositional change found here was influenced by a significant decrease in relative abundance (RA) of phylum Proteobacteria and increase of Bacteroidetes (Gu et al., 2018). At genera and species levels, *Pseudomonas* species were still dominant. Other major components included *Flavobacterium succinicans*, *Shewanella* spp., *Spingobacterium faecum* and *Chryseobacterium* spp. were reported to have the highest RA. *Pseudomonas* spp., *Erwinia* spp., *Pseudomonas viridiflava, Paenibacillus* spp., *Spingomonas* spp., *Pedobacter* spp. and family *Oxalobacteraceae* spp. were observed to have lowest RA as per amplicon sequence analysis. When 16s rDNA qPCR was used for quantification of the bacterial composition, unlike the pyrosequencing approach it did not observe the proliferation of *Pseudomonas* spp. *and Chryseobacterium* spp. except for *Flavobacterium* spp., *Pedobacter* spp.,

Flavobacterium spp., *Wautersiella* spp. and family *Bacillaceae.* 16S rDNA copies showed the lowest proliferation for *Cupriavidus* spp., *Ralstonia* spp., family *Comamonadaceae* and *Acinitobacter Iwoffii.*

The fresh produce bacterial community interacts by competing for limited available nutrients and antagonising some strains (e.g. foodborne pathogens) by producing growth inhibitor (Babic, Watada, & Buta, 1997; Schuenzel & Harrison, 2002). On the other hand, some bacterial species facilitate pathogenic organism growth through the metabolism of different sources of carbon (Lopez-Velasco, 2010). Soderqvist et al. (2017) studied the effect of *L. monocytogenes, Y. enterocolitica* and *E. coli* O157:H7 inoculated RTE salads on the native microbiota composition and diversity and observed an increase in RA of order Lactobacillales with increased counts of all inoculated strains. The increase in Enterobacteriales and Bacillales abundance also correlated positively to increased *L. monocytogenes* and *Y. enterocolitica* viable counts. By contrast, orders Rhizobiales, Burkholderiales, and Flavobacteriales were negatively correlated to all viable counts. Only the counts of *E. coli* were negatively correlated to Pseudomonadales RA increase. In case of spinach microbiota, some proportion of spinach leaf bacterial isolates, 99 % represented by Gram-negative bacilli, are known to have inhibitory activity against *E. coli* O157:H7 (Johnston et al., 2009). The most common isolates in this study belonged to the genera *Pantoea, Pseudomonas, Klebsiella, Enterobacter, Aeromonas, Burkholderia, Serratia* and *Kluyvera*. Furthermore, Lopez-Velasco et al. (2012) characterized most of these genera to species level and suggested competition for nutrients and products of metabolism (peptides) are important factors causing antagonism.

The quality of fresh produce is judged based on visual characteristics (appearance and texture), taste, nutritional quality and safety (Kader, 2002; Rico, Martin-Diana, Barat, & Barry-Ryan, 2007). Harvest techniques and processing injuries to the plant tissues affect the sensorial and microbiological quality. The harvested product is rapidly cooled for further processing because harvested spinach become highly perishable due to increased respiration rate (Rubatzky & Yamaguchi, 1997) as a subset of the bacterial population proliferation contributes to product spoilage through further tissue deterioration at ambient temperatures (Rico et al., 2007). Moreover, to prevent foodborne outbreaks similar to 2006 caused by *E. coli* O157:H7

in the US due to RTE spinach (Charatan, 2006), it is mostly further washed with a disinfectant solution before packaging for marketing.

Native microbiota of plants has positive, negative or neutral effects on survival of foodborne pathogens (if present). Some sanitisation chemicals employed to control spoilage bacteria are known to enhance pathogen growth by inadvertently reducing competitive microbiota composition (Johnston et al., 2009). A study conducted by Johnston's group (2009) reported a reduction in the prevalence of highly effective inhibitory bacterial isolates in chlorine-washed spinach leaves when compared to that of the nonchlorine washed controls. But the inhibitory activity of the bacterial species was not adversely affected by chlorine washing. The majority of bacteria exhibiting a negative effect on *E. coli* O157:H7 growth, through the production of acid and antimicrobial peptides, were Gram-negative, with *Pantoea* spp. showing the most significant degree of inhibition (Johnston et al., 2009). The effect of chlorine sanitisation on spinach bacterial microbiota community analysed by Gu and associates (2018) resulted in a shift in bacteria community structure in the chlorine-washed spinach leaves, with PCoA plot of OTUs clearly delineating chlorine-washed spinach from the control group. In their study, amplicon sequencing results showed an increase in the RA of the phylum Proteobacteria and a decrease of the phyla Actinobacteria, Firmicutes and Bacteroidetes. But the decrease of the copy numbers of 16S rRNA gene of all the four phyla was reported for the chlorine-treated spinach leaves. At genus and species level, a significant rise in the RA (> 10.0) of *Pseudomonas* sp. 1, *Pseudomonas* sp. 2 and *Erwinia* spp was observed. Other RA increases (> 2.0 - < 6.0) identified were in *Cupriavidus* spp., *Ralstonia* spp., *Psychrobacter* spp., family *Oxalobacteriaceae* and *Pedobacter* spp. 1. While a dramatic reduction in RA of *Sphingomonas* spp. (RA of -25.6) and *Acinetobacter* spp. (-12.6) were observed. The RA fall of between -3.0 and -7.0 was observed in bacterial OTUs assigned to *Flavobacterium succinicans, Shewanella* spp., *Pseudomonas viridiflava, Acinetobacter* spp., family *Microbacteriaceae*, *Spingobacterium faecum, Agrobacterium* spp. *and Exiguobacterium* spp. Based on the change in copy number of 16S rRNA, significant increases of *Cupriavidus* spp., *Ralstonia* spp., and family *Comamonadaceae* spp. were reported. Considerable reduction of *Acinetobacter lwoffii*, family *Micrococcaceae*, family *Moraxellaceae*, *Paracoccus* spp., and *Mycoplana* spp. was also observed. In another study of RTE spinach and rocket salad by Tatsika and colleagues (2019), a

reduction in richness of the bacterial community but not bacterial diversity after conventional household washing treatments (vinegar) was observed.

Gu and associates (2020) undertook the most recent spinach microbiome study on NaOCl and PAA sanitisation. This study reported *Pseudomonas* spp., *Janthinobacterium* spp., family *Enterobacteriales*, *Janthinobacterium lividum,* and *Agrobacterium spp.* as the five top RAs. Generally, at phyla level, both sanitiser treatments reduced the RA of Proteobacteria and Bacteroidetes and increased Firmicutes and Actinobacteria. At lower-order taxonomic level, NaOCl treatment (10 and 30 mg/L FAC) and PAA treatment (30 and 50 mg/L PAA concentration) reduced RA of all top five genera/species to minimum level but increased the RA of *Cupriavidus* spp., *Staphylococcus* spp., *Arthrobacter psychrolactophilus, Ralstonia* spp. *Bacillus* spp. and *Pseudomonas* spp. The RA rise of these bacteria after treatments was putatively attributed to disinfectant resistance shown through the growth of *Bacillus* spp. and *Pseudomonas* spp. on culture media and increased copies of 16s rRNA using PMA-qPCR for the remainder of the strains. The differentiation of NaOCl and PAA treated microbiome community observed by Gu et al. (2020) can be attributed to differential sanitation efficacies on sanitiser resistant bacteria. Besides an array of common bacterial resistance to both the disinfectants (*Cupriavidus* spp., *Staphylococcus* spp., *Arthrobacter psychrolactophilus, Ralstonia* spp.), *Bacillus* spp. was reported to have a higher resistance to NaOCl washing and *Vibrio* spp., *Pseudomonas* spp., family *Enterobacteriales* and *Micrococcaceae* to PAA washing. The shortcomings of these sanitisers on these resistant microbiotas could also be because of use of FAC at < 30 mg/L (Suslow, 2000) and PAA at < 50 mg/L, which is below optimal recommended concentration (Teixeira et al., 2020). A similar study by Daddiego's group (2018) on lettuce reported PAA to cause a higher reduction of total bacterial load. Partial grouping of the bacterial population by treatment type was observed, although quantitative species-level differentiation analysis was not available.

Among biocidal agents commonly used for sanitisation of fresh produce such as spinach, chlorine-based and PAA based biocidal agents are known to reduce the RA of beneficial inhibitory microbes in leafy vegetables. This decrease has caused a reduction in the evenness of the community structure. However, these sanitisers did not alter the diversity and richness of the RTE leafy vegetable bacterial community

structure. Therefore, in the case of neutral ECAS washing, we hypothesise that the bacterial community diversity and richness will not be affected. However, the inherent resistance of some bacteria in the microbiota to ECAS treatment might cause a reduction in bacterial evenness.

1.3 AIMS AND OBJECTIVES

Chemical disinfectant has limitations such as co-selection of antibiotic resistance (Gosling et al., 2016; Condell et al., 2012), generation of toxic residues (Amy et al., 2000), high costs (Premier, 2013) and negatively affecting the product quality (Bachelli et al. 2013). The acidic and slightly acidic forms of ECAS corrode (mild) equipment surfaces (Tanaka et al., 1999), are risk for chlorine off-gassing (Veasey & Muriana, 2016) and are less stable for storage (Abadias et al., 2008; Cao et al., 2009; Guentzel et al., 2008). pH neutral ECAS in addition to being an environmentally friendly disinfectant contains a higher concentration of HClO (Huang et al., 2008) and only traces of Cl_2 (Liao et al., 2007) making it a better antimicrobial agent with less occupational hazard concern than the acid or slightly acid forms. Therefore, the evaluation of robust disinfection regimens (ECAS as aerosolised fog, spray and solution wash) in animal farms, veterinary and horticulture products would provide vital data on this neutral anolyte in adopting disinfectant that fulfils environmental and health hazard safety regulations.

This thesis aimed to evaluate the efficacy of pH-neutral ECAS in the form of aerosol fog, spray and solution in disinfecting autochthonous and pathogenic bacteria in a swine farm environment, shelled eggs and RTE spinach leaves. Additionally, this thesis also aimed to assess the effect of ECAS on quality parameters and microbiota composition of spinach leaves and shelled eggs cuticle layer. The findings from these studies will provide valuable information on the effectiveness of the use of neutral anolyte in disinfecting general bacteria and pathogens in livestock and horticulture industries. Moreover, further information also will be available on its effect on the quality attributes and microbiota composition in RTE spinach sanitisation, and cuticle layer of shelled eggs.

Chapter 2: Decontamination of aerosolised bacteria from a pig farm environment using a pH neutral electrochemically activated solution (Ecas4 anolyte)

2.1 STATEMENT OF AUTHORSHIP

Statement of Authorship

Title of Paper	Decontamination of aerosolised bacteria from a pig farm environment using a pH neutral electrochemically activated solution (Ecas4 anolyte)
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Tenzin S, Ogunniyi AD, Khazandi M, Ferro S, Bartsch J, Crabb S, et al. (2019). PLoS ONE 14(9): e0222765. https://doi.org/10.1371/journal.pone.0222765 Submitted: December 17, 2018, Accepted: September 3, 2019 and Published: September 25, 2019

Principal Author

Name of Principal Author (Candidate)	Sangay Tenzin		
Contribution to the Paper	Developed methodology, conducted all experimental procedures (optimized live/dead qPCR and cyclonic air sampling procedure, farm environment decontamination of aerosolized bacteria, and laboratory procedures), analyzed data, wrote original draft and edited the manuscript.		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	16/02/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Dr. Abiodun D. Ogunniyi		
Contribution to the Paper	Project conceptualization, funding acquisition and assisted on development of some laboratory methodology.		
Signature		Date	17/02/2020

Name of Co-Author	Professor Darren J Trott for Dr. Manouchehr Khazandi
Contribution to the Paper	Project conceptualization and assisted in development of some laboratory methodology.

Signature		Date	18/02/2020

Name of Co-Author	Dr. Sergio Ferro		
Contribution to the Paper	Conceptualization, acquired funding, resources and manuscript review and editing.		
Signature		Date	18/02/2020

Name of Co-Author	Mr. Jonathon Bartsch		
Contribution to the Paper	Conceptualization and assisted in development of farm decontamination methodology.		
Signature		Date	

Name of Co-Author	Dr. Sergio Ferro for Mr. Simon Crabb		
Contribution to the Paper	Project conceptualization, funding acquisition and resources.		
Signature		Date	18/02/2020

Name of Co-Author	Dr. Sam Abraham		
Contribution to the Paper	Resources and bacterial strain identification (MRSA).		
Signature		Date	12/02/2020

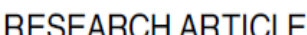
Name of Co-Author	Dr. Permal Deo		
Contribution to the Paper	Associate supervisor, assisted in project administration, resources and manuscript revision and editing.		
Signature		Date	18/02/2020

Name of Co-Author	Professor Darren J. Trott		
Contribution to the Paper	Principal supervisor, administer the project, acquired funding, assisted in experimental design and manuscript revision and editing		
Signature		Date	18/02/2020

RESEARCH ARTICLE

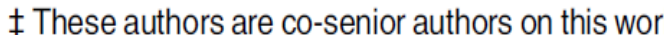
Decontamination of aerosolised bacteria from a pig farm environment using a pH neutral electrochemically activated solution (Ecas4 anolyte)

Sangay Tenzin[1]*, Abiodun David Ogunniyi[1], Manouchehr Khazandi[1], Sergio Ferro[2], Jonathon Bartsch[3], Simon Crabb[2], Sam Abraham[4], Permal Deo[5‡], Darren J. Trott[1‡]*



1 Australian Centre for Antimicrobial Resistance Ecology, School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy SA, Australia, 2 Ecas4 Australia Pty. Ltd., Mile End South SA, Australia, 3 Dr Barry Lloyd Pty. Ltd., Gawler SA, Australia, 4 School of Veterinary and Life Sciences, Murdoch University, Murdoch WA, Australia, 5 School of Pharmacy and Medical Sciences, University of South Australia, City East Campus, Playford P1-25, Adelaide SA, Australia

‡ These authors are co-senior authors on this work.
* darren.trott@adelaide.edu.au (DJT); sangay.tenzin@adelaide.edu.au (ST)



OPEN ACCESS

Citation: Tenzin S, Ogunniyi AD, Khazandi M, Ferro S, Bartsch J, Crabb S, et al. (2019) Decontamination of aerosolised bacteria from a pig farm environment using a pH neutral electrochemically activated solution (Ecas4 anolyte). PLoS ONE 14(9): e0222765. https://doi.org/10.1371/journal.pone.0222765

Editor: Ginny Moore, Public Health England, UNITED KINGDOM

Received: December 17, 2018

Accepted: September 3, 2019

Published: September 25, 2019

Copyright: © 2019 Tenzin et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files. However, the exact location of the piggery cannot be made public due to the terms agreed upon during the project proposal submission and implementation. The authors agreed to protect the identity of the location. Interested researchers may contact the following people in regards to farm location data: Mr. Derrick Prowse, Farm Manager, dw.prowse@bigpond.com or piggeryfarmer1@

Abstract

An electrochemically activated solution (ECAS), generated by electrolysis of a dilute sodium chloride solution in a four-chamber electrolytic cell (Ecas4), was tested as a sanitising aerosol in eliminating bacteria from the environment of a weaning room vacated 24-48h earlier, at a continuous flow pig farm. An ultrasonic humidifier was used to fill the environment with a fog (droplets with diameters of 1–5 μm) containing 0.25 ppm of hypochlorous acid. The weaning room was fogged for 3 min at 30 min intervals during five hours of aerosol disinfection. An innovative sample treatment with propidium monoazide dye in conjunction with cyclonic air sampling was optimised and adapted for discerning live/dead bacteria in subsequent molecular quantification steps. Without fogging, total bacterial load ranged from 5.06 ± 0.04 to 5.75 ± 0.04 Log_{10} CFU/m^3. After the first hour of fogging, a 78% total bacterial reduction was observed, which further increased to > 97% after the second hour, > 99.4% after the third and 99.8% after the fourth hour, finally resulting in a 99.99% reduction from the farm environment over five hours. Unlike the current formaldehyde spray disinfection protocol, which requires a long empty period because of its hazardous properties, this economically viable and environmentally friendly disinfection protocol may significantly lower downtime. Moreover, ECAS fogging can be easily adapted to a variety of applications, including the elimination of pathogens from livestock farm air environment for disease prevention, as well as decontamination after disease outbreaks.

Introduction

Bacterial and/or viral respiratory diseases are a major economic cost for pork production systems. Prevalence of swine respiratory diseases are influenced by interaction of multiple factors

bigpond.com; Dr. Jon Bartsch, Veterinary Consultant, jonb.barryvet@bigpond.com.

Funding: This project was funded by the Pork CRC innovation project no. 2C-121. Sangay Tenzin is supported by Endeavour Postgraduate Scholarship.

Competing interests: Ecas4 Australia Pty Ltd contributed in cash and in-kind towards this Pork CRC Innovation project no. 2C-121. Dr. Sergio Ferro is the Technical Manager and Mr. Simon Cribb is the National Business Manager of Ecas4 Australia Pty Ltd. Ecas4 Australia Pty Ltd had patent for Ecas4 anolyte production apparatus (Patents - PCT/IT 2006 000 829 and PCT/EP 2009 065 077). Dr. Jonathon Bartsch is employed by Dr Barry Lloyd Pty. Ltd. Dr. Barry Llyod Pty. Ltd. is the consulting veterinary firm for the farm where we carried out our fogging experiment. This does not alter our adherence to all the PLOS ONE policies on sharing data and materials.

such as the aetiological microorganisms, environmental factors of the farm such as temperature and moisture or the presence of particulate matters and ammonia, and management factors such as animal density, vector control, and disinfection strategies [1–5]. The level of production losses varies depending on the causative agents involved and their virulence. Bacterial pathogens, such as *Actinobacillus pleuropneumoniae*, may cause chronic or acute respiratory disease requiring prophylactic and/or metaphylactic antimicrobial use for adequate control [6]. Cleaning and disinfection of the animal's environment, in conjunction with quarantine, vaccination and antibiotic treatment, constitute an important component of prevention and control of respiratory diseases [7]. In association with vaccination and the use of antibiotics, the prevention of porcine respiratory disease complex has been achieved by managing the environmental stressors through usage of disinfectants such as sodium hypochlorite, hydrogen peroxide, chlorine dioxide, formaldehyde, quaternary ammonium compounds, peroxyacetic acid and iodophors. While all these chemicals are very effective in reducing microbial load, there are limitations including differential effectiveness against a variety of bacteria [8], co-selection of antibiotic resistance [9], corrosiveness [10], concerns with skin irritation, and cross resistance to other disinfectants and antibiotics [11].

As an alternative, an electrochemically activated solution (ECAS) may provide efficacy without detrimental side effects. ECAS is a broad-spectrum bactericidal "metastable" solution containing free available chlorine (FAC) species such as hypochlorous acid and hypochlorite [12] generated from a dilute brine solution (~ 0.5% w/v). It has a disinfection activity equivalent to 80% ethanol and is superior to 0.1% chlorhexidine or 0.02% povidone iodine [13]. However, applications of ECAS produced with the original two-chamber systems have been limited due to corrosion of processing equipment [14]. Advancements in the technique for generating ECAS with slightly acidic pH (5–6.5) has allowed the use of ECAS in sanitisation of vegetables with excellent efficacy [15–17]. Furthermore, ECAS with neutral pH having excellent oxidative properties and drastically reduced corrosiveness is increasingly being employed as a sanitiser in the food industry [18, 19] and as a wound dressing product [20]. ECAS is also employed in preventing or reducing rates of infection with MRSA in post-operative infections [21, 22] in place of broad-spectrum antibiotics whose use is limited due to the development of antibiotic resistance. In addition, ECAS is being employed in disinfection and eradication of *Legionella* biofilms in hospitals and aged care facilities water systems [23, 24].

The present work was undertaken to investigate the efficacy of an electrochemically activated solution, Ecas4 anolyte, applied as a fog, in reducing the total bacterial load in a pig barn environment. In this study, we performed *in vitro* kill kinetics of Ecas4 anolyte on the swine pathogen *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*) and livestock associated methicillin-resistant *Staphylococcus aureus* (MRSA) that could be transmitted through aerosols. A propidium monoazide (PMA) live/dead quantitative polymerase chain reaction (qPCR) for *A. pleuropneumoniae* was developed for detection and quantification. Moreover, to assess the effectiveness of the Ecas4 fogging technique in decontamination of the emptied weaning room, we optimised an air sampling technique for bacteria detection and quantification using a Coriolis cyclonic air sampler.

Materials and methods

In vitro kill kinetics of Ecas4 anolyte on Australian *A. pleuropneumoniae* and MRSA isolates

A. pleuropneumoniae serovars 1, 5, 7, 12 and 15 were obtained from Ace Laboratories (Bendigo, Victoria, Australia) and stored in culture collections at the School of Animal and Veterinary Sciences, University of Adelaide, and School of Pharmacy and Medical Sciences,

University of South Australia. MRSA isolates ST30, ST93, ST398 recently isolated within a piggery environment in Australia [25] were obtained from the School of Veterinary and Life Sciences, Murdoch University. *A. pleuropneumoniae* ATCC 27090 and *S. aureus* ATCC 29213 were used as controls. *A. pleuropneumoniae* and MRSA isolates were originally collected by veterinarians for disease surveillance and clinical diagnosis.

In vitro kill kinetics were determined for each of the *A. pleuropneumoniae* serovars and MRSA (sequence types ST30, ST93, ST398, and *S. aureus* ATCC 29213) grown overnight (20–24 h) on chocolate and sheep blood agar, in 5% CO_2 and aerobic conditions at 37 ± 1˚C, respectively. Subsequently, bacteria from overnight cultures were suspended in 5 mL of phosphate buffered saline (PBS) to obtain A_{600nm} of 0.1 (10^4–10^5 CFU/mL). Based on our preliminary data, where Ecas4 anolyte at lower concentrations of FAC has shown to be readily quenched by the presence of organic material in Veterinary Fastidious Medium (VFM) and cation-adjusted Mueller-Hinton (CA-MH) broth, a slightly modified Clinical and Laboratory Standards Institute [26] method was used for the kill-time assay. Briefly, PBS (1×) was used for the antimicrobial activity of various concentrations of Ecas4 anolyte (2-fold dilution from 25% to 0.49% v/v) versus contact time. A bacterial suspension (5 μL) (A_{600nm} = 0.1) was added to wells 2 to 12 in duplicate for all isolates and treated for various contact times, ranging from 30 s to 10 min. Well 1 contained only PBS whereas well 2 contained bacterial suspension without any Ecas4 anolyte treatment. For each contact time point, an aliquot (20 μL) from each well was plated onto chocolate and Sheep blood agar (SBA) plates for *A. pleuropneumoniae* and MRSA, respectively. SBA plates were incubated at 37˚C for 24 h in normal environmental conditions, whereas chocolate plates were incubated at 37˚C for 24 h in 5% CO_2. Ampicillin was used as bactericidal compound with a minimum inhibitory concentration (MIC) breakpoint of 0.5–2 μg/mL as a reference for *A. pleuropneumoniae* ATCC 27090 and *S. aureus* ATCC 29213. Test validity was determined based on acceptable growth in the control well, and MIC results determined as the lowest concentration of Ecas4 anolyte that totally inhibited the growth of organisms.

Development of live/dead real time PCR for *A. pleuropneumoniae* and total bacterial load detection

A previously published [27] TaqMan real-time PCR primer for detection of the *apxIVA* gene was optimised using 10-fold dilution series (from 0.005 pg/μL to 5,000 pg/μL) of *A. pleuropneumoniae* ATCC 27090 genomic DNA and the KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems, Inc., Cape Town, South Africa). The reaction mix consisted of a 6 μL aliquot of master enzyme reaction super mix, various volumes of sense and antisense primers to achieve 0.25 μM, 0.5 μM, 0.75 μM, 1 μM or 2 μM concentrations, and nuclease free water to obtain 10 μL including 1 μL of DNA template. The real time PCR run parameters were set up with initial denaturing step at 95˚C for 5 min followed by 40 cycles of denaturation at 95˚C for 15 s, primer annealing and extension at 60˚C for 30 s, where DNA amplification was acquired on green channel, with a final extension step at 40˚C for 60 s (Bio Molecular Systems, NSW 2011, Australia).The specificity of the *A. pleuropneumoniae* optimised primers was tested against DNA from the following bacterial strains: *A. lignieressi*, *A. suis*, *Mannheimia haemolytica*, *Pasteurella multocida*, *S. suis*, *Bordetella broncheseptica*, and *Haemophilus parasuis*.

Similarly, a set of universal primers for amplification of bacterial 16S rDNA [28] was optimised for detection and quantification of recoverable bacterial load by qPCR with a quantification curve generated using 10-fold serial dilutions up to 1:10,000 dilutions (from 0.012 pg/μL to 12,000 pg/μL) of *E. coli* ATCC 35218 gDNA. A 10 μL total reaction volume containing 6 μL of master enzyme reaction super mix from KAPPA fast qPCR SYBR green kit, 0.25 μM,

0.5 μM, 0.75 μM and 1 μM each of forward and reverse primers, varying volumes of nuclease free water and 1 μL DNA template per sample were used to perform qPCR. The qPCR amplification conditions mentioned above were used.

Since qPCR detects all dsDNA sequences specific to the primers used, samples were treated with propidium monoazide (PMA) (a photoreactive membrane-impermeable dye that selectively penetrates bacterial cells with compromised membranes considered dead and binds covalently to dsDNA), prior to DNA extraction for qPCR described above. To optimise the detection and differentiation of live/dead *A. pleuropneumoniae* as well as the recoverable bacterial load in air samples, 24h growth *A. pleuropneumoniae* and *E. coli* were suspended in PBS to an A_{600nm} of 0.58. Bacterial suspension (50 μL) was added to Ecas4 anolyte (5 mL, 0, 5% and 10% diluted in milliQ water) and incubated at room temperature for 5 min. After incubation, aliquots (100 μL) were plated onto either chocolate agar for *A. pleuropneumoniae* or SBA for *E. coli* and incubated as detailed above.

Additionally, aliquots (4 × 500 μL) of *A. pleuropneumoniae* and *E. coli* suspension in PBS were heated at 85˚C for 10 min to kill cells by compromising their bacterial membrane integrity and used as additional bacterial death controls. All bacterial suspensions (Ecas4 anolyte and heat treated samples) were further treated with 6.25, 12.5, 25 and 50 μL of 2 mM PMA to obtain final concentrations of 25, 50, 75 and 100 μM per reaction, vortexed briefly, and incubated for 15 min at room temperature in the dark. After incubation, samples were exposed to light using a PMA-Lite LED photolysis device (Biotium, Inc., Fremont, CA 94538, USA) for 15 min to allow PMA photoactivation. An untreated PMA bacterial suspension from each treatment was used as control. All samples were centrifuged at 12,400 × *g* for 10 min, discarding the supernatant and resuspending the pellet in 70 μL of nuclease free water.

For DNA extraction, 280 μL of lysis buffer were added to each sample, vortexed and incubated for 10 min. Samples were then pipetted into silicone membrane filter tubes and centrifuged at 9,500 × *g* for 60 s. The supernatant was discarded and pre-wash buffer (200 μL) was added to all tubes, mixed and centrifuged (1 min at 9,500 × *g*), before discarding the supernatant again. The procedure was repeated with wash buffer (500 μL). Elution buffer (35 μL) was added to all the tubes and then samples were incubated for 3 min at room temperature before centrifuging at 12,400 × *g* for 60 s. DNA concentration was measured by using a DeNovix DS-11+ spectrophotometer. qPCR runs to determine the PMA concentration required for the discernment of live and killed bacteria were performed using the optimised parameters mentioned above.

Air sampling from the farm–Optimisation study

The air sampling procedure was optimised in a commercial piggery in South Australia; consent was obtained from farm management and all experiments were carried out under the supervision of the farm manager. The piggery shed contained seven weaning rooms of equal size (145 m^3) with partially slated floors. Each room was ventilated by means of two automatically controlled exhaust fans with temperature set at 23˚C (Fig 1). Air coolers were mounted on the side walls of the entrance and exit corridor of the shed, and the cool air sucked into the individual rooms through the air vents located in the upper part of the walls located opposite to exhaust fans (Fig 1). Humidity ranged from 24 to 58%. The air vents were designed to guide the incoming air downwards, and the opening of air vents regulated by the differential pressure created in the room by two exhausts fans operating in synchrony.

Air samples from an empty weaning room were collected from the centre of the room (1.15 m height above the floor level–Fig 1) using a Coriolis cyclonic air sampler (Bertin technologies, Montigny-le-Bretonneux—France). Various collection volumes (5, 7.5, 10, and 12.5 mL) of

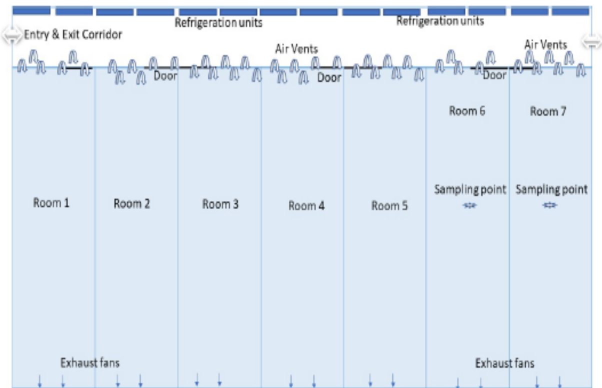


Fig 1. Scheme of the weaning shed with seven weaning rooms and locations of exhaust fans, air vents, refrigeration units and sampling points.

https://doi.org/10.1371/journal.pone.0222765.g001

VFM for different collection times (2, 3, 4, 5, and 6 min) with air sample volumes of 250 and 300 L/min were used during the optimisation trial. The volume of VFM and the collection time were optimised in order to obtain the best detection and quantification of *A. pleuropneumoniae* and other bacteria. Collected samples were immediately refrigerated on ice, transported to the laboratory and processed for total recoverable bacterial counts as well as live/dead qPCR.

Sample aliquots (100 μL) in VFM were used for the preparation of serial dilutions ranging from 10 to 10^{-6} in 0.1% peptone water, inoculated onto plate count agar plates and incubated overnight at 37 ± 1˚C in aerobic condition to estimate bacterial counts. The results obtained in CFU/mL were then converted into CFU/m^3.

Air sampling in the weaning room with and without Ecas4 anolyte fogging

Prior to Ecas4 anolyte fogging experiments, the total recoverable bacterial load in the empty weaning room (within 24–48 h of the animal transfer) was assessed by collecting samples of farm air, using the Coriolis air sampler and the optimised parameters for air collection (250 L/min for 3 min, in 10 mL of sterile VFM). Five air samplings were undertaken over five hours with a one-hour interval. After each sampling, the sampler was purged with 0.1% hydrogen peroxide (H_2O_2) before the next sample collection. Samples were transported on ice to the laboratory as described above. Aliquots (1 mL) of all the samples in VFM were centrifuged at 12,400 × *g* for 10 min, the supernatant discarded and pellet resuspended in 100 μL of nuclease free water. Then, each sample was treated with propidium monoazide (PMA) dye (Biotium Inc. CA 945, USA) at 100 μM concentration per sample. PMA binding, DNA extraction and live/dead qPCR for detection and quantification of *A. pleuropneumoniae* and total bacterial load were carried out as mentioned earlier. In addition, 100 μL aliquots of samples in VFM were also used to prepare serial dilutions ranging from 10 to 10^{-6} in 0.1% peptone water, inoculated onto plate count agar plates and incubated overnight at 37 ± 1˚C in aerobic conditions to estimate total recoverable bacterial counts.

For fogging, a 50% v/v Ecas4 anolyte containing 150 ppm of FAC was used to generate a fog containing 0.25 ppm (0.75 mg/m^3) of FAC. Within 24–48 h of the animal transfer, the room was fogged for 3 min at 30-min intervals for 5 h, using an ultrasonic humidifier (Aqua Aircon M18K, Shang-E International Resources Development Corporation, Taiwan) that produces a mist with droplets having a diameter between 1 and 5 μm. Air samples were collected

before fogging (sample 0) and then every hour for five hours (samples 1–5) during fogging using the Coriolis air sampler at a rate of 250 L/min for 3 min and with 10 mL of sterile VFM. Samples were collected and analysed in duplicate and the experiment trialled independently over 2 days. Samples were transported to the laboratory on ice to undertake total recoverable bacterial counts as well as live/dead qPCR as described above.

Results

In vitro kill kinetics of Ecas4 anolyte on *A. pleuropneumoniae* and MRSA

The *in vitro* antimicrobial susceptibility testing of Ecas4 anolyte against field strains of *A. pleuropneumoniae* (representing major serotypes 1, 5, 7, 12, 15, and ATCC 27090) and MRSA (representing sequence type 398, the main livestock-associated MRSA and the important human-disease associated clone ST93) recently isolated from Australian pigs, showed that very low concentrations of Ecas4 anolyte in water are effective in killing both *A. pleuropneumoniae* (0.39% v/v, 1.17 μg/mL) and MRSA isolates (0.78% v/v, 2.34 μg/mL) within 30 s of exposure. Ampicillin at 2.0 μg/mL was effective in killing 99.99% of both *A. pleruopneumoniae* and *S. aureus* ATCC strains.

Development of live/dead real time PCR for *A. pleuropneumoniae* and total bacterial load estimation

Reverse and forward primers for *A. pleuropneumoniae apxIVA* gene at concentrations of 1.0 μM in a reaction volume of 10 μL could detect and quantify as little as 0.05 pg/μL of *A. pleuropneumoniae* ATCC 27090 and other *A. pleuropneumoniae* serovars. Primer pairs of lower concentration (0.25 μM to 0.75 μM) could not detect *A. pleuropneumoniae* at a concentration of 0.05 pg/μL. However, a primer pair concentration of 2.0 μM could quantify 0.05 pg of *A. pleuropneumoniae* gDNA, although it caused primer-dimer formation at around the 30th quantification cycle (Cq). Quantification curves of serial dilutions of *A. pleuropneumoniae* ATCC 27090 from 5,000 ng to 0.05 pg per reaction and their corresponding Cq values for *A. pleuropneumoniae* ATCC 27090 DNA serial dilutions are shown in Fig 2 and Table 1; the corresponding calibration curve is shown in Fig 3. The specificity of *apxIVA* gene primers was high as no nonspecific amplification occurred with other tested porcine nasal bacteria.

Regarding the 16S rDNA universal bacterial gene primers, 0.5 nM and 0.75 nM of forward and reverse primers in a final volume of 10 μL were able to detect *E. coli* DNA concentrations as low as 1.2 pg/μL. The qPCR reaction efficiency of the 0.75 nM primer set is 1.17% and the correlation coefficient is 0.99 for the total bacterial qPCR. 1.0 nM primer set caused primer dimerization at the 32nd quantification cycle (Cq). The quantification curve of 10-fold serial dilutions of *E. coli* and corresponding Cq values are presented in Table 2 and Fig 4, respectively.

Amplification of *A. pleuropneumoniae* ATCC 20790 killed with 5% and 10% Ecas4 anolyte, dead control (500 μL of aliquot killed by boiling at 85˚C for 15 min and treated with 50 μM PMA) and viable (live) controls quantified employing *apxIVA* gene primers at 1.0 μM concentrations are shown in Fig 5. Cq values of viable control and dead control were 15.3 and 27.7, respectively; Cq values of amplified DNA of viable (live) *E. coli* (10.5), dead *E. coli* control (23.5—treated with 50 μM PMA) and 5% ECAS-killed (22.0) samples quantified using universal bacterial primers at 0.75 nM are shown in Fig 6. The treatment of dead control samples with PMA 50 μM resulted in more than 10,000 fold reduction in DNA amplification in comparison to that of the viable (live) controls in both *A. pleuropneumoniae* and *E. coli*. With 75 μM and 100 μM PMA, Cq value differences between the dead and viable controls observed were similar to those of the treatment with PMA 50 μM. However, with 25 μM PMA no differentiation of live and dead control for both bacteria were recorded.

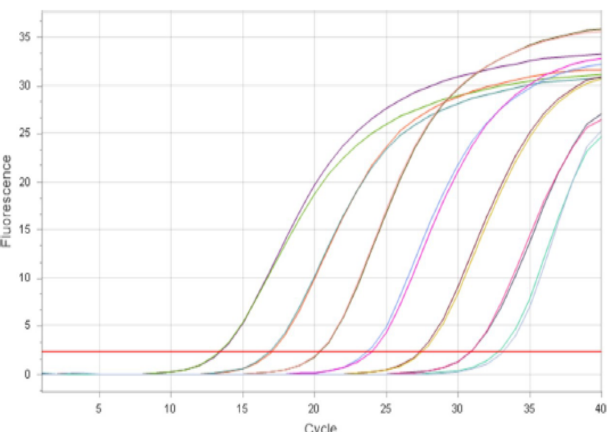


Fig 2. Amplification curves of 10-fold serial dilutions of *A. pleuropneumoniae* ATCC DNA (from 0.05 to 5,000 pg).

https://doi.org/10.1371/journal.pone.0222765.g002

Detection of *A. pleuropneumoniae* and total bacterial load in air samples–Optimisation study

The optimised parameters for the collection of the farm's environmental air samples were 10 mL of VFM as the collection liquid and a pre-set collection air volume of 250 L/min for 3 min. These parameters allowed detection of *A. pleuropneumoniae* at 200 pg (5.1×10^6 GU) in 2 mL of VFM and total recoverable bacterial gDNA of 1,200 pg (1.5×10^8 GU) in 2 mL of VFM. However, the quantity of detectable *A. pleuropneumoniae* increased to about 1,600 pg (2.1×10^8 GU) in 2 mL when samples were incubated in VFM overnight in 5% CO_2. Total recoverable bacterial load, in terms of genomic units of DNA present, also increased to 3.6×10^9 GU after overnight incubation in aerobic conditions.

Bacterial quantification in the air sampled in the weaning room before Ecas4 anolyte fogging

Total recoverable bacteria loads and bacterial DNA concentrations quantified in an empty weaning shed prior to fogging with Ecas4 anolyte are presented in Table 3. The total

Table 1. Cq values of *A. pleuropneumoniae (A. pp)* ATCC 20790 DNA serial dilutions.

Well	Sample	DNA conc. (pg/μL)	Cq	R^2
1	*A. pp* ATCC 1:1	5,000	13.41	0.99
2	*A. pp* ATCC 1:1	5,000	13.37	0.99
3	*A. pp* ATCC 1:10	500	17.04	0.99
4	*A. pp* ATCC 1:10	500	16.96	0.99
5	*A. pp* ATCC 1:100	50	20.37	0.99
6	*A. pp* ATCC 1:100	50	20.33	0.99
7	*A. pp* ATCC 1:1,000	5.0	23.69	0.99
8	*A. pp* ATCC 1:1,000	5.0	23.97	0.99
9	*A. pp* ATCC 1:10,000	0.5	27.35	0.99
10	*A. pp* ATCC 1:10,000	0.5	27.54	0.99
11	*A. pp* ATCC 1:100,000	0.05	30.92	0.99
12	*A. pp* ATCC 1:100,000	0.05	30.88	0.99

https://doi.org/10.1371/journal.pone.0222765.t001

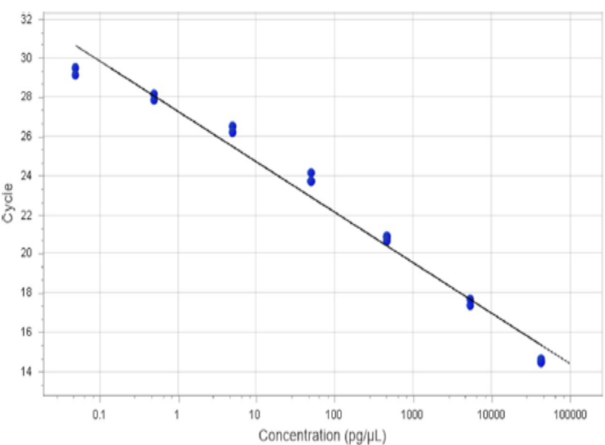


Fig 3. Calibration curve of serial dilutions of *A. pleuropneumoniae* ATCC DNA.

https://doi.org/10.1371/journal.pone.0222765.g003

recoverable bacterial load ranged from 5.06 ± 0.04 Log_{10} CFU/m^3 to 5.75 ± 0.04 Log_{10} CFU/m^3. Bacterial DNA concentrations evaluated using real-time qPCR ranged from 2,021 ± 146 pg/μL to 3,218 ± 117 pg/μL. Interestingly, *A. pleuropneumoniae* was not detected in any of the collected air samples.

Bacterial quantification in the air sampled in the weaning room during Ecas4 anolyte fogging

A. pleuropneumoniae could not be detected using SYBR green-based real time qPCR for detection of *apxIVA* gene in DNA from samples collected from the weaning shed and incubated overnight in VFM at 37 ± 1˚C in 5% CO_2.

Total recoverable bacterial load was 5.77 ± 0.01 Log_{10} CFU/m^3 in the weaning room environment at the commencement of the experiment. Bacterial counts enumerated on a plate count agar and converted into CFU/m^3 of air provided a 0.67 Log_{10} reduction in total recoverable bacterial count after the first hour of fogging, which increased to 5.12 Log_{10} after 5 hours (Fig 7). A total bacterial reduction amounting to 99.998% (Fig 8) was estimated after Ecas4 anolyte fogging (Table 4).

Table 2. Cq values of *E. coli* DNA serial dilutions.

Sl. No.	Sample	*E. coli* DNA conc. (pg/μL)	Cq	R^2
1	*E. coli* 1:10	4,100	15.14	0.99
2	*E. coli* 1:10	4,100	14.71	0.99
3	*E. coli* 1:10	4,100	15.24	0.99
4	*E. coli* 1;100	151	19.91	0.99
5	*E. coli* 1;100	151	19.33	0.99
6	*E. coli* 1;100	151	19.59	0.99
7	*E. coli* 1:1,000	15	23.42	0.99
8	*E. coli* 1:1,000	15	22.52	0.99
9	*E. coli* 1:1,000	15	22.61	0.99
10	*E. coli* 1:10,000	1.2	25.77	0.99
11	*E. coli* 1:10,000	1.2	25.17	0.99
12	*E. coli* 1:10,000	1.2	25.57	0.99

https://doi.org/10.1371/journal.pone.0222765.t002

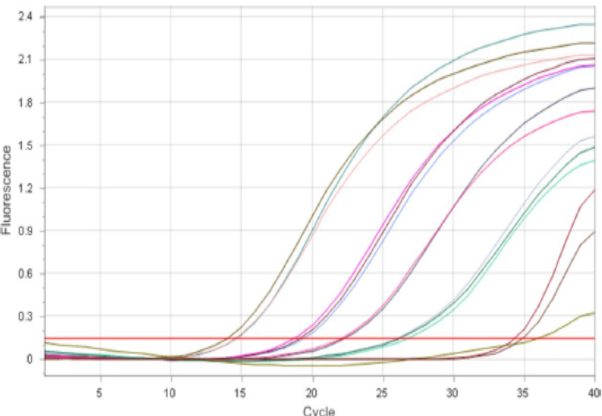


Fig 4. Amplification curves of 10-fold serial dilutions of *E. coli* DNA.

https://doi.org/10.1371/journal.pone.0222765.g004

Real time live/dead qPCR for detection and quantification of total bacterial DNA before and after the various decontamination time points showed a 1.13 Log_{10} reduction during the first 2 h of fogging, the reduction further increased to 4.79 Log_{10} in DNA concentration (pg/μL) after 5 h of fogging (Table 5). Total bacterial load reduction was 99.998% (Fig 8) after 5 h of Ecas4 fogging at 0.25 ppm of FAC in the air.

Discussion

Various forms of electrolysed oxidising (EO) waters are becoming increasingly popular as effective and safer disinfection/sanitation options in healthcare settings and in the food industry [29]. An EO water with neutral pH, such as the Ecas4 anolyte, provides an additional level of safety (since there is no dissolved chlorine gas, the solution is not corrosive), without compromising its effectiveness. As EO water is expected to kill bacteria by breaking down covalent bonds in proteins through oxidative reactions [30], we found that a lower concentration of Ecas4 anolyte was required to kill *A. pleuropneumoniae* (Gram-negative bacteria, with a

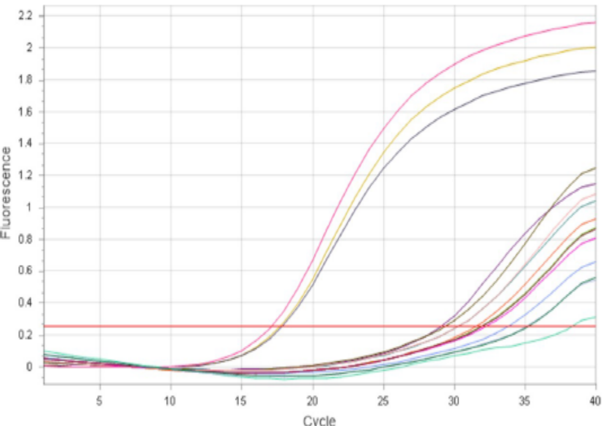


Fig 5. Amplification curve of *A. pleuropneumoniae* ATCC 27090 DNA: (1) from cells killed by boiling (PMA treated– 27.5 Cq); (2, 3) from cells killed with Ecas4 analyte at 5% (no PMA treatment– 26.0 Cq) and 10% (no PMA treatment– 30.4 Cq); (4) from a viable (live) control (15.3 Cq). qPCR was performed with 1.0 μM *apxIVA* gene primer.

https://doi.org/10.1371/journal.pone.0222765.g005

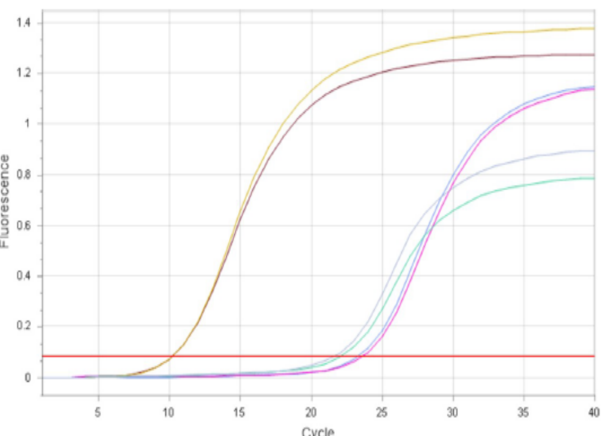


Fig 6. Amplification curves of *E. coli* DNA: (1) from viable (live) cells (10.5 Cq); (2) from cells killed with Ecas4 at 5%, without PMA treatment (22.0 Cq); (3) from dead *E. coli* control treated with 50 μM PMA (23.7 Cq–Aliquot of 500 μL killed by boiling at 85˚C for 15 min). All qPCR tests were performed using universal bacterial primers at 0.75 nM.

https://doi.org/10.1371/journal.pone.0222765.g006

single peptidoglycan layer in the cell membrane) compared to the slightly higher concentrations needed to kill MRSA (Gram-positive bacteria with a peptidoglycan multilayer).

A. pleuropneumoniae primers and reaction optimum conditions were determined to detect *A. pleuropneumoniae* DNA concentrations as low as 0.05 pg/μL in 10 μL total reaction volume, with 100% specificity when tested against other porcine nasal bacteria. For universal bacterial primers, optimal conditions were determined by exploiting their increased reproducibility and increased reaction efficiency, which allowed quantification of as low as 1.2 pg/μL of gDNA of *E. coli* without primer-dimer formation before 35.0 quantification cycles.

The use of a Coriolis cyclonic air sampler was optimised to detect and quantify *A. pleuropneumoniae* and total bacterial load in the farm's environment. It showed excellent repeatability for collection volumes between 500 and 1,500 L providing plate counts per cubic meter of air comparable to those of a conventional plate impactor system (Millipore M air T) [31] and higher efficiency than the AGI-impinger in a poultry farm study [32]. As in previous studies [31–32], the air sampling protocol adopted in this study proved successful in detecting very low levels of *A. pleuropneumoniae* in the growing/finishing and weaning sheds with pigs present, during the optimisation steps; however, no *A. pleuropneumoniae* was isolated from the empty weaning rooms during the fogging experiments. Recoverable bacterial counts from swine ambient air samples can vary depending on farm production type and management systems, with a total recoverable bacterial load ranging from 3.4 to 5.9 Log_{10} CFU/m^3 in studies to date [33, 34]. The maximum recoverable bacterial load quantified in the empty weaning

Table 3. Total bacteria and DNA concentration in the air sampled in an empty weaning room over five hours. Data reported as mean ± standard error of mean (SEM), number of replicates (n) = 2.

Samples	Total bacteria (Log_{10} CFU/m^3)	DNA concentration (pg/μL)
First hour	5.74 ± 0.01	2,021 ± 146
Second hour	5.07 ± 0.02	2,412 ± 159
Third hour	5.06 ± 0.04	2,492 ± 17
Fourth hour	5.32 ± 0.01	3,046 ± 49
Fifth hour	5.75 ± 0.04	3,218 ± 117

https://doi.org/10.1371/journal.pone.0222765.t003

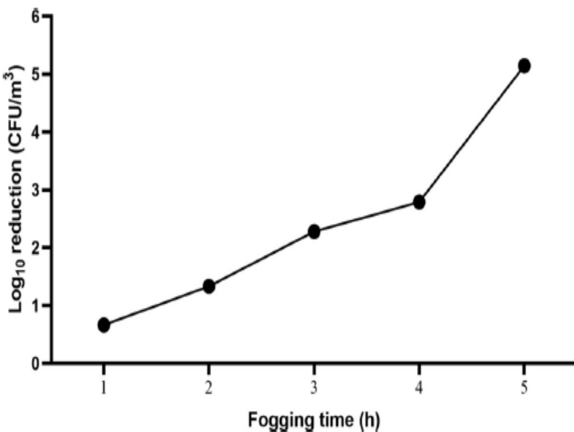


Fig 7. Reduction of bacterial load (Log_{10} CFU/m^3) over time enumerated using plate count technique.

https://doi.org/10.1371/journal.pone.0222765.g007

shed in this study was equal to 5.75 ± 0.04 Log_{10} CFU/m^3. This relatively high bacterial load in an empty weaning room could be due to the fact that the Coriolis sampler captures bacteria with greater efficiency than the conventional impinger technique [32], but it may also reflect the high bacterial load on this particular continuous flow farm.

The initial bacterial reduction of 0.67 Log_{10} CFU/m^3 after fogging for one hour is comparable to that achieved in our previous study, where Ecas4 anolyte at 45 ppm FAC wash sanitisation of fish fillets achieved reduction of 0.5 Log_{10} [18]. Likewise, the 1.34 Log_{10} CFU/m^3 reduction after two hours of fogging was superior to washing fish fillets with 150 ppm FAC (1.0 Log_{10}) [18]. The different effectiveness observed between the two fish fillet wash experiments and the two fogging time points could be attributed to the presence of organic matter quenching the anolyte active components, since even Ecas4 with low FAC (0.5 ppm) was able to totally inactivate bacterial cells (5 Log_{10}) in the absence of organic material. The high disinfection potential of Ecas4 anolyte may be attributed to the high oxidation-reduction potential of the solution (> 1100 mV), which contains powerful oxidants that sequester electrons from the bacterial structure, damage the cell membrane and inactivate intracellular proteins, lipids and nucleic acids, making the bacterial cells non-functional [29]. The efficacy may also have

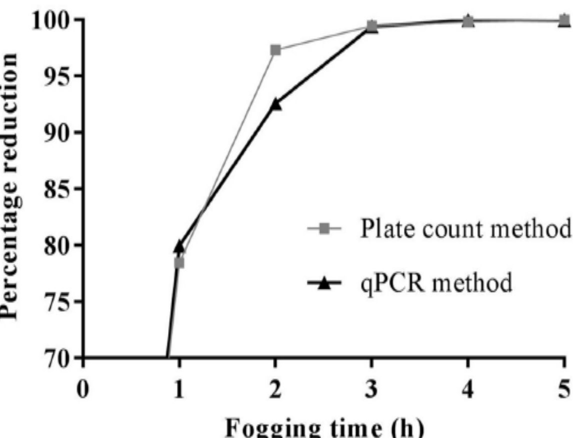


Fig 8. Percentage of killed bacteria enumerated using plate count and quantitative PCR methods.

https://doi.org/10.1371/journal.pone.0222765.g008

Table 4. Total bacteria and Log_{10} reduction on air samples collected from an empty weaning room during Ecas4 anolyte fogging. Data reported as mean ± SEM, n = 4.

Samples	Total Bacteria (Log_{10} CFU/m^3)	Log_{10} reduction	Percent reduction
Before fogging (0)	5.77 ± 0.01		
1 h after fogging	5.10 ± 0.02	0.67	78.326
2 h after fogging	4.43 ± 0.05	1.34	97.361
3 h after fogging	3.49 ± 0.07	2.28	99.459
4 h after fogging	2.98 ± 0.03	2.79	99.837
5 h after fogging	0.65 ± 0.07	5.12	99.998

https://doi.org/10.1371/journal.pone.0222765.t004

been improved by applying the ECAS as a fog, as the tiny mist particles containing aerosolised oxidative moieties bind more efficiently to the bacterial surface causing more rapid cell lysis.

Increased effectiveness of aerosol disinfection by fogging was observed when pedestal fans were used to distribute the fog more evenly and rapidly in a fogging experiment [35] and this could therefore be an important factor contributing to the high efficacy in reducing bacterial load. A similar effect could be attributed to the design of the airflow in the weaning shed, whereby the airflow enters the shed on one side and is sucked out by an exhaust fan located on the opposite side of the room (Fig 1), thus allowing a rapid distribution of the fog. However, the effectiveness of the aerosol disinfection protocol might also have been influenced by the size and design of the pig farm. The Ecas4 fog at the current FAC content (0.75 mg/m^3) was able to rapidly disinfect the air of the farm's environment in a small closed shed with uniform air circulation. However, in larger pig sheds that do not have uniform air circulation, a longer fogging time may be needed to inactivate the greater bacterial load produced by more animals.

The live/dead qPCR total bacterial load determination optimised in this work was able to provide data on the killing of bacteria (Fig 8), similar to those obtained through conventional enumeration methods, thus validating the ability of the treatment with PMA dye to quantitatively differentiate alive and dead bacteria; however, the concentration of PMA required for treating the samples had to be adjusted to the specific collection media used. In addition, qPCR data from *A. pleuropneumoniae* and *E. coli* killed with 5% and 10% Ecas4 anolyte without PMA treatment provided Cq values similar to those of the bacteria killed by boiling at 85°C for 15 min and treated with PMA 50 μM (Figs 5 & 6), i.e. a 10,000-fold reduction in DNA amplification compared to that from viable control bacteria, suggesting that no "viable but nonculturable" (VBNC) cells are induced during the treatment, as PMA is a membrane impairment dye used for VBNC determination in conjunction with pre-mRNA reverse transcription qPCR [36, 37]. However, the lack of VBNC induction by the Ecas4 anolyte should be verified in tandem using a further viability assay, such as the 16S ribosomal RNA expression or demonstration of no metabolic activity.

Since the Ecas4 anolyte is biodegradable, certified "organic" and requires extremely short downtime during the fogging period, it could potentially lead to a reduced use of prophylactic antibiotics for the prevention of porcine respiratory disease complex by minimising the transmission of respiratory pathogens such as *A. pleuropneumoniae* between carrier and naïve animals, and thus minimising the risk of development of antibiotic resistance, without the problems associated with the use of other chemicals such as formaldehyde and H_2O_2. Moreover, the levels of chlorine in the farm's environment at 0.25 ppm (0.75 mg/m^3 of hypochlorous acid) would fall within the short-term exposure limit (0.5 ppm) and the time-weighted average limit (1.0 ppm) prescribed by the Safe Work Australia standards [38]. From an economical point of view, the reduction of the environmental bacterial load should help by

Table 5. Total bacterial load (data reported as mean ± SEM, n = 2), Log_{10} and percent reductions before and after Ecas4 anolyte fogging quantified by qPCR using universal bacterial primers and live/dead differentiation.

Samples	DNA concentration (Log_{10} pg/μL)	Log_{10} reduction	Percent reduction
Before fogging (0)	2430 ± 286		
1 hour after fogging	489 ± 205	0.72	80.004
2 hours after fogging	193 ± 18	1.13	92.637
3 hours after fogging	14 ± 3	2.24	99.426
4 hours after fogging	0.051 ± 0.004	4.62	99.998
5 hours after fogging	0.039 ± 0.002	4.79	99.998

https://doi.org/10.1371/journal.pone.0222765.t005

reducing medication costs and improving food conversion and growth rate, since an increase in the concentration of bacteria in the air is a main factor in the decline of the growth rate of pigs in a high-density stocking environment, leading to high incidence of pleurisy and pneumonia [39, 40].

In conclusion, proof-of-concept data on Ecas4 anolyte as a cost-effective air decontamination agent were obtained when the anolyte was administered as a fog to prevent the transmission of bacterial pathogens. This protocol can now be adapted to a model implementable in various farm settings for the control and prevention of the transmission of aerosolised pathogenic bacteria.

Supporting information

S1 Fig. Melting curves of *Actinobacillus pleuropneumoniae* (App) DNA for qPCR performed with *apxIVA* gene primers. a. App ATCC viable control (V)–no kill b. App ATCC killed by boiling (PMA treated- D). c. Ecas4 5% killed (5EAppATCC) without PMA treatment. d. 10% Ecas4 killed (E10App ATCC)- no PMA treatment and e. NTC- no template control. 5% Ecas4–15 ppm free available chlorine (FAC), 10% Ecas4–30 ppm FAC.
(TIF)

S2 Fig. Melting curves of *E. coli* DNA for qPCR performed using universal bacterial primers. a. E. coli ATCC viable control (V1)–no kill b. E. coli ATCC killed by boiling (PMA treated- D1) c. Ecas4 5% killed (E5 E. coli ATCC) without PMA treatment. d. 10% Ecas4 killed (E10E. coli ATCC)- no PMA treatment and e. NTC- no template control. 5% Ecas4–15 ppm free available chlorine (FAC), 10% Ecas4–30 ppm FAC.
(TIF)

Acknowledgments

This project was funded by the Pork CRC innovation project no. 2C-121. ST is supported by the Endeavour Postgraduate Scholarship.

Author Contributions

Conceptualization: Abiodun David Ogunniyi, Manouchehr Khazandi, Sergio Ferro, Simon Crabb, Darren J. Trott.

Formal analysis: Sangay Tenzin.

Funding acquisition: Darren J. Trott.

Investigation: Sangay Tenzin.

Methodology: Sangay Tenzin, Abiodun David Ogunniyi, Manouchehr Khazandi, Jonathon Bartsch.

Project administration: Permal Deo, Darren J. Trott.

Resources: Sergio Ferro, Jonathon Bartsch, Simon Crabb, Sam Abraham, Permal Deo, Darren J. Trott.

Supervision: Permal Deo, Darren J. Trott.

Writing – original draft: Sangay Tenzin.

Writing – review & editing: Sangay Tenzin, Sergio Ferro, Permal Deo, Darren J. Trott.

References

1. Fablet C, Dorenlor V, Eono F, Eveno E, Jolly JP, Portier F, et al. Noninfectious factors associated with pneumonia and pleuritis in slaughtered pigs from 143 farrow-to-finish pig farms. Prev Vet Med. 2012 May 1; 104(3–4):271–280. https://doi.org/10.1016/j.prevetmed.2011.11.012 PMID: 22196500
2. Gardner IA, Willeberg P, Mousing J. Empirical and theoretical evidence for herd size as a risk factor for swine diseases. Anim Health Res Rev. 2002 Jun; 3(1): 43–55. PMID: 12400869
3. Jirawattanapong P, Stockhofe-Zurwieden N, van Leengoed L, Wisselink H, Raymakers R, Cruijsen T, et al. Pleuritis in slaughter pigs: relations between lung lesions and bacteriology in 10 herds with high pleuritis. Res Vet Sci. 2010 Feb 1; 88(1):11–5. https://doi.org/10.1016/j.rvsc.2009.06.007 PMID: 19836811
4. Michiels A, Piepers S, Ulens T, Van Ransbeeck N, Sacristán RD, Sierens A, et al. Impact of particulate matter and ammonia on average daily weight gain, mortality and lung lesions in pigs. Prev Vet Med. 2015 Sep 1; 121(1–2):99–107. https://doi.org/10.1016/j.prevetmed.2015.06.011 PMID: 26148844
5. Stärk K.D. Epidemiological investigation of the influence of environmental risk factors on respiratory diseases in swine—a literature review. Vet J. 2000 Jan 1; 159(1):37–56. https://doi.org/10.1053/tvjl.1999.0421 PMID: 10640410
6. European Platform for the Responsible Use of Medicines in Animals, [cited 2017 Dec 17]. Document on veterinary medicinal product terminology. Available from: http://www.epruma.eu/.
7. Fotheringham VJ. Disinfection of livestock production premises. Rev Sci Tech Title(s). 1995 Mar 1; 14: 191–205.
8. Meakin NS, Bowman C, Lewis MR, Dancer SJ. Comparison of cleaning efficacy between in-use disinfectant and electrolysed water in an English residential care home. J Hosp Infect. 2012 Feb 1; 80(2): 122–7. https://doi.org/10.1016/j.jhin.2011.10.015 PMID: 22196853
9. Loughlin MF, Jones MV, Lambert PA. Pseudomonas aeruginosa cells adapted to benzalkonium chloride show resistance to other membrane-active agents but not to clinically relevant antibiotics. J Antimicrob Chemother. 2002 Apr 1; 49(4): 631–9. https://doi.org/10.1093/jac/49.4.631 PMID: 11909837
10. Nicklas W, Böhm KH, Richter B. Studies on the corrosive action of some disinfectants suitable for aerosol-disinfection (author's transl). Zentralbl Bakteriol Mikrobiol Hyg A. 1981; 173(5): 374–81.
11. Chapman JS. Disinfectant resistance mechanisms, cross-resistance, and co-resistance. Int Biodeterior Biodegradation. 2003 Jun 1; 51(4): 271–6.
12. Prilutsky V, Bakhir V, 1997. Electrochemically activated water: anomalous properties, mechanism of biological action. VNIII of Med Eng. 232 pp.
13. Tanaka N, Fujisawa T, Daimon T, Fujiwara K, Tanaka N, Yamamoto M, et al. The effect of electrolyzed strong acid aqueous solution on hemodialysis equipment. J Artif Organs. 1999 Dec; 23(12): 1055–62.
14. Wang H, Duan D, Wu Z, Xue S, Xu X, Zhou G. Primary concerns regarding the application of electrolyzed water in the meat industry. J. Food Saf. 2019 Jan 1; 95: 50–6.
15. Hao J, Liu H, Liu RU, Dalai W, Zhao R, Chen T, et al. Efficacy of slightly acidic electrolyzed water (SAEW) for reducing microbial contamination on fresh-cut cilantro. J. Food Saf. 2011 Feb 1; 31(1): 28–34.
16. Koide S, Takeda JI, Shi J, Shono H, Atungulu GG. Disinfection efficacy of slightly acidic electrolyzed water on fresh cut cabbage. Food Control. 2009 Mar 1; 20(3): 294–7.
17. Rahman SM, Ding T, Oh DH. Inactivation effect of newly developed low concentration electrolyzed water and other sanitizers against microorganisms on spinach. Food Control. 2010 Oct 1; 21(10): 1383–7.

18. Khazandi M, Deo P, Ferro S, Venter H, Pi H, Crabb S, et al. Efficacy evaluation of a new water sanitizer for increasing the shelf life of Southern Australian King George Whiting and Tasmanian Atlantic Salmon fillets. Food Microbiol. 2017 Dec 1; 68: 51–60. https://doi.org/10.1016/j.fm.2017.06.008 PMID: 28800825

19. Ozer NP, Demirci A. Electrolyzed oxidizing water treatment for decontamination of raw salmon inoculated with Escherichia coli O157: H7 and Listeria monocytogenes Scott A and response surface modeling. J Food Eng. 2006 Feb 1; 72(3): 234–41.

20. Dalla Paola LMD, Brocco E, Senesi A. Feature: Super-Oxidized Solution (SOS) Therapy for Infected Diabetic Foot Ulcers. Wounds. 2006 Sept; 18(9):262–270.

21. Ichihara T, Fujii G, Eda T, Sasaki M, Ueda Y. The efficacy of function water (electrolyzed strong acid solution) on open heart surgery; postoperative mediastinitis due to methicillin-resistant Staphylococcus aureus. Kyobu geka. Jpn J Thorac Surg. 2004 Nov; 57(12): 1110–2.

22. Ohno H, Higashidate M, Yokosuka T. Mediastinal irrigation with superoxidized water after open-heart surgery: the safety and pitfalls of cardiovascular surgical application. Surg. Today. 2000 Nov 1; 30(11): 1055–6. https://doi.org/10.1007/s005950070035 PMID: 11110409

23. Migliarina F, Ferro S. A modern approach to disinfection, as old as the evolution of vertebrates. In Healthcare 2014 Dec 19 (Vol. 2, No. 4, pp. 516–526). Multidisciplinary Digital Publishing Institute. https://doi.org/10.3390/healthcare2040516 PMID: 27429291

24. Institute of Healthcare Engineering, Australia. The Aus Hosp Eng. [cited 2017 May 02] Available from: https://issuu.com/adbourne/docs/hospital_summer_2016_issuu_opt/60

25. Sahibzada S, Abraham S, Coombs GW, Pang S, Hernández-Jover M, Jordan D, et al. Transmission of highly virulent community-associated MRSA ST93 and livestock-associated MRSA ST398 between humans and pigs in Australia. Sci Rep. 2017 Jul 13; 7(1): 5273. https://doi.org/10.1038/s41598-017-04789-0 PMID: 28706213

26. CLSI. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals. 5th ed. CLSI standard VET01. Wayne, PA: Clinical and Laboratory Standards Institute; 2018.

27. Schaller A, Djordjevic SP, Eamens GJ, Forbes WA, Kuhn R, Kuhnert P, et al. Identification and detection of Actinobacillus pleuropneumoniae by PCR based on the gene apxIVA. Vet Microbiol. 2001 Mar 2; 79(1): 47–62. https://doi.org/10.1016/s0378-1135(00)00345-x PMID: 11230928

28. Nadkarni MA, Martin FE, Jacques NA, Hunter N. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiol. 2002 Jan 1; 148(1): 257–66.

29. Thorn RM, Lee SW, Robinson GM, Greenman J, Reynolds DM. Electrochemically activated solutions: evidence for antimicrobial efficacy and applications in healthcare environments. Eur. J. Clin. Microbiol. Infect. Dis. 2012 May 1; 31(5):641–53.

30. Zinkevich V, Beech IB, Tapper R, Bogdarina I. The effect of super-oxidized water on Escherichia coli. J Hosp Infect. 2000 Oct 1; 46(2): 153–6. https://doi.org/10.1053/jhin.2000.0819 PMID: 11049710

31. Marenda M, Markham P, Watt A, 2014. [cited June 16, 2017]. Real-time detection of airborne pathogens in the piggery. Available from: http://porkcrc.com.au/.

32. El Metwaly Ahmed MF, Schulz J, Hartung J. Air samplings in a Campylobacter jejuni positive laying hen flock. Ann Agric Environ Med. 2013;20(1).

33. Bilić V, Habrun B, Barač I, Humski A. Distribution of airborne bacteria in swine housing facilities and their immediate environment. Arh Hig Rada Toksiko. 2000 Nov 6; 51(2): 199–205.

34. Popescu S, Borda C, Diugan EA, Oros D. Microbial air contamination in indoor and outdoor environment of pig farms. J Anim Sci Biotechnol. 2014 May 31; 47(1):182–7.

35. Pork CRC Australia. Investigating the use of aerosol disinfection during the weaner period on APP prevalence in fogged grower/finisher pigs. [cited 2016 May 23] Available from: http://porkcrc.com.au/

36. Lee S, Bae S. Molecular viability testing of viable but non-culturable bacteria induced by antibiotic exposure. Microb Biotechnol. 2018 Nov; 11(6):1008–16. https://doi.org/10.1111/1751-7915.13039 PMID: 29243404

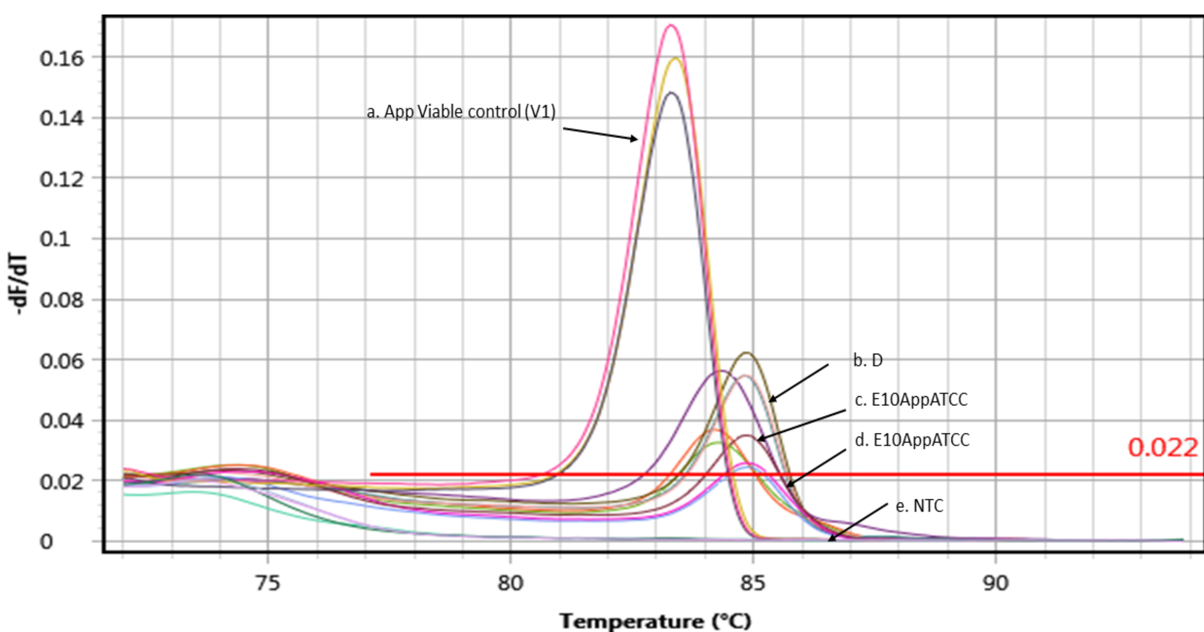
37. Ferro S, Amorico T, Deo P. Role of food sanitising treatments in inducing the 'viable but nonculturable'-state of microorganisms. Food Control. 2018 Sep 1; 91:321–9.

38. Safe Work Australia. 2018. Workplace exposure standards for airborne contaminants. [cited 2018 March 15]. Available from: https://www.safeworkaustralia.gov.au/

39. Donham KJ. Association of environmental air contaminants with disease and productivity in swine. Am J Vet Res. 1991 Oct; 52(10):1723–30. PMID: 1767997

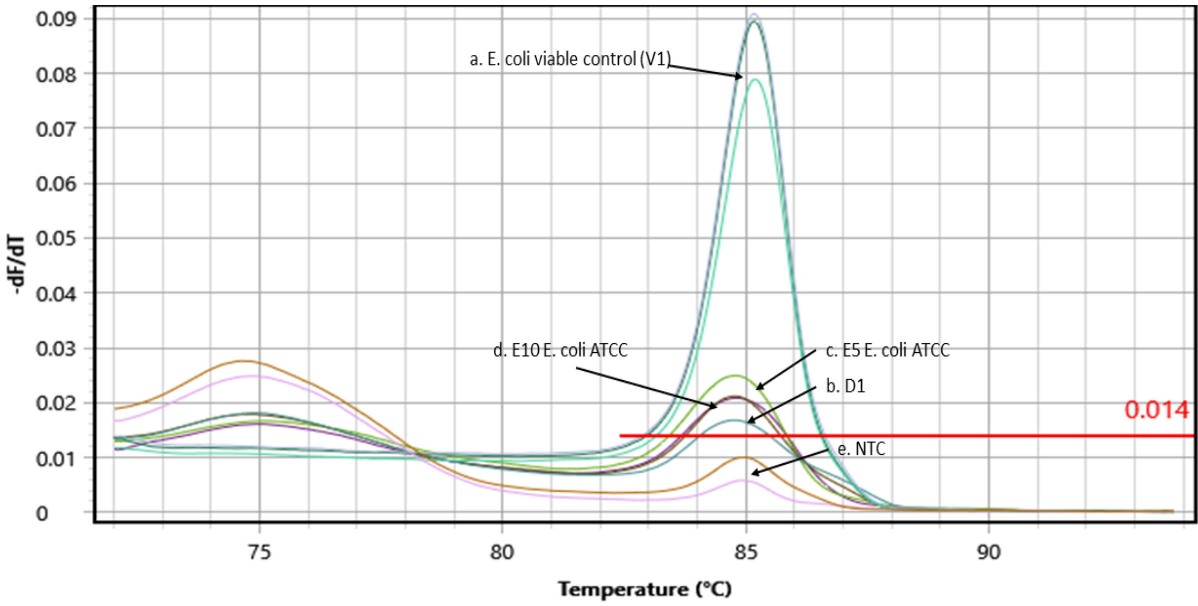
40. Stärk KD, Pfeiffer DU, Morris RS. Risk factors for respiratory diseases in New Zealand pig herds. N Z Vet J. 1998 Feb 1; 46(1):3–10. https://doi.org/10.1080/00480169.1998.36043 PMID: 16032003

Supplementary information



Supplementary Figure 1. *a.* App ATCC viable control (V) – no kill b. App ATCC killed by boiling (PMA treated- D) c. Ecas4 5% killed (5EAppATCC) without PMA treatment. c. 10% Ecas4 killed (E10App ATCC)- no PMA treatment and e. NTC- no template control

App- Actinobacillus pleuropneumoniae, 5% Ecas4 – 15 ppm free available chlorine (FAC), 10% Ecas4 – 30 ppm FAC



Supplementary Figure 2. *E. coli* ATCC viable control (V1) – no kill b. E. coli ATCC killed by boiling (PMA treated- D1) b. Ecas4 5% killed (E5 E. coli ATCC) without PMA treatment. C. 10% Ecas4 killed (E10E. coli ATCC)- no PMA treatment and D. NTC- no template control

E. coli- Escherichia coli, 5% Ecas4 – 15 ppm free available chlorine (FAC), 10% Ecas4 – 30 ppm FAC

Chapter 3: Reduction of *Salmonella* Enteritidis and total bacterial counts on eggs surface by a pH-neutral electrolyzed oxidizing water fog

3.1 STATEMENT OF AUTHORSHIP

Statement of Authorship

Title of Paper	Reduction of Salmonella Enteritidis and total bacterial counts on eggs surface by a pH-neutral electrolyzed oxidizing water fog
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Sangay Tenzin, Sergio Ferro, Permal Deo, Samiullah Khan and Darren J. Trott Submitted to PLOS ONE (26-02-2020)

Principal Author

Name of Principal Author (Candidate)	Sangay Tenzin		
Contribution to the Paper	Conceptualized and developed methodology, conducted all experimental procedures, analyzed data, wrote original draft and edited the manuscript.		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	18/02/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Dr. Sergio Ferro		
Contribution to the Paper	Conceptualization, resources and manuscript review and editing.		
Signature		Date	19/02/2020

Name of Co-Author	Dr. Permal Deo		
Contribution to the Paper	Associate supervisor, conceptualization, resources and manuscript revision and editing.		
Signature		Date	24/02/2020

Name of Co-Author	Dr. Samiullah Khan		
Contribution to the Paper	Methodology, resources and manuscript revision and editing.		
Signature		Date	19/02/2020

Name of Co-Author	Professor Darren J. Trott		
Contribution to the Paper	Principal supervisor, conceptualization, administer the project, acquired funding, and manuscript revision and editing		
Signature		Date	20/02/2020

3.2 ORIGINAL ARTICLE

Reduction of *Salmonella* Enteritidis and total bacterial counts on eggs surface by a pH-neutral electrolyzed oxidizing water fog

Sangay Tenzin[1, #], Sergio Ferro[2], Permal Deo[3], Samiullah Khan[4], and Darren J. Trott[1, #, *]

[1]Australian Centre for Antimicrobial Resistance Ecology, School of Animal and Veterinary Sciences, The University of Adelaide, Mudla Wirra Rd, Roseworthy 5371, Australia

[2]Ecas4 Australia Pty. Ltd., Unit 8 / 1 London Road, Mile End South SA 5031, Australia.

[3]School of Pharmacy and Medical Sciences, University of South Australia, Adelaide 5000, Australia

[4]School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy 5371, Australia

Corresponding authors: sangay.tenzin@adelaide.edu.au and darren.trott@adelaide.edu.au

Abstract

The effectiveness of an aerosol of pH-neutral electrochemically activated solution (ECAS) containing 150 mg/L of free available chlorine in eliminating *Salmonella* Enteritidis and the total bacteria load from eggs surface has been investigated. Some eggs were deliberately contaminated by immersion in a *Salmonella* Enteritidis inoculum (10^5 CFU/mL of broth) and then incubated at 37 °C for 18-24 h before further treatments. Two treatment groups were established: sanitization of eggs with autochthonous bacterial load, and of eggs colonized with *S.* Enteritidis; both groups included no treatment, treatment with tap water (fog and spray wash), treatment with sodium hypochlorite (spray wash with NaOCl) and treatment with ECAS (fog and spray wash). For the total bacteria sanitization experiments, all the treated groups were compared with the unwashed eggs since the bacterial load on eggs sprayed or fogged with tap water was similar (2.2 ± 0.2 $\log_{10}$ CFU/egg) to that on unwashed eggs (control). Spray washing with ECAS at 150 ppm for 45 s and fogging of individual eggs for 2 min resulted in a significant reduction of total bacteria counts (> log 2.21 CFU/egg). However, eggs fogged with ECAS for 1 min showed a reduction of 0.15 $\log_{10}$ CFU/egg while the simultaneous fogging of 3 eggs for 2 min led to 1.5 $\log_{10}$ CFU/egg reduction in total bacteria load. In the case of *S.* Enteritidis experiments, fogging of individual eggs with ECAS for 1 and 2 min led to reductions of 3.2 and 4.3 $\log_{10}$, respectively. NaOCl spray sanitization for 45 s resulted in a total reduction of total bacterial load (2.41 $\log_{10}$) and *S.* Enteritidis (4.37 $\log_{10}$). The effectiveness of the ECAS spray- and fog-washes to significantly reduce both the total bacteria counts and *S.* Enteritidis from the egg's surface suggests the use of ECAS as an alternative sanitizing agent for both hatching and table eggs.

1. Introduction

Pathogenic serovars of *Salmonella* are a major cause of foodborne diseases worldwide. The number of cases of foodborne salmonellosis in Australia amounts to around 40,000 per year, out of an estimated total of about 4.1 million foodborne gastroenteritis (Kirk et al., 2014). *Salmonella* associated foodborne illnesses have risen during the past 20 years and the rate of salmonellosis in Australia is much higher than in economically similar countries (NNDSS, 2015). It has been estimated that foodborne illnesses due to *Salmonella* spp. have caused 35% of hospitalizations and 28% of cases of mortality due to foodborne diseases (Kirk et al., 2014); the cases of hospitalization and death resulting from salmonellosis are greater than those due to foodborne diseases of different origin (Kirk et al., 2014). Among the causes of foodborne salmonellosis, raw eggs and egg products have the highest frequency (Braden, 2006; Moffatt et al., 2016; Painter et al., 2013). For example, between 2001 and 2016, 50% of all foodborne *Salmonella* diseases in Australia were attributed to contaminated eggs (Ford et al., 2018); of these, 84% were caused by *S.* Typhimurium and only 3 cases by *S.* Enteritidis (Ford et al., 2018). In contrast, Salmonellosis in other countries is mainly caused by the Enteritidis serotype (Belanger et al., 2015; Jackson et al., 2013; Sasaki et al., 2011). In addition to being a public health hazard, foodborne illnesses have an estimated cost of AUD 1.25 billion per annum to the Australian economy (Kirk et al., 2014). Therefore, since egg consumption in Australia is around 245 eggs per capita and continues to grow (AECL, 2018), the industry is constantly looking for alternative means to address *Salmonella* contamination.

Unlike the one-off costs for the establishment of farm infrastructure and human resources, farms and industries incur ongoing costs for egg hygiene and egg safety management. One of the key components of safe egg production includes washing and disinfection of the eggshell surface from organic materials and bacteria to reduce the risk of egg-related foodborne illnesses and to maintain consumer confidence in the microbiological safety of eggs. Decontamination of the total bacterial population, as well as pathogenic bacteria from the eggshell surface, is achieved using several techniques and many methods of sanitizing eggshells are also used to reduce the contamination of eggs by *Salmonella* in commercial egg production premises.

The protocols used for the inactivation of *Salmonella* cells can be broadly classified into thermal and non-thermal disinfection. Thermal disinfection, such as egg

pasteurization, is a highly effective method, but negatively affects egg proteins and rheological properties (Rodriguez Romo, 2004). The most common non-thermal processes employ quaternary ammonium compounds (QACs) and chlorine-based chemicals (Al-Ajeeli, 2013) to sanitize the eggs after washing with a high pH detergent solution (close to 11) at a temperature above 40 °C, due to the easy availability and relatively low cost. In addition, bacteria can develop resistance to QACs (Sundheim et al., 1998), which in turn induces the selection of genes for co-resistance to other antimicrobials including antibiotics (Fernandez Marquez et al., 2017), thus limiting their use. In the case of chlorine-based sanitizer, besides the possible development of resistance by bacteria in case of persistent usage (Ridgway and Olson, 1982), the accumulation of organic load from dirt, manure and broken eggs reduce the chlorine concentration, which affects the effectiveness and leads to potential contamination. Moreover, due to the environmental impacts caused by chlorine by-products and problems with wastewater disposal, its use is often avoided. Other methods of decontamination include ultraviolet (UV) irradiation of eggshells after washing and formaldehyde fumigation. Unfortunately, the antibacterial activity of the UV irradiation protocol is limited to the surface of eggshell directly exposed to UV radiation (De Reu et al., 2006a), while formaldehyde is a known occupational hazard, being a carcinogenic substance (IARC, 2006).

Since occupational safety and environmental regulations continually push towards safe alternative eggshell sanitizers, electrochemically activated solutions (ECASs) could be a potential alternative for eggshell cleaning and disinfection. The three forms of ECAS (acidic, slightly acidic and neutral) have been evaluated in the sanitisation of table eggs for food safety reasons (Achiwa and Nishio, 2003; Bialka et al., 2004; Rivera-Garcia et al., 2019; Zang et al., 2019) and of hatching eggs for the production of quality chicks (Fasenko et al., 2009). Majority of the research used two-step processes to assess the effectiveness of ECAS in disinfection of eggs. In the initial washing phase, dirt and debris are removed from the egg surface with water or alkaline detergent, followed by disinfection with ECAS. Bialka et al. (2004) reported that immersion washing of eggs with acidic ECAS at 70-80 mg/L of free available chlorine (FAC) significantly inactivated *S.* Enteritidis (2.1 $\log_{10}$) and *E. coli* (2.3 $\log_{10}$) from the surface of the eggs but affected the egg cuticle layer significantly. The spray washing of eggs with slightly acidic ECAS (50 mg/L of FAC) allowed reducing the

total bacterial load (1.0 log_{10}) without affecting the cuticle layer (Fasenko et al., 2009). In another study, immersion washing in ECAS at pH-neutral and 12 mg/L of FAC was not so effective in significantly reducing the bacterial load due to inoculated *Listeria monocytogenes* (0.4-0.7 log_{10}) (Surdu et al., 2017),, while a spray wash with 46 mg/L of FAC allowed a significant decrease (2.18 log_{10}), without affecting the egg cuticle layer (Rivera-Garcia et al., 2019).

This study explored the potential of ECAS at neutral pH and 150 mg/L of FAC as an aerosol fog for the sanitization of unwashed and visibly clean eggs, with the objective of reducing the total bacterial counts and *Salmonella* (*S.*) *enterica* serovar Enteritidis, and to study its effect on the cuticle layer. The effectiveness of ECAS was compared to that of a common disinfectant sanitiser (sodium hypochlorite). The pH-neutral ECAS used in this study, produced by electrolysis of a dilute sodium chloride solution, mainly contains oxidizing agents (active chlorine compounds) such as hypochlorous acid (HOCl), hypochlorite ions and traces of dissolved gaseous chlorine (Cheng et al., 2012; Guentzel et al., 2008; Liao et al., 2007), which make it less corrosive and more durable than acidic and slightly acids forms. Sanitisation by fogging with ECAS could be incorporated into the cleaning and disinfection protocol to improve egg safety without the use of hazardous biocidal agents.

2. Materials and Methods

2.1 *Salmonella* isolate and chicken eggs

Salmonella enterica serovar Enteritidis 11RX was used in the current study. *S.* Enteritidis stored at -80 °C in 80% glycerol was plated on xylose lysine deoxycholate (XLD) agar (Oxoid, Australia) and incubated overnight at 37 °C. Fresh eggs were sourced on the day of laying from Hy-Line brown hens (aged 36 to 40 weeks) bred in conventional battery cages that house individual hens at the School of Animal and Veterinary Sciences, The University of Adelaide. The eggs, stored for 24 h at room temperature, were screened to discard those with thermal cracks and visible dirt; only relatively clean and intact eggs were selected for experimental tests, subdivided into two groups.

The first group of eggs was used to assess the effectiveness of the electrochemically activated solution (Ecas4 anolyte, from Ecas4 Australia Pty Ltd) as

a fogged disinfectant on total bacteria present on the surface of the eggs. This experimental group included unwashed eggs (control), spray-washed eggs with water, ECAS- or NaOCl-spray sanitized eggs, and water- or ECAS-fogged eggs (Experiment 1). For the second group of eggs, they were artificially inoculated with *S.* Enteritidis as described in section 2.2 below, and then used for fog- and spray-wash disinfection test (Experiment 2). Eggs used in Experiment 2 were subdivided into unwashed (control), spray-washed with water, spray-washed with ECAS or NaOCl, and fogged with water or ECAS, similarly to those of Experiment 1.

2.2. *Salmonella* Enteritidis seeding on outer eggshell surface

2.2.1. Pre-wash of eggs for S. **Enteritidis** *seeding*

To understand the true effects of ECAS on *Salmonella* load reduction, eggs were washed as previously described (Gole et al., 2014) before being inoculated with *S.* Enteritidis. Briefly, eggs were placed on an oscillating tray, which helped in exposing the entire eggshell surface, in a biosafety cabinet (BSC) and initially spray-washed with a 0.45% (v/v) solution of NaOCl (ThermoFisher Australia; pH ≈ 12 and ~200 ppm of FAC, at 40 °C) for 45 s. Then, spray-sanitized with a 0.16% (v/v) solution of NaOCl at 32 °C for 22 s, and left on the sterilized BSC bench to dry for 60 min. The eggs were sanitized to ensure the complete removal of microbiota of the egg surface and to achieve a uniform *S.* Enteritidis colonization of the egg surface.

2.2.2. Inoculum preparation and inoculation of eggs with S. **Enteritidis**

The inoculum was prepared from *S.* Enteritidis grown overnight at 37 °C on XLD agar by suspending colonies in phosphate buffered saline (1 × PBS) to obtain an absorbance (OD_{600nm}) value of 0.45. Viable *Salmonella* was enumerated by plating 10-fold serial dilutions of the inoculum on XLD agar and incubating overnight at 37 °C. After enumeration, a 200 mL inoculum containing ~10^5 CFU per mL was prepared in 1 × PBS.

For eggshell seeding, eggs were immersed and gently massaged for 90 s either in 1 × PBS (control) or in 1 × PBS containing ~10^5 CFU/mL of *S.* Enteritidis. Eggs were then placed into sterile zip lock bags and incubated at 37 °C to allow for full

colonization. After 18-24 h post-inoculation, three eggs were placed in individual Whirl-Pak bags (Nasco, USA) containing 20 mL of buffered peptone water (BPW) and massaged for 1 min. Aliquots (100 µL) from a 10-fold serial dilution were plated on XLD plates and incubated overnight at 37 °C for enumeration of *S.* Enteritidis.

2.3. ECAS spraying and fogging disinfection experiments

The physicochemical properties of ECAS, such as temperature, pH and ORP, were measured before its use with a model MC-80 handheld meter (TPS Pty Ltd, Australia). Free and total available chlorine were measured using a Free Chlorine Checker® HC-HI701 and a Total Chlorine Checker® HC-HI711 (Hanna Instruments). ECAS was stored at 4 ± 1°C and used within one week of preparation, once diluted with Milli-Q water to obtain 150 mg/L of free available chlorine (FAC) just before the sanitation experiment. All sanitization experiments were performed in a biosafety cabinet (BSC) by placings the eggs on an oscillating tray that helped exposing the entire surface of egg during the spray-wash and fogging experiments.

2.3.1. ECAS fogging disinfection of native bacteria and S. *Enteritidis* on eggshell surface

Both ECAS disinfection experiments, targeting respectively the total bacterial counts (Experiment 1) and *S.* Enteritidis (Experiment 2), included a control group (no wash), spray treatment group with ECAS or water, spray wash group with NaOCl, fogging with ECAS or water of 3 eggs simultaneously for 1 and 2 min, and fogging with ECAS or water of individual eggs for 1 and 2 min. All experiments were repeated twice.

The total bacterial count disinfection experiment was performed on visibly clean and unwashed eggs with three eggs assigned to all types of treatment, including the unwashed control. For the spray wash groups, ECAS (at 150 ppm of FAC), NaOCl (~200 mg/L of FAC), and water were sprayed for 45 s using a portable sprayer. For the fog sanitization groups, ECAS (at 150 ppm of FAC) and water were fogged for 1 and 2 min using an ultrasonic humidifier that generates a fog of droplets sized between 1 and 3 micrometres (Ultrasonic Humidifier HU-85, Contronics Engineering BV, The

Netherlands). The first fogging disinfections were performed simultaneously on three eggs placed in separate compartments on the oscillating tray for 1 and 2 min. The second fogging sanitization was performed on individual eggs (3 repetitions per sanitizer and washing group) for both durations.

Each individual egg from the control (no wash), spray washing, and fog sanitization experiments was immediately placed in a sterile Whirl-Pak bag containing 5 mL of BPW, massaged gently for 1 min and the mixture transferred into a 10-mL sterile centrifuge tube with screw cap (SARSTEDT Australia Pty Ltd). The samples were centrifuged at 5,444 × *g* (MPW-351e Centrifuge, Med Instruments, Adelab Scientific, Australia) for 10 min and the supernatant discard by gentle pipetting; the pellets were resuspended in 200 µL of 1 × PBS, and 10-fold serial dilutions prepared in 1 × PBS for each eggs. Aliquots of 100 µL of the various serial dilutions (up to 10^{-5}) were plated on plate count agar (PCA) media in duplicate and incubated overnight at 37 °C for enumeration of the number of colonies.

The *S.* Enteritidis washing and fogging sanitization experiments were performed as described above using eggs colonized with *S.* Enteritidis. *Salmonella* enumeration eggs were placed in 10 mL of BPW and massaged for 1 min; 10-fold serial dilutions were prepared in 1 × PBS and dilutions up to 10^{-7} were plated on both PCA and XLD agar media.

2.4 Eggshell cuticle assessment

Twelve eggs were selected for each group (spray washing with water or ECAS for 45 s, and fogging with water or ECAS for 2 min) based on colour intensity measured using a MiniScan EZ colourimeter (4500L Spectrophotometer, Hunter Associates Laboratory, Inc., USA). The selected eggs were treated as described in section 2.3.1 and dried in a clean biosafety cabinet for 60 min. Then, the eggs were stained with MST Cuticle Blue dye (MS Technologies, UK) following the method by Samiullah et al. (Samiullah et al., 2016). The cuticle coverage was assessed using the ΔE_{ab}^* method (Samiullah et al., 2016). The average of four readings of the L*, a* and b* values, before and after staining, were used for the calculation of ΔE_{ab}^* with the following equation:

$$\Delta E_{ab}^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$

A higher value of ΔE_{ab}^* indicates a greater affinity of the cuticle for the dye and therefore more cuticle coverage.

2.5. Statistical analysis

Samples that did not produce colonies on PCA and XLD plates were assigned a CFU value of 1 to allow transformation into log values. Bacterial count data (expressed as log_{10}/egg) were analysed using the ANOVA option in GraphPad Prism v8 (GraphPad Software, San Diego, CA, USA). Since the bacterial count was log transformed (assuming normal distribution of data) and each experiment has an equal number of samples, ordinary one-way ANOVA was performed to compare differences of means among no sanitizer wash control and different sanitizing treatments (significance at p value of < 0.05). If the ANOVA results among the groups were significant, the level of significance between the treatment groups was separated by Tukey's Honestly Significant Differences (HSD) with p value < 0.05.

3. Results

3.1. ECAS fogging reduced total bacterial counts on egg surface

For the total bacterial counts, the average bacterial count (log_{10} CFU/egg) present on the eggs before and after the different sanitizing treatments is reported in Figure 1 and Table 1. On average, the total bacterial count on unwashed control eggs was 2.2 ± 0.2, 2.4 ± 0.1 on water-sprayed eggs, and 2.4 ± 0.2 on water-fogged eggs. Since all control groups had a similar level of total bacterial counts and were not significantly different, the log_{10} bacterial reduction for the different types of sanitizing wash was assessed based on bacterial counts on unwashed eggs (Table 1). The ordinary one-way ANOVA analysis showed significant differences in the reduction of bacterial count on eggs surface among the sanitization groups ($p = 0.01$). The follow-up post hoc Tukey's honestly significant detection (HSD) test showed spraying with water, fogging with water for 1 and 2 min, and fogging with ECAS for 1 min did not significantly reduce the total bacterial load in comparison to that of unwashed eggs. When eggs were sanitized either simultaneously or individually by fogging with ECAS for 1 min, a non-significant reduction (0.1 – 0.2 log_{10}) was observed. However, an

extension of the fogging with ECAS to 2 min significantly reduced ($p \leq 0.001$) the bacterial load by 1.4 $\log_{10}$ CFU/egg when 3 eggs were fogged simultaneously. Interestingly, a higher significant reduction ($p \leq 0.001$) in bacterial load ($\geq$ 2.2 $\log_{10}$ reduction) was observed when the eggs were spray-washed with either 0.45% (v/v) NaOCl or ECAS for 45 s, or when individual eggs were fogged with ECAS for 2 min.

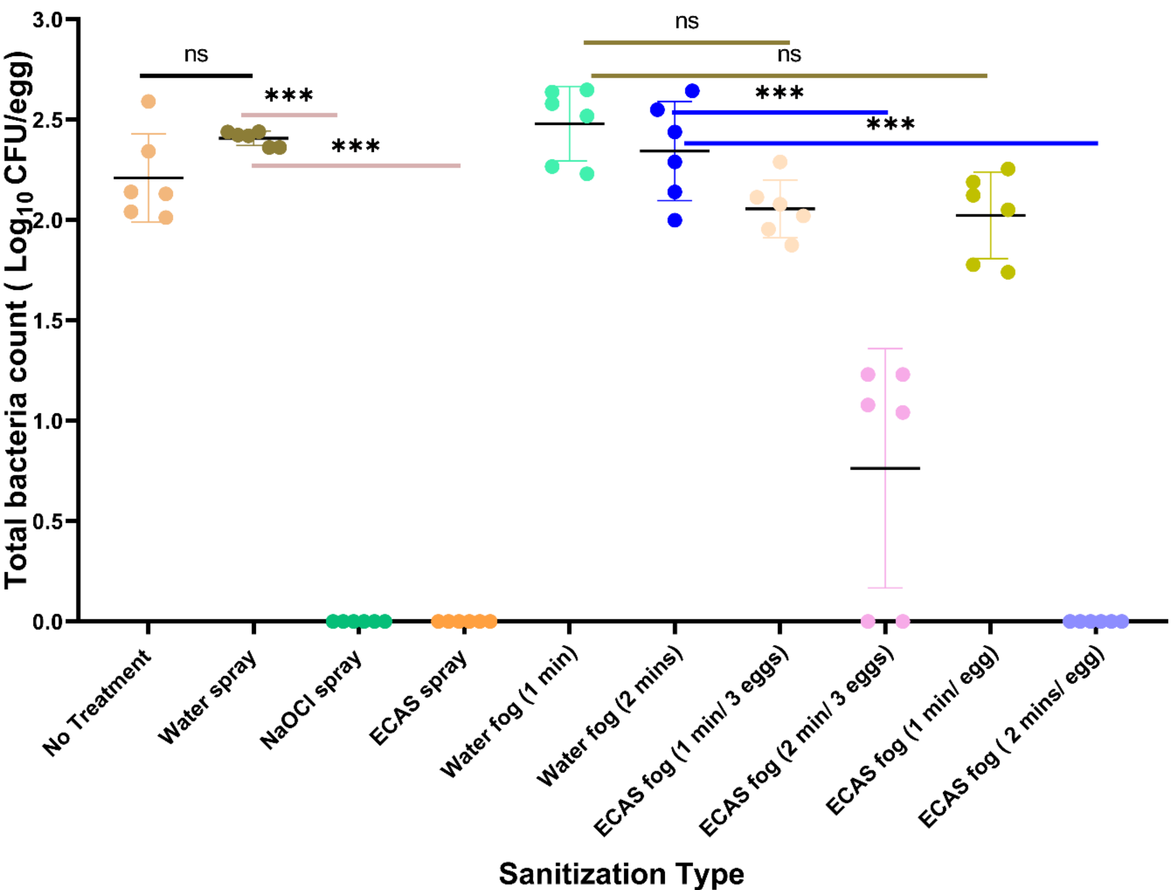


Fig 1. Scattered plot of total bacteria experiments. Each dot represents a $\log_{10}$-transformed bacterial count (CFU/egg), and each sanitization type has counts resulting from six samples. NaOCl at 0.45% (v/v) and ECAS at neutral pH and 150 ppm of free available chlorine were used for the sanitization. ns – not significant; *** - $p < 0.001$.

Table 1. Effect of ECAS on the reduction of total bacterial load on eggs surface.

Treatment	**Average bacterial count (log_{10} CFU/egg)**	**Log reduction[&]**	**Percent reduction**	**Tukey's HSD (*p*)**
Control	2.2 ± 0.2	0	0	
Water spray (45 s)	2.4 ± 0.1	+0.2	0	0.908
0.45% NaOCl spray (45 s)	0	2.2	~100	<.001
ECAS spray (45 s)	0	2.2	~100	<.001
Water fog (1 min)	2.5 ± 0.2	+0.3	0	0.624
Water fog (2 min)	2.3 ± 0.3	+0.1	0	0.992
ECAS fog (1 min / 3 eggs) *	2.1 ± 0.1	0.4	34.8	0.980
ECAS fog (2 min / 3 eggs) *	0.8 ± 0.6	1.5	94.6	<.001
ECAS fog (1 min / egg) [#]	2.0 ± 0.2	0.5	36.5	0.935
ECAS fog (2 min / egg) [#]	0	2.4	~100	0.001

Bacterial counts were calculated in log_{10} CFU/egg. There were 6 eggs in each treatment group. Values are mean ± standard deviation (SD).
* 3 eggs fogged simultaneously; [#] single egg fogged at a time; + shows a log_{10} increase in count
[&] log_{10} reduction = log_{10} counts on unwashed eggs (control) – log_{10} counts on eggs after sanitizing wash

3.2 ECAS fogging reduced *S.* Enteritidis on egg surface

S. Enteritidis counts (log_{10} CFU/egg and log_{10} reduction data) are presented in Figure 2 and Table 2. Initial *S.* Enteritidis counts, obtained from the unwashed control eggs, were $2.5 \pm 0.7 \times 10^5$ CFU/ egg (5.4 ± 0.1 log_{10} CFU/egg) after 18-24 h of incubation at 37 °C. Since spray-washing for 45 s and fogging for 2 min with water showed a significant reduction ($p < 0.001$) of *S.* Enteritidis counts, the log_{10} reductions of *S.* Enteritidis on eggs spray-washed with either NaOCl or ECAS and fogged with ECAS were assessed against the respective group controls.

One-way ANOVA test on the effectiveness of the different types of sanitation on artificially seeded *S.* Enteritidis on eggs surface showed a significant difference ($p < 0.001$) among the means of the sanitizing washing groups. Furthermore, the reduction significance between the treatment variables was evaluated using post hoc Tukey's HSD test.

Fogging with water for 1 min did not significantly reduce the *S.* Enteritidis counts compared to unwashed eggs (control), whereas water spraying and fogging for 2 min significantly reduced ($p < 0.001$) *Salmonella* counts, leading to $\log_{10}$ reductions of 1.0 and 1.1, respectively. Hence, disinfection effectiveness of spray washing, 1-min and 2-min fogging treatments were compared with water-spray washed, 1-min and 2-min fogged eggs, respectively. The *Salmonella* reductions observed for all types of treatment were statistically significant ($p < 0.001$).

The simultaneous fogging of 3 eggs with ECAS for 1 min significantly reduced ($p < 0.001$)the *S.* Enteritidis counts on the eggs surface (2.7 $\log_{10}$ reduction), and a similar reduction was obtained by extending the fogging with ECAS to 2 min (2.5 $\log_{10}$ reduction). A better result was observed when individual eggs were fogged with ECAS for 1 min (3.3 $\log_{10}$ reduction), and a 4.3 $\log_{10}$ reduction of *S.* Enteritidis was obtained when individual eggs were fogged for 2 min with ECAS, an outcome comparable to that obtained for eggs spray-washed with either NaOCl or ECAS (4.4 $\log_{10}$ reduction).

Overall, these results suggest that a spray-wash with ECAS (at 150 mg/L of FAC) for 45 s or a fogging for 2 min could be used to reduce total bacterial and *S.* Enteritidis counts on eggs surface. On the other hand, fogging with ECAS for 1 min did not lead to a significant reduction in bacterial load, suggesting that the desired outcome is achieved only when the treatment is more prolonged.

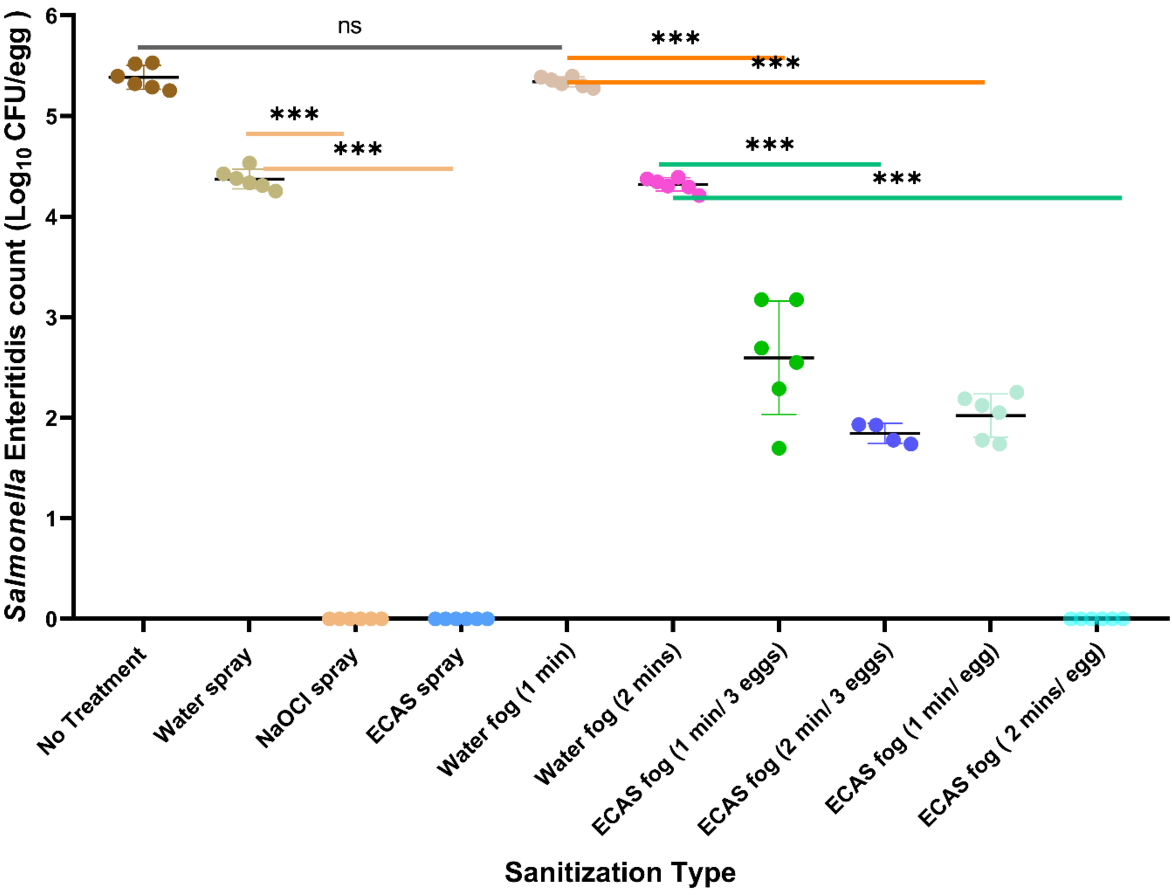


Fig 2. Scattered plot of *S.* Enteritidis experiments. Each dot represents a $\log_{10}$-transformed bacterial count (CFU/egg), and each sanitization type has counts resulting from six samples. NaOCl at 0.45% (v/v) and ECAS at neutral pH and 150 ppm of free available chlorine were used for the sanitization. ns - not significant, *** - $p < 0.001$.

Table 2. Effect of ECAS treatment on the reduction level of *S.* Enteritidis counts on egg surface.

Treatment	**Average *S.* Enteritidis counts (log_{10} CFU/egg)**	**Log reduction[&]**	**Percent reduction**	**Tukey's HSD (*p*)**
Control	5.4 ± 0.1	0	0	
Water spray (45 s)	4.4 ± 0.1	1.0	90.4	<.001
0.45% NaOCl spray (45 s)	0	4.4	100	<.001
ECAS spray (45 s)	0	4.4	100	<.001
Water fog (1 min)	5.3 ± 0.1	0.1	12.2	<.999
Water fog (2 min)	4.3 ± 0.1	1.1	91.6	<.001
ECAS fog (1 min / 3 eggs) *	2.6 ± 0.6	2.7	99.7	<.001
ECAS fog (2 min / 3 eggs) *	1.2 ± 1.0	3.1	99.8	<.001
ECAS fog (1 min / egg) [#]	2.0 ± 0.2	3.3	99.9	<.001
ECAS fog (2 min / egg) [#]	0	4.3	100	<.001

S. Enteritidis counts were calculated in log_{10} CFU/egg. There were 6 eggs in each treatment groups. Values are mean ± standard deviation (SD).
* 3 eggs fogged simultaneously; [#] single egg fogged at a time
[&] log_{10} reduction: see text for details

3.4 ECAS fogging did not affect the egg cuticle

All eggs, after the dye treatment, had a consistent light green colour. A one-way ANOVA for the effect of wash treatment on cuticle coverage (ΔE_{ab}^*) was performed to compare the effect of spray washing and fogging with ECAS, with the analogous treatments realized using tap water.

The analysis, which compared the group means of ΔE_{ab}^* among all wash types, did not reveal any significant differences ($p = 0.39$). The mean and standard deviation of the mean ΔE_{ab}^* for all the treatment options are presented in Figure 3.

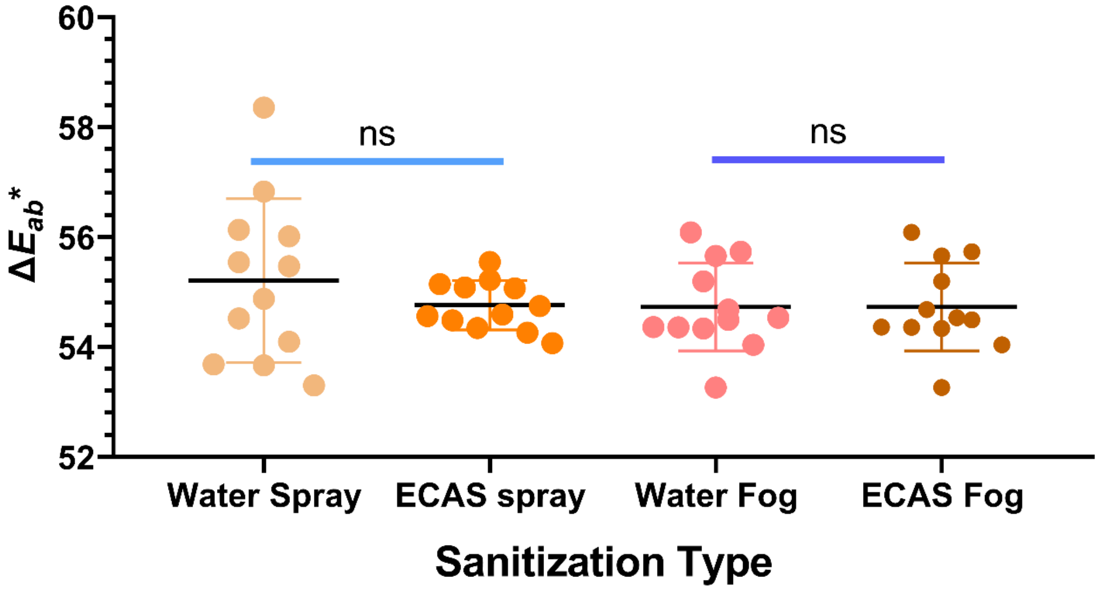


Fig 3. Scattered plot showing values for the mean of ΔE_{ab}^{*} as a measure of cuticle coverage. The thick line marks the mean, while the whiskers above and below denote SD of the mean (n = 12 eggs in each group); ns - not significant.

4. Discussion

This study assessed the effectiveness of a spray-wash and fogging with ECAS in reducing the total bacterial load and *S.* Enteritidis (inoculated) on eggs surfaces and their effect on the cuticle layer. Spray-washing of eggs with ECAS for 45 s and fogging of individual eggs for 2 min completely reduced the native total bacterial load and *S.* Enteritidis on eggshell surfaces. These two sanitization approaches showed a disinfection efficacy similar to that of washing with sodium hypochlorite for 45 s. Moreover, the efficacy of ECAS in reducing the total bacterial load and *S.* Enteritidis from egg surfaces was greater than the bacterial reduction observed in previous studies on the sanitization of eggshells with electrolysed water at nearly neutral pH and acidic pH (Bialka et al., 2004; Fasenko et al., 2009; Ni et al., 2014).

The bacterial load on eggshells is usually acquired through contamination from the farm environment; therefore, the type of breeding and the poultry housing system influence the total bacterial count on egg surfaces (Alvarez-Fernandez et al., 2012; De Reu et al., 2005; De Reu et al., 2006b; De Reu et al., 2009). Eggshells from conventional caged hens usually harbour lower total bacterial counts compared to

other housing systems (Đukić-Stojčić et al., 2009). The total bacterial load of 2.2 ± 0.2 log_{10} CFU/egg found in this study was slightly lower than those observed by Wall et al. (2008) (2.7 log_{10}) and Alvarez-Fernandez et al. (2012) (2.34 log_{10}) on eggshells from conventional-caged hens, probably because the eggs considered in this study were laid by individually housed hens with a low density of hens in the shed (49 hens/shed).

The unwashed, spray-washed with water, and fogged with water eggs had a similar (i.e. no significant difference) total bacterial load (2.2 – 2.5 log_{10} CFU/egg). Fogging of multiple eggs (3 simultaneously) as well as of a single egg at a time with ECAS for 1 min reduced the total bacterial count by 0.1 and 0.2 log_{10} CFU/egg, respectively, but the reduction was not significant (p values of 0.980 and 0.935, respectively) compared to the non-sanitized samples. In contrast, ECAS fogging of 3 eggs simultaneously for 2 min led to a statistically significant reduction (1.4 log_{10}) in the total bacterial count, while a 2.2 log_{10} reduction was found by treating a single egg at a time. The slightly lower reduction found in treating multiple eggs could be due to an uneven distribution of the fog, as fogging was performed in a biological safety cabinet that quickly sucked out the surrounding air, including the fog. A complete reduction of the total bacterial load was also achieved by spraying eggs with ECAS or with sodium hypochlorite solution (control). Although no literature information is available on the sanitization of egg surfaces with an ECAS fog, previous research has shown a significant reduction in total bacterial counts on egg surfaces when spray-washed with neutral (Ni et al., 2014) or acidic ECAS (Fasenko et al., 2009; Ni et al., 2014).

Numerous studies have discussed the effectiveness of the various forms of electrolysed water (acidic, slightly acidic, alkaline) by immersion or spray-washing of egg surfaces inoculated with *S.* Enteritidis (Cao et al., 2009; Park et al., 2005; Venkitanarayanan et al., 1999). In this study, we assessed a neutral pH ECAS in the form of a fog, in addition to spray washing. The log_{10} reductions observed for eggs sprayed with water (control) and fogged with water (2 min) were probably due to a dislodgment of inoculated *S.* Enteritidis from the egg surfaces. The fogging of eggs with ECAS at 150 mg/L of FAC for 1 min, either individually or simultaneously, led to a reduction of *S.* Enteritidis of about 3 log_{10} CFU/egg. These findings are different from those from a previous study (Cao et al., 2009), in which a total reduction of

Salmonella Enteritidis (6.5 log_{10} CFU/g) after washing eggs with acidic and slightly acidic EO water at 15 mg/L of FAC was reported. As previously mentioned, the lower efficacy of the disinfection in the current study is most likely due to the fog being quickly sucked out by the BSC and not moistening the egg surface with sufficient FAC to reduce the bacterial counts. A total reduction of *S.* Enteritidis was observed when the eggs were spray-washed with either ECAS at 150 mg/L of FAC for 45 s or with the sodium hypochlorite control solution. The sterilization efficacy of the latter is attributable to its higher concentration of FAC (200 mg/L) and higher pH (12.0) (Cao et al., 2009).

Although electrolysed water is an environmentally friendly, non-hazardous sanitizer with proven antibacterial efficacy against foodborne pathogens such as *Escherichia coli* O157:H7, *Listeria monocytogenes* (Park et al., 2004) and *S.* Enteritidis on eggshells (Cao et al., 2009; Park et al., 2005), its use for decontamination in commercial environments, to obtain pathogen-free eggs, has not yet been taken into consideration. The reasons are probably related to the production cost of the solution, its corrosiveness towards steel surfaces (especially in the case of acid products), and the lack of consumer knowledge on the impact of chemicals for disinfecting the environment. The reasons are due to the cost of producing the solution, its corrosiveness towards steel surfaces and the lack of consumer knowledge on the impact of chemicals for disinfecting the environment.

The current scenario shows a shift in table egg production towards free-range system (Parisi et al., 2015). For instance, in Australia, this production has increased from 39% in 2015 (AECL, 2015) to 45% in 2018 (AECL, 2018), driven by consumer demand for bird welfare and access to a range area. However, this approach poses risks to the health of the human population as the eggshell bacterial load, including the *Campylobacter* and *Salmonella* spp. counts (Parisi et al., 2015), in such a production system, is higher (De Reu et al., 2008; Parisi et al., 2015), compared to conventional battery cage eggs. The problem is further aggravated by the increase in the consumption of meals consisting of raw egg products (Kretser et al., 2014). Therefore, egg producers should consider cleaning and disinfecting the surface of table eggs as a priority for producing safe eggs and maintaining consumer confidence. Besides issues with wastewater disposal, washing eggs with commonly used chlorine-based products

requires intensive monitoring of the water temperature, pH and chlorine concentration to retain its optimal effectiveness and the integrity of the eggshell cuticle.

In conclusion, the reduction of the total bacterial load and *S.* Enteritidis through the use of ECAS at neutral pH in the form of aerosol, and without affecting the cuticle layer, show that this form of ECAS could be used as an alternative sanitising agent in the cleaning and disinfection of eggs. Moreover, this disinfection protocol is easily implemented because ECAS can be easily generated on-site and already has automatic controls for FAC concentration and pH measurements. In order for this process to lead to large-scale testing and industrial implementation, further tests, such as internal egg quality and a sensory evaluation by consumers (as per the regulatory requirements), need to be performed.

Acknowledgements

Sangay Tenzin received Endeavour Postgraduate Scholarship Program, Australia for his PhD.

Author contributions

S.T. (Conceptualization, Methodology, Formal analysis, Investigation, Writing-Original, Review and Editing, Resources, Visualization), S.F. (Conceptualization, Resources and Writing- Review and Editing), P.D. (Conceptualization, Resources, Supervision and Writing- Review and Editing), S.K. (Resources, Methodology, Writing- Review and Editing) and D.J.T. (Conceptualization, Resources, Supervision, Writing- Review and Editing).

Declaration of interest

Dr S. Ferro is the Technical Manager of Ecas4 Australia Pty Ltd; however, Ecas4 Australia had no role in the study design, collection, and interpretation of data, writing, and decision to submit the article for publication.

References

Achiwa, N., Nishio, T., 2003. The use of electrolyzed water for sanitation control of eggshells and GP center. Food science and technology research 9, 100-103. doi: 10.3136/fstr.9.100

AECL 2015. Australian Eggs Corporation. Annual Report 2015. North Sydney, NSW; 2015 [cited 2019 July 21] Available from: https://www.australianeggs.org.au/dmsdocument/751-annual-report-2015

AECL 2018. Australian Egg Corporation. Annual Report 2018. North Sydney, NSW; 2018 [cited 2019 July 20]. Available from: https://www.australianeggs.org.au/dmsdocument/881-annual-report-2018

Al-Ajeeli, M.N., 2013. Development of Best Practices for Shell Egg Disinfection Based upon Efficacy and Egg Quality. https://oaktrust.library.tamu.edu/handle/1969.1/151915

Alvarez-Fernandez, E., Dominguez-Rodriguez, J., Capita, R., Alonso-Calleja, C., 2012. Influence of housing systems on microbial load and antimicrobial resistance patterns of Escherichia coli isolates from eggs produced for human consumption. J Food Prot 75, 847-853. doi: 10.4315/0362-028X.JFP-11-182

Belanger, P., Tanguay, F., Hamel, M., Phypers, M., 2015. An overview of foodborne outbreaks in Canada reported through Outbreak Summaries: 2008-2014. Can Commun Dis Rep 41, 254-262. doi: 10.14745/ccdr.v41i11a01

Bialka, K.L., Demirci, A., Knabel, S.J., Patterson, P.H., Puri, V.M., 2004b. Efficacy of electrolyzed oxidizing water for the microbial safety and quality of eggs. Poult Sci 83, 2071-2078. doi: 10.1093/ps/83.12.2071

Braden, C.R., 2006. Salmonella enterica serotype Enteritidis and eggs: a national epidemic in the United States. Clin Infect Dis 43, 512-517. doi: 10.1086/505973

Cao, W., Zhu, Z.W., Shi, Z.X., Wang, C.Y., Li, B.M., 2009. Efficiency of slightly acidic electrolyzed water for inactivation of Salmonella enteritidis and its contaminated shell eggs. Int J Food Microbiol 130, 88-93. doi: 10.1016/j.ijfoodmicro.2008.12.021

Cheng, K.-C., Dev, S.R., Bialka, K.L., Demirci, A. 2012. Electrolyzed oxidizing water for microbial decontamination of food, In: Microbial decontamination in the food industry. Elsevier, 563-591. doi: 10.1533/9780857095756.3.563

De Reu, K., Grijspeerdt, K., Herman, L., Heyndrickx, M., Uyttendaele, M., Debevere, J., Putirulan, F.F., Bolder, N.M., 2006. The effect of a commercial UV disinfection system on the bacterial load of shell eggs. Lett Appl Microbiol 42, 144-148. doi: 10.1111/j.1472-765X.2005.01825.x

De Reu, K., Grijspeerdt, K., Heyndrickx, M., Zoons, J., De Baere, K., Uyttendaele, M., Debevere, J., Herman, L., 2005. Bacterial eggshell contamination in conventional cages, furnished cages and aviary housing systems for laying hens. Br Poult Sci 46, 149-155. doi: 10.1080/00071660500065359

De Reu, K., Grijspeerdt, K., Messens, W., Heyndrickx, M., Uyttendaele, M., Debevere, J., Herman, L., 2006b. Eggshell factors influencing eggshell penetration and whole egg contamination by different bacteria, including Salmonella enteritidis. Int J Food Microbiol 112, 253-260. doi: 10.1016/j.ijfoodmicro.2006.04.011

De Reu, K., Messens, W., Heyndrickx, M., Rodenburg, T., Uyttendaele, M., Herman, L., 2008. Bacterial contamination of table eggs and the influence of housing systems. Worlds Poult Sci J 64, 5-19. doi: 10.1017/S0043933907001687

De Reu, K., Rodenburg, T.B., Grijspeerdt, K., Messens, W., Heyndrickx, M., Tuyttens, F.A., Sonck, B., Zoons, J., Herman, L., 2009. Bacteriological contamination, dirt, and cracks of eggshells in furnished cages and noncage systems for laying hens: an international on-farm comparison. Poult Sci 88, 2442-2448.

Đukić-Stojčić, M., Perić, L., Bjedov, S., Milošević, N., 2009. The quality of table eggs produced in different housing systems. Biotechnol Anim Husb 25, 1103-1108.

Fasenko, G.M., O'Dea Christopher, E.E., McMullen, L.M., 2009. Spraying hatching eggs with electrolyzed oxidizing water reduces eggshell microbial load without compromising broiler production parameters. Poult Sci 88, 1121-1127.

Fernandez Marquez, M.L., Burgos, M.J.G., Pulido, R.P., Gálvez, A., López, R.L., 2017. Biocide tolerance and antibiotic resistance in Salmonella isolates from hen eggshells. Foodborne Path Dis 14, 89-95. doi: 10.1089/fpd.2016.2182

Ford, L., Moffatt, C.R., Fearnley, E., Miller, M., Gregory, J., Sloan-Gardner, T.S., Polkinghorne, B.G., Bell, R., Franklin, N., Williamson, D.A., 2018. The

Epidemiology of Salmonella enterica Outbreaks in Australia, 2001–2016. Front Sustain Food Syst 2, 86. doi: 10.3389/fsufs.2018.00086

Gole, V.C., Chousalkar, K.K., Roberts, J.R., Sexton, M., May, D., Tan, J., Kiermeier, A., 2014. Effect of egg washing and correlation between eggshell characteristics and egg penetration by various Salmonella Typhimurium strains. PLoS One 9, e90987.

Guentzel, J.L., Liang Lam, K., Callan, M.A., Emmons, S.A., Dunham, V.L., 2008. Reduction of bacteria on spinach, lettuce, and surfaces in food service areas using neutral electrolyzed oxidizing water. Food Microbiol 25, 36-41. doi: 10.1016/j.fm.2007.08.003

IARC. 2006. Formaldehyde, 2-Butoxyethanol and 1-tert-Butoxypropan-2-ol, In: Formaldehyde, 2-butoxyethanol and 1-tert-butoxypropan-2-ol. IARC Monogr. Eval. Carcinog. Risks Hum., 1-287. PMID: 17366697

Jackson, B.R., Griffin, P.M., Cole, D., Walsh, K.A., Chai, S.J., 2013. Outbreak-associated Salmonella enterica serotypes and food Commodities, United States, 1998-2008. Emerg Infect Dis 19, 1239-1244. doi: 10.3201/eid1908.121511

Kirk, M., Ford, L., Glass, K., Hall, G., 2014. Foodborne illness, Australia, circa 2000 and circa 2010. Emerg Infect Dis 20, 1857. doi: 10.3201/eid2011.131315

Kretser, A., Dunn, C., DeVirgiliis, R., Levine, K., 2014. Utility of a new food value analysis application to evaluate trade-offs when making food selections. Nutr Today 49, 185-195. doi: 10.1097/NT.0000000000000040

Liao, L.B., Chen, W.M., Xiao, X.M., 2007. The generation and inactivation mechanism of oxidation–reduction potential of electrolyzed oxidizing water. J Food Eng 78, 1326-1332. doi: 10.1016/j.jfoodeng.2006.01.004

Moffatt, C.R.M., Musto, J., Pingault, N., Miller, M., Stafford, R., Gregory, J., Polkinghorne, B.G., Kirk, M.D., 2016. Salmonella Typhimurium and Outbreaks of Egg-Associated Disease in Australia, 2001 to 2011. Foodborne Pathog Dis 13, 379-385. doi: 10.1089/fpd.2015.2110

Ni, L., Cao, W., Zheng, W.-c., Chen, H., Li, B.-m., 2014. Efficacy of slightly acidic electrolyzed water for reduction of foodborne pathogens and natural microflora on shell eggs. Food Sci Tech Res 20, 93-100. doi: 10.3136/fstr.20.93

NNDSS 2015. National notifiable diseases: Australia's notifiable diseases status 2015: Annual report of the National Notifiable Diseases Surveillance System (Department of Health: Canberra, Australia). doi: 10.33321/cdi.2019.43.6

Painter, J.A., Hoekstra, R.M., Ayers, T., Tauxe, R.V., Braden, C.R., Angulo, F.J., Griffin, P.M., 2013. Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998-2008. Emerg Infect Dis 19, 407-415. doi: 10.3201/eid1903.111866

Parisi, M., Northcutt, J., Smith, D., Steinberg, E., Dawson, P., 2015. Microbiological contamination of shell eggs produced in conventional and free-range housing systems. Food Control 47, 161-165. doi: 10.1016/j.foodcont.2014.06.038

Park, C.-M., Hung, Y.-C., Lin, C.-S., Brackett, R.E., 2005. Efficacy of electrolyzed water in inactivating Salmonella enteritidis and Listeria monocytogenes on shell eggs. J Food Prot 68, 986-990. doi: 10.4315/0362-028x-68.5.986

Park, H., Hung, Y.-C., Chung, D., 2004. Effects of chlorine and pH on efficacy of electrolyzed water for inactivating Escherichia coli O157:H7 and Listeria monocytogenes. Int J Food Microbiol 91, 13-18. doi: 10.1016/S0168-1605(03)00334-9

Ridgway, H.F., Olson, B.H., 1982. Chlorine resistance patterns of bacteria from two drinking water distribution systems. Appl Environ Microbiol 44, 972-987. PMID: 7149722

Rivera-Garcia, A., Santos-Ferro, L., Ramirez-Orejel, J.C., Agredano-Moreno, L.T., Jimenez-Garcia, L.F., Paez-Esquiliano, D., Andrade-Esquivel, E., Cano-Buendia, J.A., 2019. The effect of neutral electrolyzed water as a disinfectant of eggshells artificially contaminated with Listeria monocytogenes. Food Sci. Nutr. doi: 10.1002/fsn3.1053

Rodríguez Romo LA (2004). Control of Salmonella enterica serovar Enteritidis in shell eggs by ozone, ultraviolet radiation, and heat. 2004. Doctoral dissertation, The Ohio State University. Available from: https://etd.ohiolink.edu/!etd.send_file?accession=osu1078887311&disposition=inline

Samiullah, S., Omar, A.S., Roberts, J., Chousalkar, K., 2016. Effect of production system and flock age on eggshell and egg internal quality measurements. Poultry science 96, 246-258. doi: 10.3382/ps/pew289

Sasaki, Y., Tsujiyama, Y., Asai, T., Noda, Y., Katayama, S., Yamada, Y., 2011. Salmonella prevalence in commercial raw shell eggs in Japan: a survey. Epidemiol Infect 139, 1060-1064. doi: 10.1017/S0950268810002153

Sundheim, G., Langsrud, S., Heir, E., Holck, A.L., 1998. Bacterial resistance to disinfectants containing quaternary ammonium compounds. Int Biodeter Biodegr 41, 235-239. doi: 10.1016/S0964-8305(98)00027-4

Surdu, I., VĂTUIU, D., JURCOANE, Ş., Olteanu, M., VĂTUIU, I., 2017. The antimicrobial activity of neutral electrolyzed water against germs and fungi from feedstuffs, eggshells and laying hen house. Romanian Biotechnol Letters.

Venkitanarayanan, K.S., Ezeike, G.O., Hung, Y.-C., Doyle, M.P., 1999. Efficacy of electrolyzed oxidizing water for inactivating Escherichia coli O157: H7, Salmonella enteritidis, and Listeria monocytogenes. Appl. Environ. Microbiol. 65, 4276-4279.

Wall, H., Tauson, R., Sørgjerd, S., 2008. Bacterial Contamination of Eggshells in Furnished and Conventional Cages. J Appl Poultry Res 17, 11-16. doi: 10.3382/japr.2006-00058

Zang, Y.T., Bing, S., Li, Y.J., Shu, D.Q., Huang, A.M., Wu, H.X., Lan, L.T., Wu, H.D., 2019. Efficacy of slightly acidic electrolyzed water on the microbial safety and shelf life of shelled eggs. Poultry Science 98, 5932-5939. doi: 10.3382/ps/pez373

Chapter 4: Effect of high concentration pH neutral electrochemically activated solution (ECAS) fogging on the cuticle layer of a chicken egg

4.1 STATEMENT OF AUTHORSHIP

Statement of Authorship

Title of Paper	Effect of high concentration pH neutral electrochemically activated solution (ECAS) fogging on the cuticle layer of a chicken egg
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Sangay Tenzin, Sergio Ferro, Permal Deo, Samiullah Khan and Darren J. Trott

Principal Author

Name of Principal Author (Candidate)	Sangay Tenzin		
Contribution to the Paper	Developed methodology, conducted all experimental procedures and analyzed data, wrote original draft and edited the manuscript.		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	18/02/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Dr. Sergio Ferro		
Contribution to the Paper	Resources and manuscript review and editing.		
Signature		Date	19/02/2020

Name of Co-Author	Dr. Permal Deo		
Contribution to the Paper	Associate supervisor, assisted in project administration		
Signature		Date	24/02/2020

Name of Co-Author	Dr. Samiullah Khan		
Contribution to the Paper	Methodology development and Manuscript review and edition		
Signature		Date	19/02/2020

Name of Co-Author	Professor Darren J. Trott		
Contribution to the Paper	Principal supervisor, administer the project		
Signature		Date	20/02/2020

4.2 ORIGINAL ARTICLE

Effect of high concentration pH neutral electrochemically activated solution (ECAS) fogging on the cuticle layer of a chicken egg

Sangay Tenzin[1, #], Sergio Ferro[2], Permal Deo[3], Samiullah Khan[4], and Darren J. Trott[1, #, *]

[1]Australian Centre for Antimicrobial Resistance Ecology, School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy 5371, Australia

[2]Ecas4 Australia Pty. Ltd., Unit 8 / 1 London Road, Mile End South SA 5031, Australia.

[3]School of Pharmacy and Medical Sciences, University of South Australia, Adelaide 5000, Australia

[4]School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy 5371, Australia

[*] Senior author

[#] For correspondence: sangay.tenzin@adelaide.edu.au and darren.trott@adelaide.edu.au

Australian Centre for Antimicrobial Resistance Ecology

School of Animal and Veterinary Sciences

The University of Adelaide, Roseworthy Campus

Mudla Wirra Rd, Roseworthy, AUSTRALIA 5371

Abstract

The first protective layer on the eggshell is a cuticle, and it is made up of antibacterial proteins, that prevent bacterial penetration and colonisation. The factors that affect cuticle thickness are hen genetics, farming type and hen age. Increased frequency of bacterial penetration was observed in older and free-range hens, and eggs with abrased eggs. So, additional preventive measures such as chemical sanitisation of shelled eggs or coating of eggs with mineral oil or edible material are practised reduce incidences of food poisoning. Some chemical washing causes cuticle layer damage facilitating bacterial trans-shell transmission. Acidic form of the electrochemically activated solution (ECAS) caused cuticle erosion, whereas a pH neutral spray washing at moderate free chlorine concentration (FAC) did not. But, the effect of neutral ECAS spray and fogging washing with a high level of FAC (100-200 mg/L) on cuticle was not evaluated. The cuticle coverage was quantified in terms of ΔE^*_{ab}, which is the square root of the sums of colour space values (ΔL^*, Δa^* and Δb^*). Colour intensity values of before and after MST cuticle blue dye staining of eggs treated with high concentration ECAS and tap water was obtained using a spectrophotometer. The cuticle coverage of eggs spray treated (45s) with a high concentration of ECAS did not differ significantly from tap water washed. Similarly, the cuticle coverage of eggs fogged with the high level ECAS for 2 minutes did not differ from the eggs fogged washed with tap water for 2 minutes. Therefore, ECAS at high levels of FAC could be a one-step eco-friendly sanitiser cleaning and disinfection alternative to two-step cleaning and sanitisation that use chemical disinfectants that cause an environmental hazard and co-select for bacterial antibiotic resistance.

Keywords: Electrolysed oxidising water; eggshell washing; cuticle layer; aerosol fogging; MST cuticle blue dye; spectrophotometer; colour space values

1. Introduction

Eggs and egg products are a significant vehicle for transmission and cause of foodborne illnesses worldwide (Ford et al., 2018). Eggs get contaminated with pathogens at different stages of production from the farm to preparation for consumption. Contamination occurs via transovarian transfer during the formation of the egg in the hen reproductive tract or trans-eggshell transmission (Miyamoto et al., 1998) in the contaminated environment. Bacterial penetration of an egg depends on multiple factors categorised in general as extrinsic and intrinsic factors. External factors that affect bacterial penetration are bacterial strain, and environmental factors such as temperature, moisture and pH. Whereas, intrinsic factors include cuticle coverage, eggshell strength and shell membranes quality. The cuticle is an *in utero* deposition of a thin inner zone of hydroxyapatite crystals and outer superficial non-calcified water-insoluble organic pigments (Dennis et al., 1996; Nys et al., 2004; Nys et al., 1991; Parsons, 1982). The cuticle is the first protective protein layer (0.5-12.8 µm thickness) on an eggshell (Parsons, 1982) that prevents bacterial penetration to internal egg contents. The cuticle contains antibacterial proteins and hydrophobic nanostructure (D'Alba et al., 2014; Wellman-Labadie et al., 2008) that closes the shell pores (Williams et al., 1968).

The cuticle is a moderately inheritable trait (Dunn et al., 2019), where its coverage is affected by factors, such as hen age and production system. For example, eggs from a cage production system have better cuticle cover compared when compared to free-range eggs farming (Samiullah et al. 2013). Eggs from older hens were reported to have significantly depleted polysaccharides and lipid components of the cuticle (Rodríguez-Navarro et al., 2013). Moreover, the abrasion on eggshell was observed to increase substantially bacterial penetration across the eggshell (Board et al., 1979; Gole et al., 2014). Hence, additional preventive measures such as wash and sanitisation are employed for safe and hygienic production of eggs to decrease the incidences of human food poisoning.

Many countries including Australia use quaternary ammonium compounds (QACs), sodium hypochlorite (NaOCl) and sodium carbonate (Na_2CO_3) for egg washing to reduce the eggshell pathogenic bacterial load (Hutchison et al., 2003). However, egg washing often results in the damage of the cuticle layer (Bialka et al., 2004; Wang and Slavik, 1998), which enhances the horizontal transmission of

pathogens. The effect on the cuticle integrity varies with different sanitisers (Wang and Slavik 1998) Whereas, some chemicals leave residues on eggshell surfaces that promote co-selection of antibiotic resistance (Fernandez Marquez et al., 2017) and adversely affect consumer acceptability (Wang and Slavik, 1998). Therefore, an eco-friendly sanitiser ECAS with various pH levels is currently being trialled in egg sanitisation.

pH-neutral anolyte at 150 mg/L of free-available chlorine (FAC) effectively decontaminated native bacteria (Surdu et al., 2017) and inoculated *Salmonella enterica* serovar Enteritidis and *Listeria monocytogenes* (Rivera-Garcia et al., 2019) on eggs. Therefore, the objectives of this study are to examine the effect of high concentration ECAS anolyte spray and fogging disinfection on the integrity of cuticle. The outcome of the study will provide additional data on a cost-effective and simplified eco-friendly egg sanitisation at farm level.

2. Materials and Methods

2.1 Preparation of egg wash solutions

Fresh 100% pH neutral electrochemically activated solution (ECAS anolyte) (Ecas4 Australia Pty Ltd) was used as a stock solution. A 50% (v/v) ECAS anolyte containing ~150 ppm of free available chlorine (FAC) was prepared for spray washing and fogging experiments. Tap water was used as a control wash group. Free available chlorine concentration (HI 701, Free Chlorine Checker, Hanna Instruments), temperature and oxidation-reduction potential (ORP) (HI Waterproof pH / ORP & Temperature Meter, Hanna Instruments) were measured before the experiments.

2.2 Selection of eggs

Sixty visibly clean eggs, devoid of calcareous material, were selected. Their shell pigment was quantified using a colourimeter (MiniScan EZ, 4500L Spectrophotometer, Hunter Associates Laboratory, Inc., USA) to obtain L*, a* and b* colour space data around the equator at four points, before washing. The MiniScan Ez colourimeter functions on the L∗a∗b∗ space system, where L∗ represents the grading between black (0) and white (100). The higher the value for L∗, the lighter is the shell

colour. The value for chromatic component a∗ indicates the colour grading between green and red, where green is towards the negative end of the scale and red towards the positive end. The b∗ component is the grading between yellow and blue, where blue is towards the negative end and yellow towards the positive end of the scale. The average of L*, a* and b* values were calculated, and shell colour intensity was (E^*_{ab}) quantified for each egg as follows:

$$E_{ab}^* = \sqrt{[(L^*)^2 + (a^*)^2 + (b^*)^2]}$$

The eggs median and standard deviation (SD) of E_{ab}^* were calculated. The lowest value of E_{ab}^* was calculated by subtracting SD from the median value and highest E_{ab}^* by adding median value and SD. The eggs selected for the experiment were within the highest and the lowest E_{ab}^*.

2.3 Sanitisation and Cuticle assessment

For cuticle assessment, the ΔE_{ab}^* method (Leleu et al., 2011) was followed and L*, a* and b* colour space values were obtained by using MiniScan EZ spectrophotometer. In the ΔE_{ab}^* method, shell colour of an egg is measured before and after staining with MST cuticle blue dye and the value is calculated based on the below formula.

$$\Delta E_{ab}^* = \sqrt{[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]}$$

A higher value of ΔEab* denotes a higher cuticle staining affinity and hence more cuticle cover.

EO water spray and fog treatments of the eggs. Fresh eggs visibly free from dirt were sourced from backyard chickens. Shell colour (L*a*b*) of the eggs was measured before EO water treatment and cuticle staining. Twelve eggs each for tap water spray and fogging (control group), and EO water spray and fogging (treatment group), were assessed for the effect of sanitisation on cuticle integrity. Tap water and EO water (150 mg/L of FAC) spray washing was performed by spraying eggs for 45 seconds. Next, the eggs were left in a clean biosafety cabinet for 60 min to dry. For fogging control, an ultrasonic humidifier (HU-85, Contronics Engineering, The Netherlands) was used to generate fog from tap water as per the manufacturer's guideline. Each egg was treated with fog for 2 minutes, and eggs dried as above. For fogging treatment, EO water at 150 FAC was used to generate fog and dried as described earlier.

Cuticle assessment. The MST cuticle blue dye (MS Technologies, Europe Ltd, Kettering, Northamptonshire, UK) was prepared according to the manufacturer's recommendation. Eggs from the control and treatments groups were immersed in MST cuticle blue dye for 1 min, rinsed with distilled water for 3 secs and allowed to dry thoroughly. Shell colour (L*, a*, b*) of the stained eggs around the equator at four points was measured as described previously. Average of the above measurement values were used to obtain ΔL*, Δa* and Δb* to quantify the cuticle cover (ΔE^*_{ab}) for individual eggs.

3. Statistical analysis

The statistical analysis was performed in Prism v8 (GraphPad Software, San Diego, CA, USA). A one-way ANOVA was used to compare the differences in tap water spray washed, tap water fogged, EO water spray washed and EO water fogged eggs with confidence level set at 95%. The Tukey's comparison test, to discern differences between the treatment variables, was performed at the 95% confidence level.

4. Results

Wash solutions. The FAC, ORP, pH and temperature of 50% (v/v) ECAS anolyte was 152 mg /L, 703 mV, 6.84 and 6 ºC, respectively. FAC, ORP, pH and temperature of tap water was 2 mg/L, 269 mV, 7.2 and 5 ºC, respectively.

Selection of eggs. In total, sixty visibly clean randomly selected eggs colour space value L*, a* and b* were measured, and colour difference (E^*_{ab}) quantified. Figure 1 shows the E^*_{ab} of all eggs (Group 1) and eggs selected for experiments (Group 2) with their group mean and SD. Mean, SD, highest and lowest E^*_{ab} values of Group 1 eggs were 71.36, 3.23, 79.63 and 63.03, respectively. In Group 2, 48 Eggs with E^*_{ab} between 67 and 74 were selected and randomly assigned to control and treatment groups for further experiments. The mean and SD of eggs chosen for cuticle assessment experiment were 70.36 and 1.51, respectively. The eggs with similar colour pigments were selected to reduce sample variability.

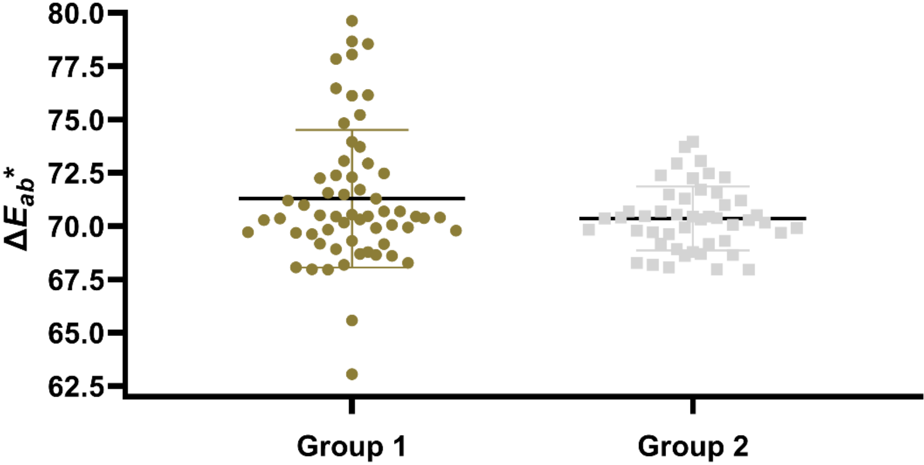


Figure 1. Scatter dot plot presenting the pigment intensity (E^*_{ab}) of 60 randomly chosen eggs (Group 1) and 48 eggs selected from Group 1 with E^*_{ab} values between 67 and 74 (Group 2). The thick line in the scattered dots marks the mean, while the whiskers above and below denote SD of the mean.

Cuticle assessment. The qualitative analysis of eggs dried after dye treatment had a consistent light green colour for all the control and treatment groups. The colour space measurement was performed for the quantification of the green colour intensity (ΔE^*_{ab}), as a measure of cuticle coverage. A one-way ANOVA for the effect of wash treatment on cuticle coverage was performed to compare the effect of pH-neutral ECAS at 152 FAC spray and fog washes, and tap water spray and fog washes. The analysis compared the group mean of ΔE^*_{ab} among all the wash type and were found not significantly different (p = 0.39). The mean and standard deviation of the mean ΔE^*_{ab} for all the treatment options are presented in figure 2.

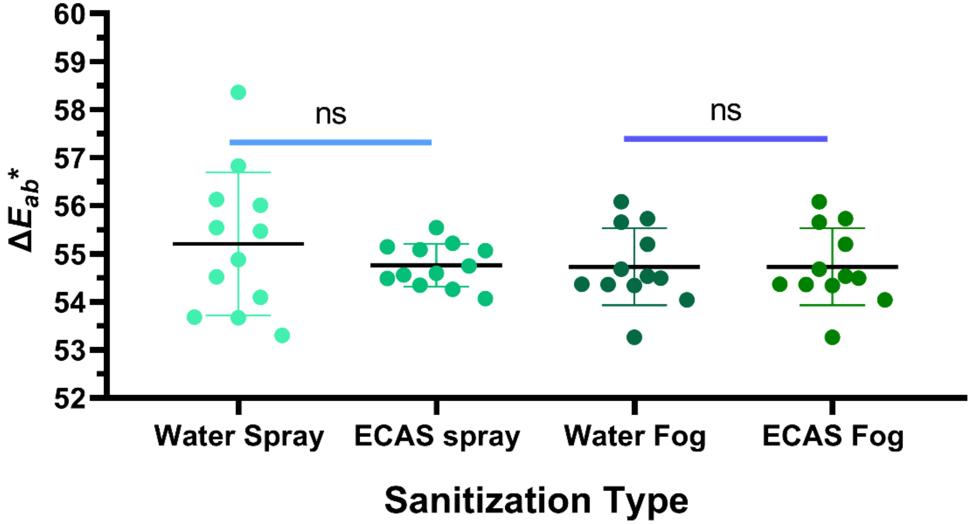


Figure 2. Figure showing values for the mean of ΔE^*_{ab} as a measure of cuticle coverage. The thick line in the scattered dots marks the mean, while the whiskers above and below denote SD of the mean. n ~ 12 eggs in each group. ns – not significant

5. Discussion

The cuticle structure in the hen is moderately inheritable (Dunn et al., 2019) and factors, such as hen housing (Samiullah et al., 2013), hen age (Leleu et al., 2011; Roberts and Chousalkar, 2013) and commercial washing affect its coverage. Moreover, as the cuticle is deposited while egg-laying, about 3.5% percentage of eggs were found without cuticle coverage (Board and Halls, 1973). Therefore, in the current study, the colour pigment of the outer layer of egg, that mostly consists of the cuticle, was quantified for selection of eggs that had similar cuticle integrity. As 8% of eggs are without cuticle on the apex or blunt end of the egg (Board and Halls, 1973), the cuticle measurement was made around the equator and average of four measures were considered for all the cuticle assessment. The selected eggs for the subsequent cuticle cover assessment showed less variability, and therefore, a minimum error was expected in measuring the true effects of the sanitisers treatment applied.

The use of contemporary chemical sanitiser solutions is, besides its effect on the cuticle layer, based on environmental impact, consumer acceptability and co-selection of antimicrobial resistance. Some alkaline solution is known to cause the cuticle erosion (Wang and Slavik, 1998; Gole et al., 2014), while others such as QAC causes

for co-selection of antibiotic resistance (Fernandez Marquez et al., 2017). And others leave residual chlorine on the shell surface affecting egg aesthetics (Wang and Slavik, 1998). So, effective eco-friendly sanitisers are currently being evaluated for effectiveness in the washing of eggs. In particular, three forms of ECAS solutions have shown promising results in terms of bacterial load reduction. Currently, the immersion or spray form of ECASs has been tested for the possible application in the poultry industry for egg washing. The immersion of the acidic ECAS method for egg washing is not very effective as it significantly affects the cuticle cover (Bialka et al. 2004). In the case of a slightly acidic form, Zhang et al., (2019) indicated to have reduced corrosion of cuticle as it preserved egg quality parameters better than acidic ECAS washing. The pH neutral ECAS at 46 mg/L FAC spray sanitisation was reported not to affect the cuticle (Rivera-Garcia et al., 2019). The current study showed that a high concentration ECAS of neutral pH (152 mg/L FAC) spray and fog did not affect the cuticle coverage.

In eggshell sanitisation, usually two-step cleaning and disinfection are employed. In the initial washing step, where dirt and debris are washed off the eggshell with water or alkaline detergent, followed by sanitiser disinfection. Since pH neutral ECAS at a high concentration significantly reduced total bacteria load and pathogens from the eggshell surface without an initial washing step (Rivera-Garcia et al., 2019; Surdu et al., 2017). And as it did not erode the cuticle coverage, therefore ECAS at this concentration would reduce wash and sanitisation time in egg safety management. Moreover, ECAS would quickly fulfil the regulatory requirement for egg washing as it is a sanitiser approved for food safety applications in major egg-producing countries (Venturini, 2013).

6. Acknowledgement

We want to thank Dr John Carragher, School of Food, Agriculture and Wine, The University of Adelaide, Waite Campus for loaning us the MiniScan EZ, 4500L Spectrophotometer, Hunter Associates Laboratory, Inc., the USA and showing us how to operate it.

7. References

Bialka, K.L., Demirci, A., Knabel, S., Patterson, P., Puri, V., 2004. Efficacy of electrolyzed oxidizing water for the microbial safety and quality of eggs. Poultry Science 83, 2071-2078.

Board, R., Halls, N., 1973. The cuticle: a barrier to liquid and particle penetration of the shell of the hen's egg. British Poultry Science 14, 69-97.

Board, R., Loseby, S., Miles, V., 1979. A note on microbial growth on hen egg-shells.

D'Alba, L., Jones, D.N., Badawy, H.T., Eliason, C.M., Shawkey, M.D., 2014. Antimicrobial properties of a nanostructured eggshell from a compost-nesting bird. Journal of Experimental Biology 217, 1116-1121.

Dennis, J.E., Xiao, S.Q., Agarwal, M., Fink, D.J., Heuer, A.H., Caplan, A.I., 1996. Microstructure of matrix and mineral components of eggshells from white leghorn chickens (Gallus gallus). Journal of Morphology 228, 287-306.

Dunn, I.C., Woolliams, J.A., Wilson, P.W., Icken, W., Cavero, D., Jones, A.C., Quinlan-Pluck, F., Williams, G.O., Olori, V., Bain, M.M., 2019. Genetic variation and potential for genetic improvement of cuticle deposition on chicken eggs. Genetics Selection Evolution 51, 25.

Fernandez Marquez, M.L., Burgos, M.J.G., Pulido, R.P., Gálvez, A., López, R.L., 2017. Biocide tolerance and antibiotic resistance in Salmonella isolates from hen eggshells. Foodborne pathogens and disease 14, 89-95.

Ford, L., Moffatt, C., Fearnley, E., Miller, M., Gregory, J., Sloan-Gardner, T., Polkinghorne, B.G., Bell, R., Franklin, N., Williamson, D.A., 2018. The Epidemiology of Salmonella enterica Outbreaks in Australia, 2001-2016. Frontiers in Sustainable Food Systems 2, 86.

Gole, V.C., Chousalkar, K.K., Roberts, J.R., Sexton, M., May, D., Tan, J., Kiermeier, A., 2014a. Effect of egg washing and correlation between eggshell characteristics and egg penetration by various Salmonella Typhimurium strains. PLoS One 9, e90987.

Hutchison, M., Gittins, J., Walker, A., Moore, A., Burton, C., Sparks, N., 2003. Washing table eggs: a review of the scientific and engineering issues. World's poultry science journal 59, 233-248.

Leleu, S., Messens, W., De Reu, K., De Preter, S., Herman, L., Heyndrickx, M., De Baerdemaeker, J., Michiels, C., Bain, M., 2011. Effect of egg washing on the cuticle quality of brown and white table eggs. Journal of Food Protection 74, 1649-1654.

Miyamoto, T., Horie, T., Baba, E., Sasai, K., Fukata, T., Arakawa, A., 1998. Salmonella penetration through eggshell associated with freshness of laid eggs and refrigeration. Journal of Food Protection 61, 350-353.

Nys, Y., Gautron, J., Garcia-Ruiz, J.M., Hincke, M.T., 2004. Avian eggshell mineralization: biochemical and functional characterization of matrix proteins. Comptes Rendus Palevol 3, 549-562.

Nys, Y., Zawadzki, J., Gautron, J., Mills, A., 1991. Whitening of brown-shelled eggs: mineral composition of uterine fluid and rate of protoporphyrin deposition. Poultry Science 70, 1236-1245.

Parsons, A., 1982. Structure of the eggshell. Poultry Science 61, 2013-2021.

Rivera-Garcia, A., Santos-Ferro, L., Ramirez-Orejel, J.C., Agredano-Moreno, L.T., Jimenez-Garcia, L.F., Paez-Esquiliano, D., Andrade-Esquivel, E., Cano-Buendia, J.A., 2019. The effect of neutral electrolyzed water as a disinfectant of eggshells artificially contaminated with Listeria monocytogenes. Food Science & Nutrition.

Roberts, J., Chousalkar, K., 2013. Egg quality and age of laying hens: implications for product safety. Animal Production Science 53, 1291-1297.

Rodríguez-Navarro, A.B., Domínguez-Gasca, N., Muñoz, A., Ortega-Huertas, M., 2013. Change in the chicken eggshell cuticle with hen age and egg freshness. Poultry Science 92, 3026-3035.

Samiullah, Chousalkar, K.K., Roberts, J.R., Sexton, M., May, D., Kiermeier, A., 2013. Effects of egg shell quality and washing on Salmonella Infantis penetration. Int J Food Microbiol 165, 77-83.

Surdu, I., Vătuiu, D., Jurcoane, Ş., Olteanu, M., Vătuiu, I., 2017. The antimicrobial activity of neutral electrolyzed water against germs and fungi from feedstuffs, eggshells and laying hen house.

Venturini, M., 2013. Acqua elettrolizzata: tecnologia emergente del settore ortofrutticolo. Alim Bev 4, 50-55.

Wang, H., Slavik, M.E., 1998. Bacterial penetration into eggs washed with various chemicals and stored at different temperatures and times. Journal of Food Protection 61, 276-279.

Wellman-Labadie, O., Picman, J., Hincke, M., 2008. Antimicrobial activity of cuticle and outer eggshell protein extracts from three species of domestic birds. British poultry science 49, 133-143.

Williams, J., Dillard, L., Hall, G.O., 1968. The penetration patterns of Salmonella typhimurium through the outer structures of chicken eggs. Avian Diseases 12, 445-466.

Zang, Y., Bing, S., Li, Y., Shu, D., Huang, A., Wu, H., Lan, L., Wu, H., 2019. Efficacy of slightly acidic electrolyzed water on the microbial safety and shelf life of shelled eggs. Poultry science.

Chapter 5: A pH-neutral electrolyzed oxidizing water significantly reduces microbial contamination of fresh spinach leaves

5.1 STATEMENT OF AUTHORSHIP

Statement of Authorship

Title of Paper	A pH-neutral electrolyzed oxidizing water significantly reduces microbial contamination of fresh spinach leaves
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Abiodun D. Ogunniyi, Sangay Tenzin, Sergio Ferro, Henrietta Venter, Hongfei Pi, Tony Amorico, Permal Deo, Darren J. Trott Submitted to Food Microbiology (25-02-2020).

Principal Author

Name of Principal Author (Candidate)	Sangay Tenzin		
Contribution to the Paper	Conceptualization and methodology, conducted experimental procedures, curated and analyzed data, visualization, Writing- edited the draft manuscript.		
Overall percentage (%)	40 %		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary co-author of this paper.		
Signature		Date	18/02/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Dr. Abiodun D. Ogunniyi		
Contribution to the Paper	Conceptualization, methodology (experiment), conducted experimental procedures, curated and analyzed data, visualization, Writing- original draft, Writing- Review and Editing		
Signature		Date	19/02/2020

Name of Co-Author	Dr. Sergio Ferro		
Contribution to the Paper	Acquired funding, resources and Writing- review and editing.		
Signature		Date	19/02/2020

Name of Co-Author	Dr. Henrietta Venter		
Contribution to the Paper	Acquired funding, resources and Writing- review and editing.		
Signature		Date	20/02/2020

Name of Co-Author	Sangay Tenzin for Hongfei Pi		
Contribution to the Paper	Methodology and conducted experiment		
Signature		Date	03-03-2020

Name of Co-Author	Dr. Permal Deo		
Contribution to the Paper	Associate supervisor, assisted in project administration, and manuscript revision and editing.		
Signature		Date	18/02/2020

Name of Co-Author	Professor Darren J. Trott		
Contribution to the Paper	Principal supervisor, administered the project, acquired funding, and manuscript revision and editing		
Signature		Date	04/03/2020

5.2 ORIGINAL ARTICLE

A pH-neutral electrolyzed oxidizing water significantly reduces microbial contamination of fresh spinach leaves

Abiodun D. Ogunniyi[1,¶,#], Sangay Tenzin[1,¶], Sergio Ferro[2], Henrietta Venter[3], Hongfei Pi[1], Tony Amorico[2], Permal Deo[3,*#], Darren J. Trott[1,*]

[1]Australian Centre for Antimicrobial Resistance Ecology, School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy 5371, Australia

[2]Ecas4 Australia, Unit 8 / 1 London Road, Mile End South 5031, Australia

[3]School of Pharmacy and Medical Sciences, University of South Australia, Adelaide 5000, Australia

¶ Co-first author

* Co-senior author

For correspondence: david.ogunniyi@adelaide.edu.au or Permal.Deo@unisa.edu.au

Abstract

There are growing demands globally for the use of safe, efficacious and environmentally friendly sanitizers for post-harvest treatment of minimally-processed fruits and vegetables to reduce or eliminate spoilage and foodborne pathogens. Here, we compared the effectiveness of an emerging pH-neutral electrolyzed oxidizing water (Ecas4 Anolyte) with that of an approved peroxyacetic acid-based sanitizer (Ecolab Tsunami® 100) in reducing the microbial load of inoculated *Escherichia coli*, *Salmonella* Enteritidis and *Listeria innocua* on post-harvest baby spinach leaves over 10 days. The impact of both sanitizers on the overall quality of the spinach leaves during storage was also assessed through the evaluation of the shelf life and vitamin C content. We show that Ecas4 Anolyte at 50 ppm and 85 ppm significantly reduced the bacterial load, compared to leaves treated with tap water or untreated (control). The reductions in microbial loads were similar (approx. 10-fold reduction) to those achieved using 50 ppm of Ecolab Tsunami®. No deleterious effects of the treatment with tap water or Anolyte at 50 ppm and 85 ppm on the appearance of the leaves were detected; on the contrary, there have been evident negative effects of Tsunami® on the appearance of the leaves such as yellowing and browning. Given its safety, efficacy and environmentally friendly characteristics, Ecas4 Anolyte could be a valid alternative to the chemical-based sanitizers currently used for the post-harvest treatment of minimally processed vegetables.

Keywords: Food safety; electrolyzed oxidizing water; peroxyacetic acid; post-harvest sanitation; baby spinach; foodborne pathogens

Running title: Post-harvest disinfection of fresh spinach leaves

1. Introduction

There is growing demand worldwide for the production and consumption of low-risk, fresh minimally processed fruits and vegetables as an integral part of a 'one health' approach to achieving better public health outcomes (WHO, 2015). Areas of particular interest include the implementation of safe, environmentally sustainable and economically sustainable food safety practices, the prevention of zoonotic diseases and the fight against the rise of antibiotic resistance in human and animal populations. In the context of food safety, microbial contamination in irrigation water (pre-harvest) or in washing water (post-harvest) are the dominant sources of contamination of fresh produce by opportunistic human pathogens (FSANZ, 2011). For example, outbreaks of *Salmonella* spp. and *Listeria monocytogenes* have been associated with cantaloupes, pre-packaged baby spinach and lettuce leaves, leading to a major recall of these products (FSANZ, 2016; Zhu et al., 2017).

From the above, it is apparent that the quality of irrigation water directly affects the safety of edible fresh produce, and there is sufficient evidence to suggest that contaminated irrigation water acts as a conduit for transferring pathogens onto the leaf surface (De Keuckelaere et al., 2015; Jongman and Korsten, 2018; Markland et al., 2017; Uyttendaele et al., 2015). Microbial pathogens found in irrigation water and most commonly associated with disease outbreaks in fresh produce include toxin-producing *Escherichia coli*, *Salmonella* spp., *Yersinia enterocolitica*, and *L. monocytogenes*. However, to date, farm management practices have mainly focused on the post-harvest treatment of fresh produce (Mahajan et al., 2014), although effective removal of bacteria from leaf surfaces through post-harvest washing is difficult once the bacteria are firmly attached (Banach et al., 2017).

A number of post-harvest treatments of fresh produce have been described in the literature (see e.g. the review by Mahajan et al. (2014)). These include physical treatments (e.g. heat, gamma irradiation), gaseous treatments (e.g. ozone, modified atmosphere packaging), chemical sanitizers (e.g. chlorine-based solutions, peroxyacetic acid (PAA), organic acids, hydrogen peroxide and electrolyzed oxidizing (EO) water). A report (Premier, 2013) on the various post-harvest chemical treatments used for commercial vegetables in Australia concluded that PAA-based sanitizers are

more effective for treating post-harvest leafy vegetables than organic-based sanitizers (such as Citrox, Aussan and CitroFresh) but are more expensive and result in lower shelf-life of the vegetables. Noteworthy, the report pointed out that emerging technologies such as EO water are safe, economical and could offer superior efficacy compared to other sanitization methods, while also leading to an increase in the shelf life of the fresh produce. Cheng et al. (2012) also reported that EO water is highly effective in reducing the levels of major human pathogens such as *E. coli* O157:H7, *L. monocytogenes* and *S.* Enteritidis in fruit and vegetable products.

In 2017, the US Department of Agriculture, Food Safety and Inspection Services (FSIS) approved a specific type of EO water, "electrolytically generated hypochlorous acid", also known as neutral electrolyzed oxidizing water (NEW), as an antimicrobial product for sanitizing and disinfecting surfaces (USDA-FSIS, 2016). In Europe, Ecas4 supplies the same product under the name "Electro-Chemically Activated Solution" (ECAS or Ecas4 Anolyte), which is mainly used in the healthcare industry to control *Legionella* in water supplies (Migliarina and Ferro, 2014). The Ecas4 solution is also available on the Australian market, and we have shown that it significantly increases the shelf life of Southern Australian King George Whiting and Tasmanian Atlantic Salmon fillets (Khazandi et al., 2017). The pH-neutral Ecas4 Anolyte is synthesized through the electrolysis of a dilute solution of NaCl in a patented electrochemical reactor comprising 4 chambers (Ferro, 2015; Migliarina and Ferro, 2014). It is a non-hazardous, certified "organic" solution that contains active chlorine mainly in the form of hypochlorous acid.

Published studies have demonstrated the efficacy of Ecas4 Anolyte in the healthcare and seafood industries. However, there has been no report on its efficacy in the decontamination of known pathogens of fresh produce. In this study, we investigated the effects of Ecas4 Anolyte on total organoleptic properties of minimally processed baby spinach leaves and examined its effectiveness in eliminating known, non-pathogenic microorganisms and its effects on the overall reduction of total microbial load using a currently approved PAA-based sanitizer for fresh produce (Tsunami® 100), as a comparator.

2. Materials and Methods

2.1. Reagents, solutions and instruments

Freshly prepared Ecas4 Anolyte containing about 350 ppm of free chlorine was supplied by Ecas4 Australia, stored at 4±1 °C and used within a week from manufacture. Ecolab Tsunami® 100 (15.2% peroxyacetic acid, 11.2% H_2O_2 and 73.6% inert ingredients, including 30–60% acetic acid) was purchased from Ecolab USA Inc.; it is largely used as a post-harvest sanitizer in the fresh produce industry.

2.2. Fresh produce

Untreated, freshly cut baby spinach leaves were supplied by a Tasmanian commercial horticulture farm in 2-kg consignments and shipped at 4±1°C. The leaves were received within 48 h of harvest and used within 24–48 h on receipt for inoculation experiments.

2.3. Temperature, pH, oxidation-reduction potential (ORP), turbidity and chlorine level measurements

The temperature, pH and ORP of Ecas4 Anolyte, Ecolab Tsunami® 100 and tap water were measured using a model MC-80 handheld meter (TPS Pty Ltd, Australia). Turbidity measurements of the solutions were carried out on a Jenway 6320D spectrophotometer (Cole-Parmer, UK). The amounts of free and total chlorine in Ecas4 Anolyte were measured using a Free Chlorine Checker® HC-HI701 and a Total Chlorine Checker® HC-HI711 (Hanna Instruments). The amount of active agent in the Tsunami® 100 solution was determined using a PAA titration kit (Ecolab).

2.4. Bacterial strains and growth conditions

The bacterial strains used in this study were *E. coli* (ATCC 25922), *L. innocua* 6a (ATCC 33090) and *S.* Enteritidis 11RX (Ogunniyi et al., 1994; Ushiba et al., 1959). *L. innocua* is commonly used as a surrogate of *L. monocytogenes*, a pathogen of fresh produce, since it displays similar behaviour; the main advantage is that it does not require biosafety level 2 containment (Rasch, 2004). Glycerol stocks were maintained at –80°C and streaked onto Luria Bertani (LB) agar (Oxoid) to obtain isolated colonies. Single colonies were streaked onto the following selective agar plates (ThermoFisher Scientific) to confirm purity: Eosin Methylene Blue (EMB) agar (PP2169) for *E. coli*; *Listeria* Selective Agar Oxford (OXF) agar (PP2141) for *L. innocua* 6a, and Xylose Lysine Deoxycholate (XLD) agar (PP2004) for *S.* Enteritidis 11RX.

For the experiments, single colonies from selective agar plates were inoculated into LB broth and grown overnight at 37°C with aeration at 150 rpm on a digital

platform mixer (Ratek Instruments). Subsequently, the bacteria were sub-cultured at a 1:10 dilution in fresh LB broth and further incubated at 180 rpm for 2–3 h until A_{600} = 1.0 (for *E. coli* and *S.* Enteritidis 11RX) or A_{600} = 0.5 (for *L. innocua* 6a) was reached (equivalent to approx. 1×10^9 colony-forming units (CFU)/mL for each strain). The bacteria were then harvested and washed extensively (3×) in autoclave-sterilized Milli-Q water (Milli-Q Academic A10, MILLIPORE) to remove residual culture medium and suspended in sterile Milli-Q water to approx. 1×10^6 CFU/mL for each strain.

2.5. Preliminary efficacy assessments and bacterial inoculation experiments

In a preliminary assessment of the effectiveness of Ecas4 Anolyte and Ecolab Tsunami® 100 (Figure 1), the damaged spinach leaves and debris were removed followed by treatment with tap water, Tsunami® 100 (50 ppm), ECAS 15% (50 ppm) or ECAS 25% (85 ppm) for 60 s. Subsequently, the excess liquid was removed from the spinach leaves using an orbital salad spinner at 70 rpm for 30 s; 200 g samples were placed in sealable bags and stored at 4±1°C for post-treatment sampling and analysis. For the bacterial inoculation experiments, leaves were briefly washed with tap water at 4±1°C (1 kg of spinach in 4 L of water) for approx. 45 s, and the excess liquid removed as described above. The leaves were then spiked submerged in *E. coli*, *S.* Enteritidis or *L. innocua* suspension at a concentration of between 5×10^5 and 1×10^6 CFU/g of sample weight and mixed intermittently by swirling in a sterile plastic container. After a contact time of 15 min, the excess liquid was removed using the salad spinner for 60 s. The inoculated samples were placed in open containers and air-dried for 2 h in a biosafety level 2 cabinet to allow complete attachment of bacteria onto the spinach leaves. Subsequently, a sub-sample of the inoculated leaves was analyzed for an initial count of *Salmonella*, *Listeria* or *E. coli*. The rest of the inoculated leaves were divided into 4 groups and submerged in tap water, Tsunami® 100 (50 ppm) or ECAS (either at 50 ppm or 85 ppm) for 60 s, with intermittent mixing. Samples were then placed in sealable bags and stored at 4±1°C for post-treatment sampling and analysis, as described above for the uninoculated leaves.

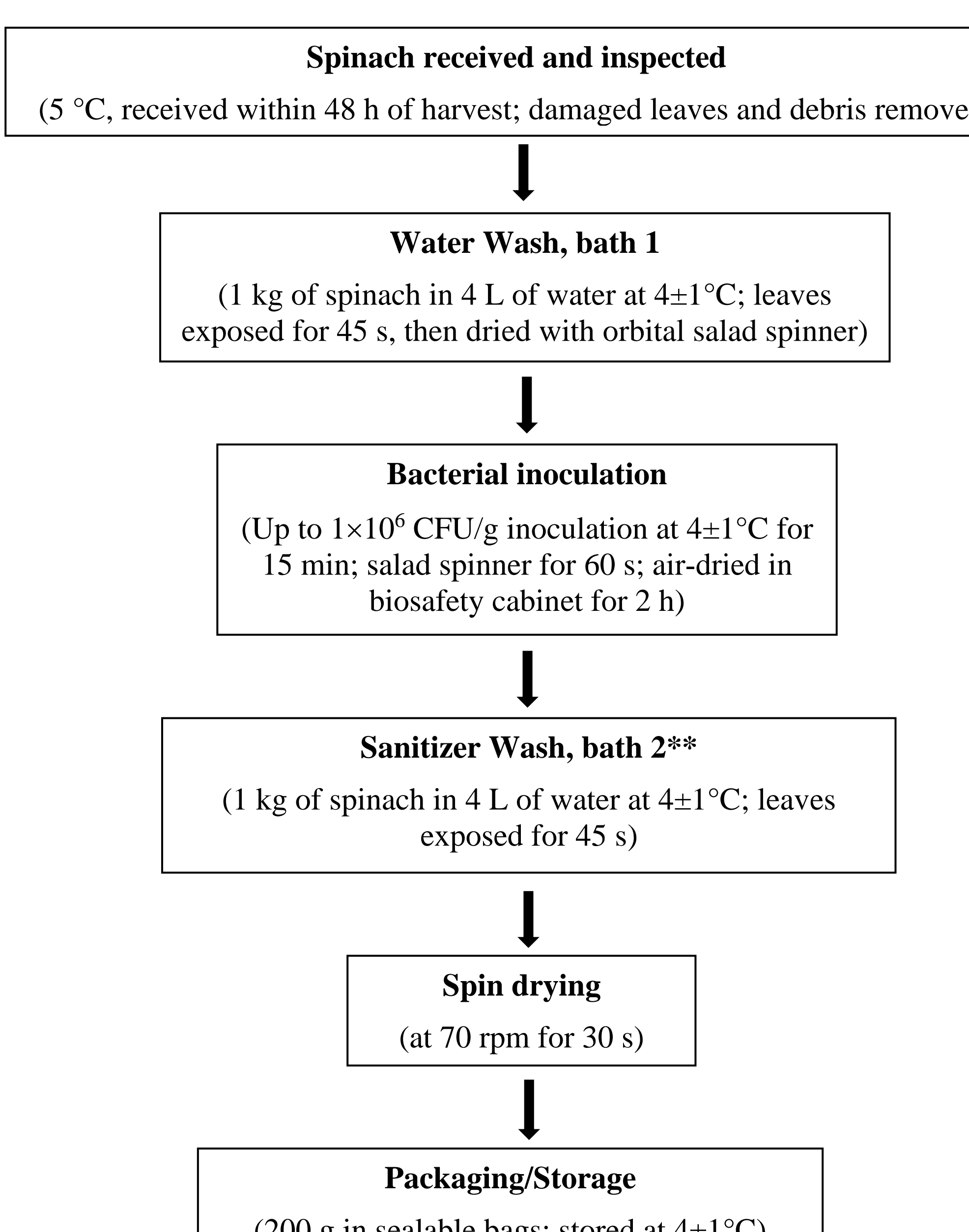


Figure 1. Schematic of the processing steps and conditions used for washing minimally processed vegetables. ** Tap water; Tsunami® 100 (50 ppm); ECAS (50 or 85 ppm).

2.6. Post-treatment sampling and analysis

2.6.1. Microbiological analysis

On days 0, 5 and 10 post-treatment, 25g samples (3–5 replicates for each treatment) were homogenized in 225 mL of 0.1% peptone water (ThermoFisher Scientific) in a Seward BA6021 stomacher (Seward Limited, Worthing, UK) for 60 s. 10-fold serial dilutions of each sample were carried out and duplicates of each dilution were plated for bacterial enumeration using the following media: Plate Count Agar for total viable counts; EMB agar and Brilliance™ *E. coli*/coliform Selective Agar for *E. coli* counts; Oxford agar for *Listeria* counts; XLD Agar for *Salmonella* counts and Compact Dry YM plate for Yeast & mold counts. Plates were incubated aerobically for 24-72 h at 35±1°C except for the Compact Dry YM plates that were incubated at 25±1°C for 72-96 h.

2.6.2. Sensory evaluation of spinach leaves

For each treatment, 5 individual leaves were packed in separate sealable plastic bags. All bags with leaves were stored in a container with ice or ice packs at 4±1°C. On day 0, 5 and 10 post-treatment, the samples were independently assessed by three trained sensory panelists. For sensory evaluation, a previously optimized shelf-life assessment sheet was used (Table 1).

Table 1. Quality assessment scheme for baby spinach leaves used in this study.

Scoring criteria	Score/Rating				
	5	4	3	2	1
Yellowing	No yellowing	slight yellowing	just acceptable	bad unacceptable yellowing	very severe yellowing
Bruising	No bruising	slight bruising	just acceptable	bad unacceptable bruising	very severe bruising
Wilting	No wilting	slight wilting	just acceptable	bad unacceptable wilting	very severe wilting
Sliming	No Sliming	No rating	sliming evident	bad sliming	very severe sliming

2.6.3. Determination of ascorbic acid content

The Vitamin C content in the spinach leaves was determined using a previously described iodometric titration technique (Spinola et al., 2012) with a slight modification. Briefly, 1 mL of 10 mg/mL starch solution and 1 mL of 100 mg/mL potassium iodide solution were mixed with accurately weighed spinach extract. The mixture was homogenized for 30 s using a magnetic stirrer before titrating with a previously standardized 0.005M potassium iodate solution until the mixture turned dark blue and the color persisted for at least 60 s. All solutions were prepared and standardized with standard ascorbic acid and sample analysis was done in triplicates. The results were expressed as mg of Vitamin C/100g sample.

2.7. In vitro comparison of the antibacterial action of ECAS and PAA

In order to compare the antibacterial action of ECAS and PAA, we measured the metabolic activity of bioluminescent *E. coli* Xen14 (PerkinElmer Inc, MA, USA) after treatment with various concentrations of freshly prepared sanitizers over a period of 10 minutes. For this assay, approx. 5×10^7 CFU of Xen14 were washed and resuspended in sterile Milli-Q water and then added to ECAS or PAA solutions containing 1, 2, 5, 10, 20 and 50 ppm of active agent. The reaction was stopped after 2, 5 or 10 min using 0.05% (v/v) sodium thiosulfate. Untreated bacteria resuspended in sterile Milli-Q water were used as control. Thereafter, samples were then serially diluted in PBS and plated on LB agar for bacterial enumeration. To measure bioluminescence, approx. 1×10^6 CFU of Xen14 from each treatment (after the addition of sodium thiosulfate) was added to 200 µL of sterile LB broth in a Nunc™ F96 MicroWell™ Black plate (ThermoFisher Scientific, 237105) which was then incubated at 37°C in a Cytation 5 Cell Imaging Multi-Mode Reader (BioTek; Winooski, VT, USA). Absorbance at OD_{600nm} and total luminescent signals were measured over a 48 h incubation period.

2.8. Statistical analyses

In all experiments, differences in microbial load between treatments were determined using unpaired t-test (two tailed). A P value of <0.05 was considered statistically significant.

3. Results

To evaluate the efficacy of ECAS in decontaminating known pathogens of fresh produce and assess its effects on the total organoleptic properties of post-harvest baby spinach leaves, we compared the outcomes of washing with two different concentrations of ECAS to those of tap water and a PAA-based sanitizer approved for fresh produce (Ecolab Tsunami® 100).

3.1. Preliminary efficacy assessments

In a preliminary investigation, the pH, temperature, ORP, turbidity and active agent levels were measured for each treatment solution (tap water, Tsunami® 100 and ECAS) before and after washing of the spinach leaves. We found that the parameters measured for each treatment were essentially similar before and after washing (Table 2). We also ascertained that the free available chlorine levels on homogenized spinach leaves treated with 50 ppm and 85 ppm ECAS had reduced to <5 ppm after 5 min contact time and activity was also quenched in 0.1% peptone water after 5 min contact time (not shown).

Next, we carried out bacterial enumeration and sensory analysis for the preliminary experiment at days 0, 5 and 10 post-treatment. The results of total plate, coliform and yeast/mold counts for tap water, 50 ppm ECAS, 85 ppm ECAS and Tsunami® 100 and the corresponding sensory attributes are presented in **Figure 2**.

Table 2. Measured pH, temperature, ORP, turbidity and active agent content in tap water, 50 ppm of Tsunami® 100, 50 ppm ECAS and 85 ppm ECAS before and immediately after washing of spinach leaves.

Treatment	Before / After spinach wash				
	pH	Temperature (°C)	ORP (mV)	Turbidity (NTU)	Active agent (ppm)
Tap water	7.4 / 7.4	4.9 / 5.0	287 / 290	0.0 / 0.0	0.38 / 0.35*
Tsunami® 100	4.2 / 4.0	5.6 / 6.4	427 / 426	0.0 / 0.0	50 / 50#
50 ppm ECAS	7.0 / 7.0	4.5 / 4.4	820 / 857	0.0 / 0.0	56 / 56*

85 ppm ECAS	7.4 / 7.0	4.8 / 4.5	868 / 867	0.0 / 0.0	89 / 89*

* = Available chlorine, # = Peroxyacetic acid

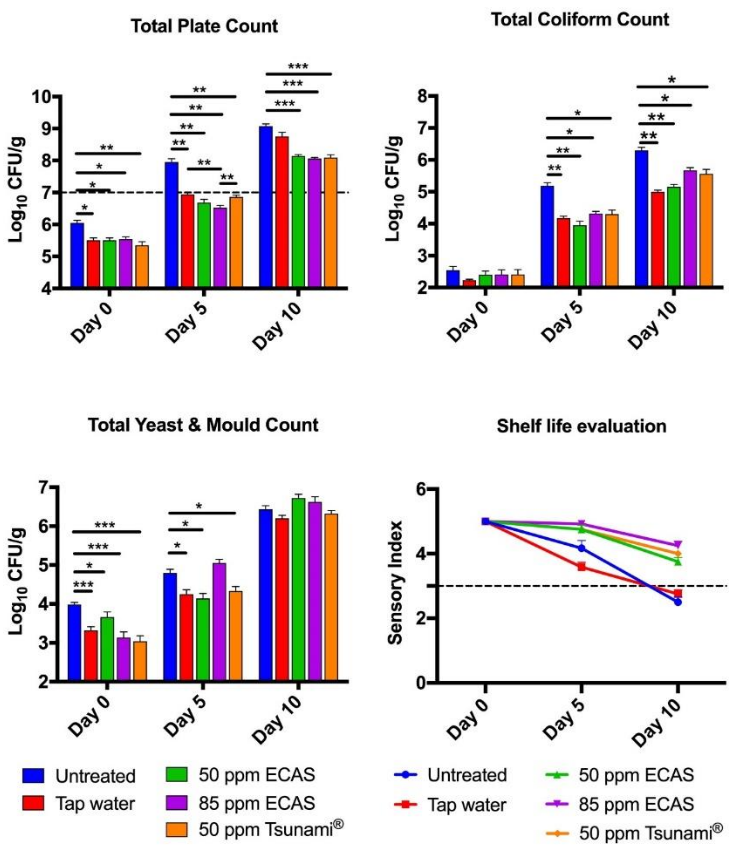


Figure 2. Effect of treatment with tap water, 50 ppm ECAS, 85 ppm ECAS and 50 ppm of Tsunami® 100 on the microbial load of spinach leaves in the preliminary experiment. Baby spinach leaves were treated with tap water, 50 ppm ECAS, 85 ppm ECAS or 50 ppm of Tsunami® 100 as described in Methods. At days 0, 5 and 10, 25 g of leaves from each treatment ($n = 5$) were assessed for total bacterial, coliform, and yeast/mold counts. Differences in microbial load between treatments were determined using unpaired *t*-test (two tailed). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

On day 0, the total bacterial load was significantly reduced in all treated groups (tap water, 50 ppm ECAS, 85 ppm ECAS and Tsunami® 100) compared to the control (untreated) group. On day 5, the total bacterial counts for 85 ppm ECAS treatment was significantly lower compared to Tsunami® 100 and tap water treatments. On day 10, the total bacterial and coliform counts for spinach leaves treated with tap water, 50 ppm ECAS, 85 ppm ECAS and Tsunami® 100 were significantly lower, being reduced

by approximately 10 times, compared to the control (untreated leaves). Notably, no *Listeria* spp., *Salmonella* spp. or *E. coli* were isolated from the spinach leaves either before or after treatment with tap water, 50 ppm ECAS, 85 ppm ECAS or Tsunami® 100. For the yeast and mold counts, spinach leaves treated with tap water, 50 ppm ECAS, 85 ppm ECAS and Tsunami® 100 had significantly lower counts in comparison to the control (untreated leaves) at day 0. On day 5, the yeast and mold count of spinach leaves treated with tap water, 50 ppm ECAS and Tsunami® 100 were significantly lower compared to untreated leaves. Surprisingly, the yeast and mold count for spinach leaves treated with 85 ppm ECAS was not different from those for the untreated leaves.

To ascertain that sanitizer treatment does not diminish the nutritional value of the baby spinach leaves during storage, the vitamin C content of the leaves was measured. The vitamin C content in the leaves treated with 50 ppm ECAS, 85 ppm ECAS and Tsunami® 100- was more stable during storage and significantly higher than that of untreated leaves or those washed with tap water or untreated (**Figure 3**). Together, these results suggest that treatment of the leaves with the sanitizers significantly reduced the total bacterial counts and improved the shelf life of leaves, and indicate that ECAS at either 50 ppm or 85 ppm was as effective as a 50ppm Tsunami® 100 treatment of leaves.

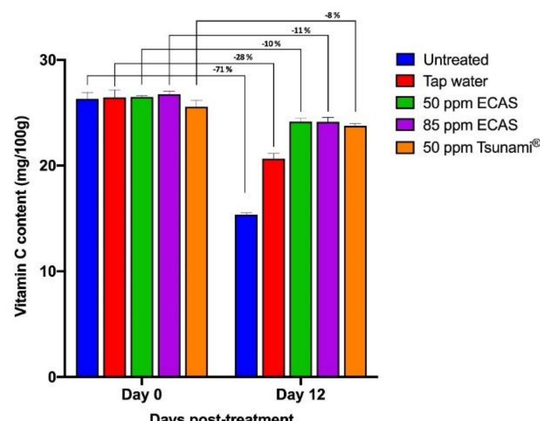


Figure 3. Vitamin C content in spinach leaves treated with tap water, 50 ppm ECAS, 85 ppm ECAS or 50 ppm of Tsunami® 100, in comparison to that in untreated leaves on day 0 and day 12.

3.2. ECAS is as effective as Tsunami® 100 in reducing *E. coli* populations on spinach leaves

On account of the promising results obtained for ECAS in preliminary investigations, we proceeded to examine its efficacy in sanitizing leaves deliberately spiked with approx. 5×10^5 CFU *E. coli* per g of sample, as illustrated earlier in Figure 1. The average measured pH, temperature, ORP, turbidity and active agent content in tap water, 50 ppm of Tsunami® 100, 50 ppm ECAS and 85 ppm ECAS before and after washing the spinach leaves in the *E. coli* inoculation experiment were similar to those shown in **Table 1**.

The analysis of bacterial counts on day 0 showed that 85 ppm ECAS and Tsunami® 100 significantly reduced levels of *E. coli* on spinach leaves by 0.3 and 1.5 log CFU/g, respectively, compared to tap water treatment (**Figure 4**). On day 5, there were no significant differences in the *E. coli* counts between tap water wash and 50 ppm ECAS or 85 ppm ECAS, but surprisingly the number of *E. coli* increased significantly for the leaves treated with Tsunami® 100. In addition, on day 10, the total *E. coli* count was significantly lower for the 85 ppm ECAS treatment compared to washing with Tsunami® 100 and tap water (**Figure 4**), suggesting that ECAS is at least as effective as Tsunami® 100 in reducing *E. coli* populations on spinach leaves.

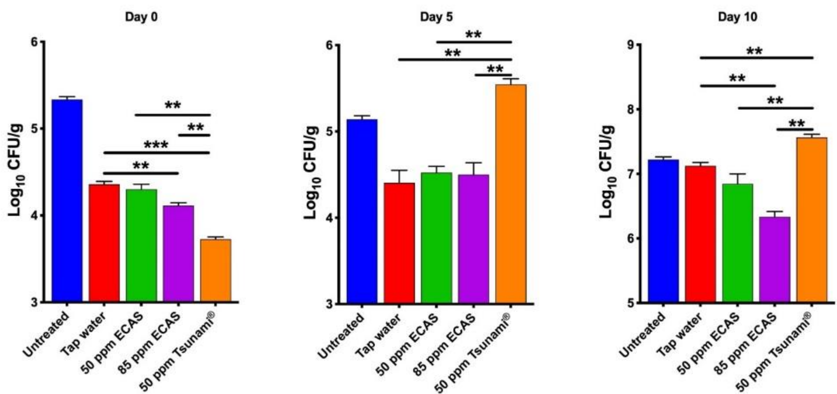


Figure 4. Effect of treatment with tap water, 50 ppm ECAS, 85 ppm ECAS and 50 ppm of Tsunami® 100 on the microbial load of spinach leaves after inoculation with *E. coli* at approx. 5×10^5 CFU/g of sample. Baby spinach leaves were treated with tap water, 50 ppm ECAS, 85 ppm ECAS, or 50 ppm of Tsunami® 100 as described in Methods. At days 0, 5 and 10, 25 g of leaves from each treatment (n = 3) were assessed for *E. coli* counts. Differences in microbial load between treatments were determined using unpaired *t*-test (two tailed). ** $P < 0.01$; *** $P < 0.001$.

3.3. ECAS is more effective than Tsunami® 100 in reducing *L. innocua* populations on spinach leaves

Following the *E. coli* inoculation experiment, we compared the efficacy of tap water, 50 ppm ECAS, 85 ppm ECAS or 50 ppm of Tsunami® 100 in sanitizing spinach leaves spiked with approx. 5×10^5 CFU of *L. innocua* per g of sample. On day 0, Tsunami® 100 significantly reduced *L. innocua* counts compared to treatments with 50 ppm and 85 ppm ECAS (**Figure 5**). On day 5, no significant difference in *L. innocua* counts between treatments was found. However, on day 10, the *L. innocua* counts were significantly lower in spinach leaves treated with either 50 ppm or 85 ppm ECAS compared to leaves treated with 50 ppm Tsunami® 100 (**Figure 5**), an indication of better sanitizing efficacy of ECAS over Tsunami® 100 at the concentrations used.

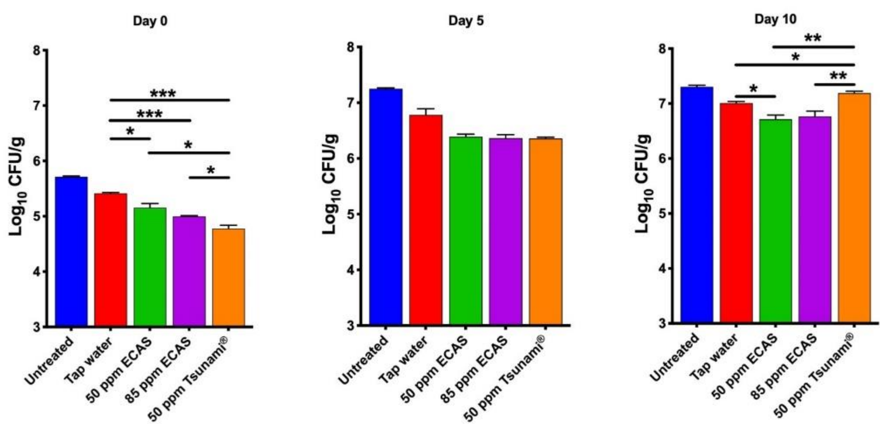


Figure 5. Effect of treatment with tap water, 50 ppm ECAS, 85 ppm ECAS and 50 ppm of Tsunami® 100 on the microbial load of spinach leaves after inoculation with *L. innocua* at approx. 5×10^5 CFU/g of sample. Baby spinach leaves were treated with tap water, 50 ppm ECAS, 85 ppm ECAS or 50 ppm of Tsunami® 100 as described in Methods. At days 0, 5 and 10, 25 g of leaves from each treatment ($n = 3$) were assessed for *L. innocua* counts. Differences in microbial load between treatments were determined using unpaired *t*-test (two tailed). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

3.4. ECAS and Tsunami® 100 are equally effective at reducing *S.* Enteritidis 11RX contamination of spinach leaves

In a third spiking experiment, we compared the efficacy of tap water, 50 ppm ECAS, 85 ppm ECAS or 50 ppm of Tsunami® 100 in sanitizing spinach leaves inoculated with approx. 1×10^6 CFU of *S.* Enteritidis 11RX per g of sample. On day

0, 85 ppm ECAS and Tsunami® 100 significantly reduced *S.* Enteritidis 11RX counts when compared to tap water (**Figure 6**). On day 5, the *S.* Enteritidis 11RX counts in spinach leaves treated with 50 ppm ECAS, 85 ppm ECAS and 50 ppm of Tsunami® 100 were significantly lower compared to leaves washed with tap water. Although there was a further reduction in *S.* Enteritidis 11RX counts on the treated spinach leaves compared to the untreated control by day 10, these differences did not reach statistical significance (**Figure 6**).

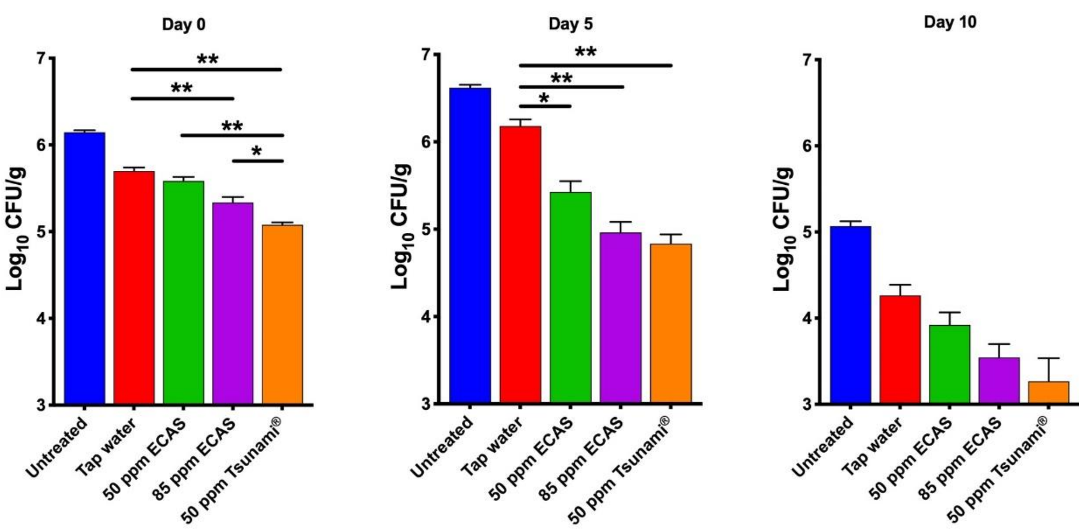


Figure 6. Effect of treatment with tap water, 50 ppm ECAS, 85 ppm ECAS and 50 ppm of Tsunami® 100 on the microbial load of spinach leaves after inoculation with *S.* Enteritidis 11RX at approx. 1×10^6 CFU/g of sample. Baby spinach leaves were treated with tap water, 50 ppm ECAS, 85 ppm ECAS or 50 ppm of Tsunami® 100 as described in Methods. At days 0, 5 and 10, 25 g of leaves from each treatment (n = 3) were assessed for *S.* Enteritidis 11RX counts. Differences in microbial load between treatments were determined using unpaired *t*-test (two tailed). * $P < 0.05$; ** $P < 0.01$.

3.5 ECAS is more effective than Tsunami® 100 at killing bacteria at low concentrations in vitro

Given the promising results obtained with the treatment of spiked spinach leaves with ECAS, we sought to determine its effective antibacterial concentration by comparing its efficacy with that of Tsunami® 100 at different concentrations over a 10-min period. For this assay, we used bioluminescent *E. coli* Xen14 to measure

metabolic activity in a manner similar to that described recently (Ogunniyi et al., 2019). We found that both sanitizers were bactericidal after 2-, 5- and 10-min contact time, and no metabolic activity was observed in the range of 10–50 ppm of active agent content over the 48-h incubation period (**Figure 7**). Interestingly, growth and detectable metabolic activity were observed for Xen14 treated with Tsunami® 100 at 1, 2 and 5 ppm of PAA, and this was consistent through the 48-h incubation period (**Figure 7**). However, under the same conditions, no metabolic activity was observed for Xen14 treated with ECAS at 1, 2 or 5 ppm of free available chlorine.

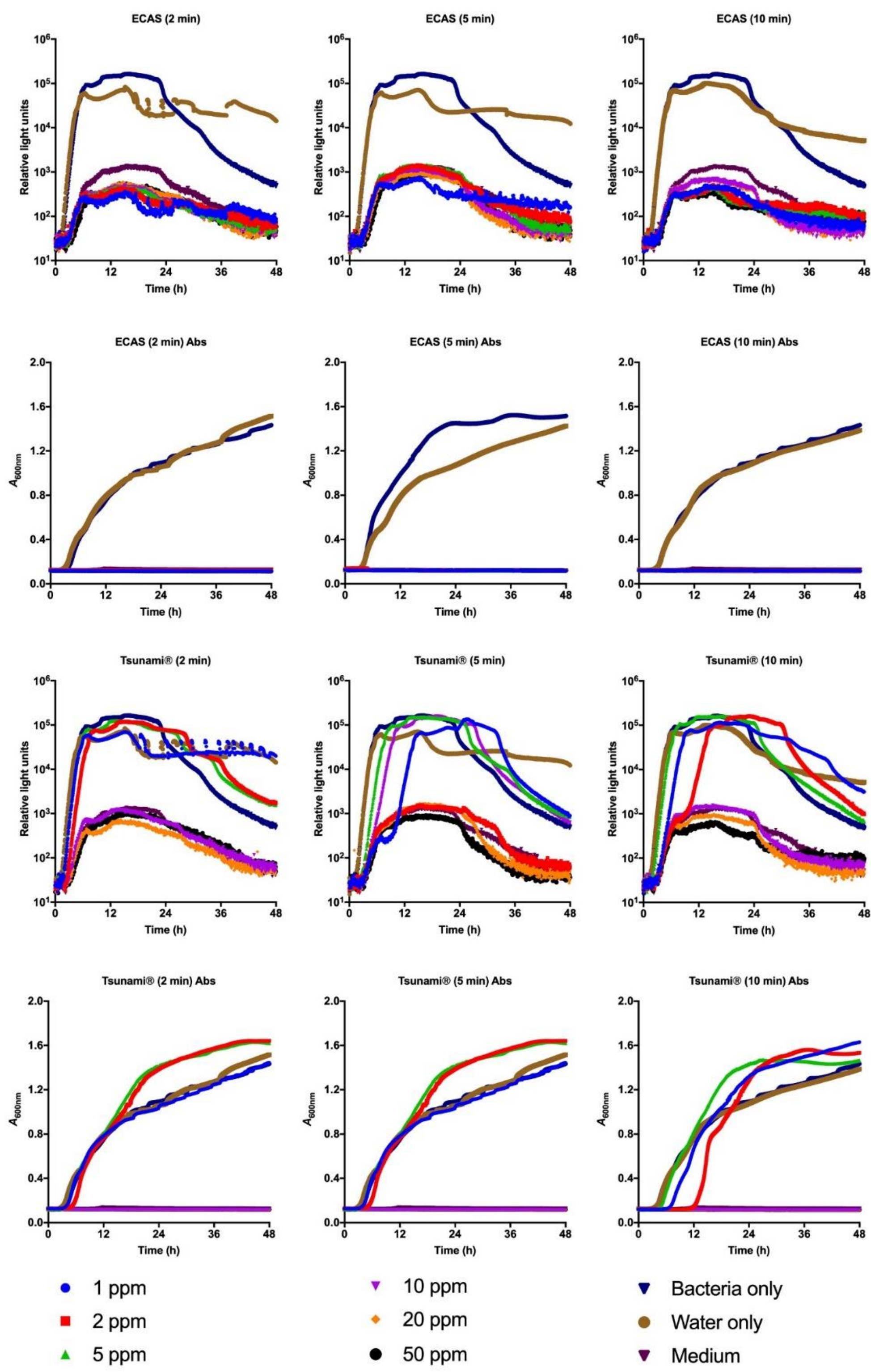


Figure 7. Bioluminescence and absorbance measurements of *E. coli* Xen14 after treatment with various concentrations of freshly prepared ECAS and Tsunami® 100 over a 10-min period. Results show growth and detectable metabolic activity for Xen14 treated with Tsunami® 100 at 1, 2 and 5 ppm, but not at 10, 20 or 50 ppm,

whereas no growth or detectable metabolic activity for Xen14 treated with ECAS could be observed at any of the concentrations tested.

4. Discussion

In this study, we have examined the potential of a pH-neutral electrolyzed oxidizing water (Ecas4 Anolyte or ECAS) in reducing the bacterial load and increasing the shelf life of post-harvest baby spinach leaves inoculated with three bacterial species, and compared its effectiveness with that of a widely used peroxyacetic acid-based sanitizer (Tsunami® 100). The study was carried out in response to the global request to use safe, effective and environmentally friendly sanitizers for the post-harvest treatment of minimally processed fruits and vegetables to reduce or eliminate spoilage and foodborne pathogens and increase the nutritional value and overall quality of fresh produce.

Tsunami® 100 has proven to be highly effective in the post-harvest treatment of fresh produce to reduce pathogen and spoilage load (Mahajan et al., 2014; Premier, 2013), and readily decomposes into harmless byproducts such as acetic acid (CH_3COOH), O_2 and H_2O (Kitis, 2004; Koivunen and Heinonen-Tanski, 2005; Sigge et al., 2016). However, the increase of the organic content in the treated water due to the presence of CH_3COOH in the mixture, the safety concerns with the handling of the stock solutions, the potential microbial regrowth after peracetic acid decomposition and the high initial purchase cost have somewhat limited its widespread use. Given that previous studies have shown the efficacy of ECAS in controlling *Legionella* in water supplies (Migliarina and Ferro, 2014) and in increasing significantly the shelf life of seafood while remaining safe at high concentrations (up to 150 ppm) (Khazandi et al., 2017), we compared its effectiveness at 50 ppm and 85 ppm with that of 50 ppm of the widely used Tsunami® 100.

Our examination of the effects of ECAS on spinach leaves indicated that treatment with 85 ppm ECAS is better than the use of 50 ppm ECAS. We also found that treatment with 85 ppm ECAS compared favourably (in terms of bacterial load reduction) with treatment using 50 ppm of Tsunami® 100, particularly over the 10 days of storage. In previous studies, neutral electrolyzed water at ≥100 ppm free chlorine was required to reduce the microbial load in fresh vegetables (Navarro-Rico et al., 2014; Rico et al., 2008) whereas 85 ppm of ECAS proved to be effective in our studies.

In a recent study, a slightly acidic electrolyzed water (4 ppm) combined with levulinic acid (3% v/v) showed bactericidal efficacy against natural microbial load and reduced survival population of *E coli* and *L. innocua* compared to acidic electrolyzed water (4 ppm) alone (Zhao et al., 2019). Furthermore, we observed that the treatment of spinach leaves with ECAS at 85 ppm did not show any apparent negative effects on leaf appearance, quality of the leaves whereas the use of 50 ppm of Tsunami® showed some yellowing and browning of the leaves as observed during sensory evaluation. It is also noteworthy that ECAS performed consistently well against all pathogens that are most likely to be found as contaminants in fresh produce, while Tsunami® 100 did not work as well against *E. coli* in this study. Additionally, our finding of a potent *in vitro* bactericidal effect of ECAS at low concentrations in the bioluminescence (metabolic activity) assay could be important in the context of using sanitizers that have the ability to potentially eliminate the induction of the viable but nonculturable state (Ferro et al., 2018), although further detailed investigations will be required to verify this proposition.

5. Conclusions

The overall desirable effects (safety, efficacy, environmentally friendly characteristics and potentially low costs of use) described here for ECAS on the post-harvest sanitization of baby spinach leaves are encouraging for the horticulture industry, especially in consideration of the drive to move away from the use of chemical-based sanitizers. Further studies extending the use of ECAS to treat other minimally processed fresh produce such as lettuce, broccoli, tomato and capsicum and assess the overall quality and shelf life of such products are therefore welcome, as they will shed light on its wide applicability. Experiments examining the combination of ECAS treatment of fresh produce with appropriate storage practices such as temperature control and modified atmosphere packaging to increase the physical, nutritional, sensory attributes and shelf life of such products are also desirable.

It is important to note that the bacterial strains used in this study were not pathogenic and were used to test the efficacy of the ECAS. Therefore, it would be essential to replicate the assays using known human pathogens, either singly or in a mixture. Moreover, it would be critical to perform on-field (pre-harvest) sanitization

of leafy greens and compare the results with post-harvest treatment to assess the best practice that allows the most relevant effects on the overall safety, nutritional value and shelf life of the products.

Acknowledgements

The Authors would like to thank Houston's Farm, Tasmania, 7170, Australia for the supply of baby spinach leaves used in this study. We would also like to thank Simon Crabb (previously national business manager at Ecas4 Australia), Daniel Vallelonga (Ecas4 Australia), Amanda Ruggero, Lora Bowes and Hong Nguyen (University of South Australia) for excellent technical support.

Conflict of Interest Statement

Sergio Ferro and Tony Amorico are technical manager and managing director, respectively, of Ecas4 Australia.

Funding Information

This work was supported by a Research Connections (AusIndustry/Department of Industry) funding (A157363) to The University of Adelaide.

6. References

Banach, J.L., van Bokhorst-van de Veen, H., van Overbeek, L.S., van der Zouwen, P.S., van der Fels-Klerx, H.J., Groot, M.N.N., 2017. The efficacy of chemical sanitizers on the reduction of Salmonella Typhimurium and Escherichia coli affected by bacterial cell history and water quality. Food Control 81, 137-146.

Cheng, K.C., Dev, S.R.S., Bialka, K.L., Demirci, A., 2012. 19 - Electrolyzed oxidizing water for microbial decontamination of food, in: Demirci, A., Ngadi, M.O. (Eds.), Microbial Decontamination in the Food Industry. Woodhead Publishing, pp. 563-591.

De Keuckelaere, A., Jacxsens, L., Amoah, P., Medema, G., McClure, P., Jaykus, L.-A., Uyttendaele, M., 2015. Zero Risk Does Not Exist: Lessons Learned from Microbial Risk Assessment Related to Use of Water and Safety of Fresh Produce. Comprehensive Reviews in Food Science and Food Safety 14, 387-410.

Ferro, S., 2015. Electrochemical activated solutions. The Australian Hospital Engineer 38, 50-53.

Ferro, S., Amorico, T., Deo, P., 2018. Role of food sanitising treatments in inducing the 'viable but nonculturable' state of microorganisms. Food Control 91, 321-329.

FSANZ, 2011. Supporting document 2: Review of foodborne illness associated with selected ready-to-eat fresh produce (December 2011). Proposal P1015. Primary Production & Processing Requirements for Horticulture. Food Standards Australia New Zealand, ACT, Australia.

FSANZ, 2016. Woolworths loose leaf lettuce product recall. Food Standards Australia New Zealand, ACT, Australia.

Jongman, M., Korsten, L., 2018. Irrigation water quality and microbial safety of leafy greens in different vegetable production systems: A review. Food Reviews International 34, 308-328.

Khazandi, M., Deo, P., Ferro, S., Venter, H., Pi, H., Crabb, S., Amorico, T., Ogunniyi, A.D., Trott, D.J., 2017. Efficacy evaluation of a new water sanitizer for increasing the shelf life of Southern Australian King George Whiting and Tasmanian Atlantic Salmon fillets. Food Microbiol 68, 51-60.

Kitis, M., 2004. Disinfection of wastewater with peracetic acid: a review. Environ Int 30, 47-55.

Koivunen, J., Heinonen-Tanski, H., 2005. Peracetic acid (PAA) disinfection of primary, secondary and tertiary treated municipal wastewaters. Water Res 39, 4445-4453.

Mahajan, P.V., Caleb, O.J., Singh, Z., Watkins, C.B., Geyer, M., 2014. Postharvest treatments of fresh produce. Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences 372, 20130309.

Markland, S.M., Ingram, D., Kniel, K.E., Sharma, M., 2017. Water for Agriculture: the Convergence of Sustainability and Safety. Microbiol Spectr 5.

Migliarina, F., Ferro, S., 2014. A Modern Approach to Disinfection, as Old as the Evolution of Vertebrates. Healthcare 2, 516-526.

Navarro-Rico, J., Artés-Hernández, F., Gómez, P.A., Núñez-Sánchez, M.Á., Artés, F., Martínez-Hernández, G.B., 2014. Neutral and acidic electrolysed water kept microbial quality and health promoting compounds of fresh-cut broccoli

throughout shelf life. Innovative Food Science & Emerging Technologies 21, 74-81.

Ogunniyi, A.D., Dandie, C.E., Ferro, S., Hall, B., Drigo, B., Brunetti, G., Venter, H., Myers, B., Deo, P., Donner, E., Lombi, E., 2019. Comparative antibacterial activities of neutral electrolyzed oxidizing water and other chlorine-based sanitizers. Sci Rep 9, 19955.

Ogunniyi, A.D., Manning, P.A., Kotlarski, I., 1994. A Salmonella enteritidis 11RX pilin induces strong T-lymphocyte responses. Infect Immun 62, 5376-5383.

Premier, R., 2013. Evaluation of vegetable washing chemicals, Final Report for Horticulture Australia Ltd Project VG09086. ISBN 0 7341 3028 7, 1-57.

Rasch, M. (2004). Experimental design and data collection. In: *Modelling Microbial Responses in Food* (pp. 1-20). CRC Press Inc. Boca Raton, FL.

Rico, D., Martín-Diana, A.B., Barry-Ryan, C., Frías, J.M., Henehan, G.T.M., Barat, J.M., 2008. Use of neutral electrolysed water (EW) for quality maintenance and shelf-life extension of minimally processed lettuce. Innovative Food Science & Emerging Technologies 9, 37-48.

Sigge, G.O., Olivier, F., Bester, C., Giddey, K.F., van rooyen, B., Kotze, M., Blom, N., Bredenhann, L., Britz, T.J., Lamprecht, C., 2016. Scoping study on different on-farm treatment options to reduce the high microbial contaminant loads of irrigation water to reduce the related food safety risk. Sigge, G.O. and Lamprecht, C. (eds), Stellenbosch University, Department of Food Science.

Spinola, V., Mendes, B., Camara, J.S., Castilho, P.C., 2012. An improved and fast UHPLC-PDA methodology for determination of L-ascorbic and dehydroascorbic acids in fruits and vegetables. Evaluation of degradation rate during storage. Anal Bioanal Chem 403, 1049-1058.

USDA-FSIS, 2016. Safe and Suitable Ingredients Used in the Production of Meat and Poultry, and Egg Products. USDA-FSIS; Washington, DC, USA. Retrieved from https://www.fsis.usda.gov/wps/wcm/connect/5776e129-96bc-4bdc-ad12-1e2ba541ef7c/Table+of+Safe+and+Suitable+Ingredients.pdf?MOD=AJPERES

Ushiba, D., Saito, K., Akiyama, T., Nakano, M., Sugiyama, T., Shirono, S., 1959. Studies on experimental typhoid: bacterial multiplication and host cell

response after infection with Salmonella enteritidis in mice immunized with live and killed vaccines. Jpn J Microbiol 3, 231-242.

Uyttendaele, M., Jaykus, L.-A., Amoah, P., Chiodini, A., Cunliffe, D., Jacxsens, L., Holvoet, K., Korsten, L., Lau, M., McClure, P., Medema, G., Sampers, I., Rao Jasti, P., 2015. Microbial Hazards in Irrigation Water: Standards, Norms, and Testing to Manage Use of Water in Fresh Produce Primary Production. Comprehensive Reviews in Food Science and Food Safety 14, 336-356.

WHO, 2015. Media centre. World Health Day 2015: From farm to plate, make food safe. Retrieved from https://www.who.int/mediacentre/news/releases/2015/food-safety/en/

Zhao, L., Zhao, M.Y., Phey, C.P., Yang, H., 2019. Efficacy of low concentration acidic electrolysed water and levulinic acid combination on fresh organic lettuce (Lactuca sativa Var. Crispa L.) and its antimicrobial mechanism. Food Control 101, 241-250.

Zhu, Q., Gooneratne, R., Hussain, M.A., 2017. Listeria monocytogenes in Fresh Produce: Outbreaks, Prevalence and Contamination Levels. Foods 6.

Chapter 6: Effects of an eco-friendly sanitizing wash on spinach leaf bacterial community structure and diversity

6.1 STATEMENT OF AUTHORSHIP

Statement of Authorship

Title of Paper	Effects of an eco-friendly sanitizing wash on spinach leaf bacterial community structure and diversity
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Sangay Tenzin, Abiodun D. Ogunniyi, Sergio Ferro, Permal Deo and Darren J. Trott Submitted to Applied Sciences of MDPI (special edition of Sustainable Environmental Solutions) on April 24, 2020.

Principal Author

Name of Principal Author (Candidate)	Sangay Tenzin		
Contribution to the Paper	Conceptualization and methodology, conducted all experimental procedures, curated and analyzed data, visualization, wrote original draft and edited the manuscript.		
Overall percentage (%)	75%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	18/02/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Dr. Abiodun D. Ogunniyi		
Contribution to the Paper	Conceptualization, acquired funding, methodology (experiment), Writing- Review and Editing		
Signature		Date	19/02/2020

Name of Co-Author	Dr. Sergio Ferro		
Contribution to the Paper	Conceptualization, acquired funding, resources and Writing- review and editing.		
Signature		Date	19/02/2020

Name of Co-Author	Dr. Permal Deo		
Contribution to the Paper	Associate supervisor, assisted in project administration, and manuscript revision and editing.		
Signature		Date	18/02/2020

Name of Co-Author	Professor Darren J. Trott		
Contribution to the Paper	Principal supervisor, administered the project, acquired funding, and manuscript revision and editing		
Signature		Date	20/02/2020

6.2 ORIGINAL ARTICLE

Article

Effects of an Eco-Friendly Sanitizing Wash on Spinach Leaf Bacterial Community Structure and Diversity

Sangay Tenzin [1,*], Abiodun D. Ogunniyi [1], Sergio Ferro [2], Permal Deo [3] and Darren J. Trott [1,*]

1 Australian Centre for Antimicrobial Resistance Ecology, School of Animal and Veterinary Sciences, The University of Adelaide, Mudla Wirra Rd, Roseworthy 5371, Australia; david.ogunniyi@adelaide.edu.au
2 Ecas4 Australia Pty. Ltd., Unit 8/1 London Road, Mile End South SA 5031, Australia; sergio@ecas4.com.au
3 Health and Biomedical Innovation, UniSA Clinical and Health Sciences, University of South Australia, Adelaide 5000, Australia; permal.deo@unisa.edu.au
* Correspondence: sangay.tenzin@adelaide.edu.au (S.T.); darren.trott@adelaide.edu.au (D.J.T.)

Received: 16 February 2020; Accepted: 21 April 2020; Published: 24 April 2020

Abstract: Ready-to-eat (RTE) spinach is considered a high-risk food, susceptible to colonization by foodborne pathogens; however, other microbial populations present on the vegetable surface may interact with foodborne pathogens by inhibiting/inactivating their growth. In addition, sanitizers applied to minimally processed salad leaves should not disrupt this autochthonous barrier and should be maintained throughout the shelf life of the product. This investigation aimed at comparing the effects of a pH neutral electrochemically activated solution (ECAS), a peroxyacetic acid (PAA)-based commercial sanitizer (Ecolab Tsunami® 100), and tap water wash on the minimally processed spinach leaf microbiome profile for 10 days after washing. The bacterial microbiota composition on spinach samples was assessed by 16S rRNA pyrosequencing and downstream analyses. Predominant phyla observed in decreasing order of abundance were Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes corresponding with the dominant families *Micrococcaceae*, *Clostridiales Family XII*, *Flavobacteriaceae*, *Pseudomonadaceae*, and *Burkholderiaceae*. Bacterial species richness and evenness (alpha diversity) and bacterial community composition among all wash types were not significantly different. However, a significant difference was apparent between sampling days, corresponding to a loss of overall heterogeneity over time. Analysis of composition of microbiome (ANCOM) did not identify any amplicon sequence variants (ASVs) or families having significantly different abundance in wash types; however, differences (17 ASVs and five families) were found depending on sampling day. This was the first bacterial microbiome composition study focused on ECAS and PAA-based wash solutions. These wash alternatives do not significantly alter microbial community composition of RTE spinach leaves; however, storage at refrigerated temperature reduces bacterial species heterogeneity.

Keywords: *Spinacia oleracea* microbiota; electrochemically activated solution; peroxyacetic acid; sanitization; 16s rRNA pyrosequencing; amplicon sequence variants; alpha diversity; bacterial community composition

---

1. Introduction

A wide range of microbes, with distinct phylogenetic structure, is associated with the aerial organs (phyllosphere) of plants through parasitic or symbiotic interactions; in particular, bacteria are the most common microorganisms colonizing plant phyllosphere in comparison to fungi and archaea. The bacterial communities associated with edible leafy vegetables are less diversified than those of farm soil and coastal seawater habitats [1]. Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria are

the predominant bacterial phyla present in ready-to-eat (RTE) leafy vegetables (which are consumed raw, either treated or minimally processed) [2–6]. The core bacterial genera identified in most studies are *Pseudomonas*, *Sphingomonas*, *Methylobacterium*, *Bacillus*, *Massilia*, *Arthrobacter*, and *Pantoea* [2,3]. Human pathogens mostly associated with RTE leafy vegetables include *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* spp. [7,8], but these are greatly affected by the vegetable type and bacterial community structure [9,10].

Lettuce and spinach are minimally processed RTE vegetables highly susceptible to colonization by foodborne pathogens [11]; therefore, various post-harvest sanitizing washing strategies are generally implemented to reduce spoilage and eliminate human pathogens. Today, the effectiveness of a post-harvest sanitizer is assessed based on its effect on the overall microbial populations, in addition to its propensity to reduce the microbial load and eliminate foodborne pathogens [12]. The composition of the microbiome community is assessed because the microbiome present on fresh produce is not only responsible for spoilage but rather acts as a natural biological barrier against spoilage organisms and pathogens, which constitute a smaller subset of the whole soil microbial population [13–15]. Furthermore, the bacterial microbiota on the surface of the plant inhibits or inactivates the growth of bacterial pathogen by producing acidic antimicrobial peptides and other secondary metabolites [16–18] that adversely affect the survival of the pathogen [19].

Bacterial population on RTE spinach is generally assessed using traditional culture-based techniques or specific polymerase chain reaction (PCR) to detect pathogens known for public health risk and quantify the population of indicator bacteria. Molecular techniques such as denaturing gradient gel electrophoresis and terminal restriction length polymorphism have been used for the analysis of 16S ribosomal RNA (rRNA) gene to understand the bacterial community of the phyllosphere on spinach leaves [20–22]. Contemporary next-generation sequencing techniques are now widely used for comprehensive analysis of the composition of bacterial community due to the increase in the depth of sequence readings and improved easier to use bioinformatics pipelines [23,24]. This method, in addition to providing information on the community structure, provides insights into the association of bacterial phyllosphere diversity with environmental factors [6,23], use of biocidal agents [6,23], and pesticides [6,25]. It also provides the interaction dynamics of the composition of the bacterial community with the various stages of plant growth, post-harvest, during processing and storage [3,23,26,27].

For leafy vegetable processing, chlorine- or peroxyacetic acid (PAA)-based sanitizers are commonly used. Chlorine is used for its effectiveness and low cost, whereas PAA for its activity over a wide pH range and limited reaction with organic matter. Electrochemically activated solution (ECAS) with an approximately neutral pH (6.5–7.5) has been suggested as a promising alternative washing solution with disinfection capability comparable to that of other commonly used disinfection chemicals such as chlorine and PAA [28–32]. Izumi [28] reported that neutral ECAS containing 50 mg/L of free available chlorine (FAC), completely inactivated the total bacteria on leaf surface. Guentzel et al. [31] reported a reduction of 4.0–5.0 Log_{10} CFU/mL of *E. coli*, *S. typhimurium*, *S. aureus*, *L. monocytogenes*, and *E. faecalis* inoculated on spinach leaves, working with 100 mg/L and 200 mg/L of FAC.

The sanitizers used in washing RTE vegetables have a different influence on bacterial microbiota. Some sections of the bacteria composition of plants affect the survival of pathogens through competition for limited nutrients or production of growth inhibitors [16,19,33], and others facilitate the growth of pathogens through the metabolism of different carbon sources [24]. Chlorine-based washing has previously been reported to reduce the number of microbes that inhibit the growth of pathogens in lettuce and spinach [18]. Gu et al. [25] observed changes in the bacteria community in spinach leaves washed with chlorine. Tatsika et al. [34] reported a reduction in the richness of the bacterial community of RTE spinach without affecting bacterial diversity after washing the spinach leaves with vinegar. However, the effect of washing with ECAS on the composition of the microbiome of RTE spinach leaves compared to that of PAA sanitizer has not previously been assessed.

This study evaluated the effect of an ECAS at neutral pH with proven efficacy against foodborne pathogens and in reducing the overall bacterial load in RTE spinach [28,35–37] focusing on the structure of the bacterial community present on RTE spinach leaves. We compared the changes in the profile of the bacterial microbiome in minimally processed fresh spinach leaves washed with tap water, PAA (50 mg/L), and ECAS (50 mg/L and 85 mg/L of FAC) on days 0, 5, and 10 after the sanitizing wash and storage at 4 ± 1 °C. Furthermore, a comparative analysis of the bacterial composition was performed through an analysis of the composition of microbiomes among all the treatment types and sampling days.

2. Materials and Methods

2.1. Sanitizers Treatment of Spinach Leaves

Freshly cut Tasmanian baby spinach leaves, grown in soil, stored and shipped at 4 ± 1 °C, were used within 24–48 h of receipt. ECAS (produced by Ecas4 Australia Pty Ltd., Mile End South, Adelaide, South Australia, Australia) was also stored at 4 ± 1 °C and used within one week of production, diluted in Milli-Q water (Milli-Q academic A10 deionizer, Millipore Corporation, Molsheim, France) to 50 mg/L and 85 mg/L of FAC. Peroxyacetic acid (Ecolab Tsunami® 100, which nominally contains 30–60% acetic acid, 10–30% peroxyacetic acid and 10–30% H_2O_2), commonly used as a post-harvest sanitization of fresh agriculture produce, was used at 50 mg/L of PAA. The temperature, pH, and oxidation-reduction potential (ORP) of ECAS, Tsunami® 100, and tap water were measured using a portable MC-80 m (TPS Pty Ltd., Brendale, Queensland, Australia). The quantities of free and total chlorine in ECAS were measured using a Free Chlorine Checker® HC-HI701 and a Total Chlorine Checker HC-HI711, both from Hanna Instruments (Keysborough, Victoria, Australia). The amount of PAA in Tsunami® 100 was measured using specific test strips (Hydrion PAA160 Peroxyacetic Acid (PAA) Sanitizer Test Strips, Brooklyn, New York, USA).

Three samples of spinach leaves (200 g each) were washed with 800 mL of either tap water (control, pH 7.4 ± 0.1) or sanitizers (52 ± 2 mg/L of PAA, ORP of 492 ± 15 mV, pH 3.6 ± 0.1; ECAS with 48 ± 4 mg/L of FAC, ORP of 833 ± 13, pH 7.1 ± 0.2; and ECAS with 82 ± 4 mg/L of FAC, ORP of 864 ± 13, pH 7.0 ± 0.2) at 4 ± 1 °C for 60 s, and the excess liquid removed using a salad spinner at 70 rpm for 30 s. Samples (3 × 25 g) from each treatment were homogenized in 225 mL of sterile 0.1% peptone water for 60 s in a stomacher (BA 6021 Stomacher, Seward Ltd., Worthing, UK) immediately after treatment (day 0) and stored at −20 °C. Spinach samples from each treatment were stored at 4 ± 1 °C and further processed on day 5 and day 10, as described by Ogunniyi et al. [37].

2.2. Samples Preparation for Variable V3-V4 Region Sequencing

Samples stored at −20 °C were thawed in a shaking incubator kept at 20 °C for about 45 min. Samples from each type of treatment and for the various sampling days were centrifuged at 15,000× *g* for 15 min; the supernatants were discarded, and the pellets were frozen at −20 °C for DNA extraction. The DNA from the samples was then isolated and purified using the Qiagen QIAamp DNA Mini Kit (Cat. #51304, Germantown, MD, USA) as per the manufacturer's instructions. DNA concentrations were measured using the multi-mode microplate reader (CLARIOstar Plus).

The amplicon-sequence PCR was performed using the 16S DNA V3-V4 region primers from Klindworth et al. [38] and following the guidelines provided in "16S Metagenomic Sequencing Library Preparation" (Part #15044223 Rev. B) [39]. PCR products were confirmed to produce a single amplicon size of ~460 bp after electrophoresis on a 2.0% agarose gel. Aliquots (25 µL) of all samples were subjected to clean-up PCR, index PCR, second clean-up PCR and MiSeq 16S metagenomic sequencing at the South Australian Health and Medical Research Institute (SAHMRI), Adelaide, South Australia. The data analyzed were based on Illumina Miseq sequences of 300 bp paired amplicon sequences from the V3 and V4 region of 16S rRNA gene from baby spinach leaf samples with and without sanitizing treatments. The profile of the demultiplexed fastq paired-end reads was assessed using FastQC [40] for

sequence quality scores and adapter contents. First, the forward reads were truncated at position 260 and the reverse at position 220 to remove low quality reads (<26 Phred). Trimming was set up for the first 20 nucleotides for forward reads and 10 nucleotides for reverse reads to remove primer sequences and low-quality reads. The trim and filter parameters were performed jointly on the paired-end read by setting a maximum of two errors expected per read [41], so that both paired-end reads passes the filter for the pair to pass. Downstream analysis to infer the amplicon sequence variants (ASVs) was performed in R version 3.5.3 [42] using the DADA2 workflow that resolves variants that differ by a single nucleotide [43]. Taxonomic assignments were made for the sequence variants data implementing the naïve Bayesian classifier method [44] using the SILVA reference data set (version 132) [45] formatted for DADA2 [46]. The DECIPHER R package [47] was used for the alignment of multiple sequences, and a phylogenetic tree was built using the phanghorn R package [48]. The phyloseq R package [49] was used to synthesize sample data, phylogeny and taxonomic assignment objects into a single phyloseq object. Further downstream analyses and graphical visualization of the microbiome data were performed in phyloseq [49] and Shiny-phyloseq [50] R packages.

2.3. Statistical Analysis

Calculations of alpha diversity indexes were performed in R versions 3.5.3 [42] with the phyloseq R package [49]. The Shannon and inverse Simpson indexes were compared among the variables since these indexes consider the richness and evenness that are powerful in providing insights into the structure of the microbial community [51,52]. In addition, the number of ASVs (species) was estimated using the observed richness and Chao1 richness estimator. The alpha diversities among the groups of samples were statistically tested using the analysis of variance (ANOVA) test to evaluate any differences in the microbial composition among treatment types and sampling days, as both variables (treatment type and sampling day) had more than two levels and the data distribution was normal according to the Shapiro–Wilk normality test. Tukey's honest significance test as a post hoc test was performed on the ANOVA results to compare within-group alpha diversity.

Measurements of samples similarity (beta diversity) with the R phyloseq and vegan packages [53] were also performed at ASV level based on non-metric multidimensional scaling (NMDS) Bray–Curtis dissimilarity [54] and Unifrac distances [55], which include abundance and phylogenetic information respectively, in addition to taxon counts. Statistical significance testing among the groups, such as the type of sanitization and the days post sanitizing treatment, was performed using permutational multivariate analysis of variance (PERMANOVA) [56] using the adonis function in the R package vegan. The community pattern of microbial composition among the groups using taxon dissimilarity information was visualized by NMDS Bray–Curtis and Unifrac ordination methods. In addition, microbiota heterogeneity, a measure of dissimilarity of the beta diversity (Bray–Curtis) of each sample with respect to the group, was compared between the various types of treatment (sanitizing and control washes) and days of sampling to evaluate the differences in homogeneity of each treatment group and homogeneity of sampling day using the R package microbiome [57]. Statistical tests for multiple variables within the type of treatment and sampling days were performed by the betadisper function on distance matrix (Bray–Curtis), and an ANOVA was performed to compare the variances between pairs of groups using the permutest function by setting the pairwise variable to true and the number of permutations to 1000 on R package vegan [56].

Analysis of differentially abundant taxa among the types of sanitization and days 0, 5, and 10 post treatment, at ASV and family level, were performed using analysis of composition of microbiomes (ANCOM) [58] plugin in QIIME 2 [59], at ASV and genus levels. For ANCOM analysis, ASVs present in less than three samples and ASV frequencies below fifty were removed before the analysis.

2.4. Data Submission

The access number for raw reads submitted to GenBank-SRA is PRJNA576552.

3. Results

We characterized the overall bacterial composition of minimally processed spinach leaves using high-throughput amplicon sequences from the V3–V4 region of the 16S rRNA gene. Moreover, changes in bacterial composition at phyla and families levels were compared for the washed samples and the control (unwashed) on day 0, day 5 and day 10 post sanitization, and between the types of washing (ECAS, Tsunami® 100, and Tap Water). In addition, the differences in bacterial diversity associated with the days post-treatment and the types of sanitizer were evaluated.

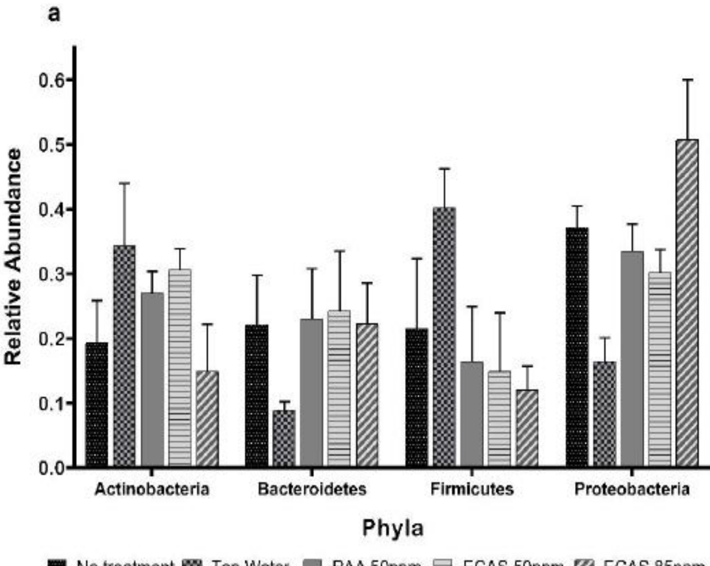
3.1. Composition of Spinach Bacterial Community

Overall, a total of 1,093,364 ASVs were observed, with a maximum of 113,737 and minimum of 39,474 reads. After removing uncharacterized phyla and contaminants and normalizing the data to the lowest number of reads (1000), the total number of ASVs reduced to 383,290 with a minimum of 1044 (observed for samples washed with ECAS at 85 mg/L of FAC on day 0) and a maximum of 50,871 (observed for samples washed with ECAS at 50 mg/L of FAC on day 5). The above reads were assigned to 12 distinct phyla, with the majority identified as Proteobacteria (2949 distinct ASVs), followed by Bacteroidetes (1876 ASVs), Actinobacteria (756 ASVs) and Firmicutes (396 ASVs). All other phyla had ≤8 ASVs (Table 1) and were excluded from further analysis [27] as the percentage abundance of these phyla were approximately 0.1% which would not affect the biological interpretation. All ASVs were assigned to one of 65 bacterial family identified and 84% of reads were further assigned to different bacterial genus with 158 genera identified. The five most abundant families identified were *Micrococcaceae* (28.2%), *Clostridiales Family XII* (19.7%), *Flavobacteriaceae* (17.9%), *Pseudomonadaceae* (12.8%), and *Burkholderiaceae* (10.1%). The five most abundant genera identified were *Exiguobacterium* (19.7%), *Flavobacterium* (17.7%), *Arthobacter* (15.4%), *Pseudomonas* (12.6%), and *Paeniglutamicibacter* (10.3%) (Table S1).

Table 1. Abundance and percentage abundances of phyla present in spinach leaf samples identified from 16S rRNA gene sequences analyzed using DADA2 package in R and taxonomic assignment performed according to the SILVA rRNA database.

Phylum	Phyla Abundance	Percentage Abundance
Actinobacteria	756	12.59
Bacteroidetes	1876	31.25
Deinococcus-Thermus	7	0.12
Firmicutes	396	6.60
Fusobacteria	4	0.07
Patescibacteria	8	0.13
Planctomycetes	7	0.12
Proteobacteria	2949	49.13

The overall relative abundances (RA) of phyla observed for all types of sanitization wash are presented in Figure 1a, and the relative abundances for the samples collected immediately after treatment (day 0) as well as on day 5 and day 10 after storage at 4 °C are presented in Figure 1b. On day 0, the phyla Proteobacteria had the highest RA (0.36 ± 0.07), while the phyla Actinobacteria had the lowest RA (0.18 ± 0.05). On day 5, Proteobacteria was still the most abundant phyla (0.35 ± 0.08), whereas phyla Bacteroidetes was the least abundant (0.09 ± 0.01). However, on day 10, Actinobacteria was the most abundant phyla (0.34 ± 0.05) and Firmicutes was the least abundant phyla (0.10 ± 0.05). The relative abundances of bacterial taxonomy at order level for sanitization wash types and sampling days are presented in Supplementary Figure S1.



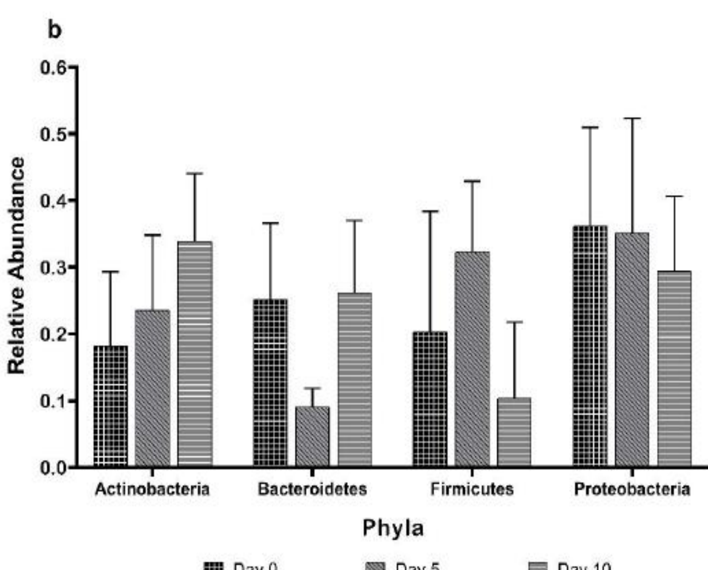


Figure 1. Relative abundance of phyla (Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria) for samples collected (**a**) after the sanitizing wash (no wash, tap water, peroxyacetic acid (PAA) at 50 mg/L, ECAS at 50 mg/L and 85 mg/L of free available chlorine (FAC)), and (**b**) immediately after the treatment (day 0) and on days 5 and 10 post sanitizing wash.

3.2. Alpha Diversity

The alpha diversity metrics (Figure S2) and the Shapiro–Wilk tests for normality showed that the data were normally distributed. The alpha diversity measures for all samples are presented in Table 2. The mean ratio between observed to expected (Chao1) richness was >0.99 for all samples. The lowest Shannon (3.4), Inverse Simpson (17.6), and richness (Chao1 = 60) indexes were recorded for samples that were not washed (control) on day 0. The highest Shannon (5.6) and richness (771) indexes were recorded for the samples that were washed in ECAS at 85 mg/L of FAC on day 0, while the highest Inverse Simpson index (134.5) was observed for the no-wash control on day 5. Species richness (Shannon diversity and Inverse Simpson indexes) and species evenness (Chao1 and abundance-based coverage estimator, ACE) measures of the bacterial community structure were assessed for the four

types of treatment plus control and the three sampling days. For all samples washed with sanitizers, the Shannon and Inverse Simpson diversity measures were higher than those found for the no-wash and tap water wash, but these measures were not significantly different (ANOVA and Tukey's honestly significant difference (HSD)). Similar results on species richness (Chao1 and ACE) were observed, with no significant differences between all types of washing (Kruskal–Wallis and pairwise Wilcox (FDR corrected) (Table S2).

Table 2. Alpha diversity metrics of species richness (Shannon and Inverse Simpson & Fisher) and evenness (Chao1 and abundance-based coverage estimator, ACE) for all samples.

Treatment Type	Sampling Day	Chao1	ACE	Shannon	InvSimpson	Fisher
Tap water	0	128.00	128.29	3.86	26.38	22.58
	5	371.08	371.89	4.45	39.49	61.56
	10	337.42	338.75	4.38	41.90	51.58
ECAS 50 mg/L	0	771.06	771.86	5.59	125.72	128.92
	5	487.38	488.94	4.47	38.84	75.21
	10	542.10	542.79	5.42	118.15	96.19
ECAS 85 mg/L	0	60.00	60.00	3.42	17.66	13.74
	5	445.04	446.17	5.31	134.56	77.66
	10	630.76	633.55	5.42	113.40	133.90
PAA 50 mg/L	0	577.32	578.44	5.28	91.59	94.20
	5	368.25	369.16	4.69	50.61	58.87
	10	468.20	469.06	5.19	88.46	83.08
No treatment (control)	0	64.00	64.00	3.54	22.51	15.04
	5	342.00	342.00	4.50	35.73	53.66
	10	415.60	416.57	5.11	80.86	73.09

3.3. Bacterial Diversity Associated with Treatment Type and Sampling Day

The PERMANOVA analyses of microbial communities for the different types of treatment were not significantly different among all the variables tested ($p = 0.053$). PERMANOVA analysis of Bray–Curtis distances for sampling days determined that the microbial communities were significantly different on sampling days ($p = 0.006$) (Table 3a). Moreover, non-metric multidimensional scaling (NMDS) cluster analysis showed that the microbial communities for different treatment groups did not cluster into distinct treatment groups (Figure 2a); however, the bacterial communities on day 5 and day 10 assembled distinctly, with a divergent microbial community observed for day 0 (Figure 2b). Also the quantification of the group divergence between the treatment types (ECAS at 50 and 85 mg/L of FAC, tap water and PAA washing) plotted as a box and whisker diagram showed that the group homogeneity among treatment types did not differ (Figure 2c). However, it shows that the microbiota of the ECAS and PAA wash treatments were more homogenous, whereas the tap water wash and the no wash (control) samples were more divergent (Figure 2c). The group divergence measurement for the sampling days shows that samples on day 0 had a higher value (>0.7), indicating that the composition was more heterogeneous. On the contrary, samples on day 5 and day 10 had lower divergence values (>0.3 and >0.2, respectively), indicating homogenous microbiota (Figure 2d).

The statistical homogeneity test of the multivariate dispersion of microbial composition among the types of treatment and the sampling days showed that the variances between the different washing treatments were not significantly different. In the case of the sampling days, the composition changes between day 5 and day 10 were not significantly different, but changes between day 0 and day 5 and between day 0 and day 10 were significantly different ($p < 0.05$) (Table 3b).

Table 3. (a) Permutational multivariate analysis of variance (PERMANOVA) results based on Bray-Curtis dissimilarities using abundance data for treatment types and sampling days. (b) Analysis of variance (ANOVA) pairwise comparison tests of dispersion of microbial composition among sampling days (significant if p value < 0.05).

	Df	Sum Sq	F.Model	R^2	P
Treatment Type	4	0.455	1.813	0.330	0.053
Sampling Day	2	0.421	3.359	0.305	0.006
Residual	8	0.502			
Total	14	1.378			

(a) Df—degrees of freedom; Sum Sq—sum of squares; F.Model—F value by permutation. R^2—the effect size. Boldface indicates statistical significance with $p < 0.05$ based on 1000 permutations.

Sampling Day	p-Value (Observed)	p-Value (Permutated)
Day 0–Day 5	0.022	0.029
Day 0–Day 10	0.031	0.027
Day 5–Day 10	0.856	0.854

(b) Boldface indicates statistical significance with $p < 0.05$ based on 1000 permutations.

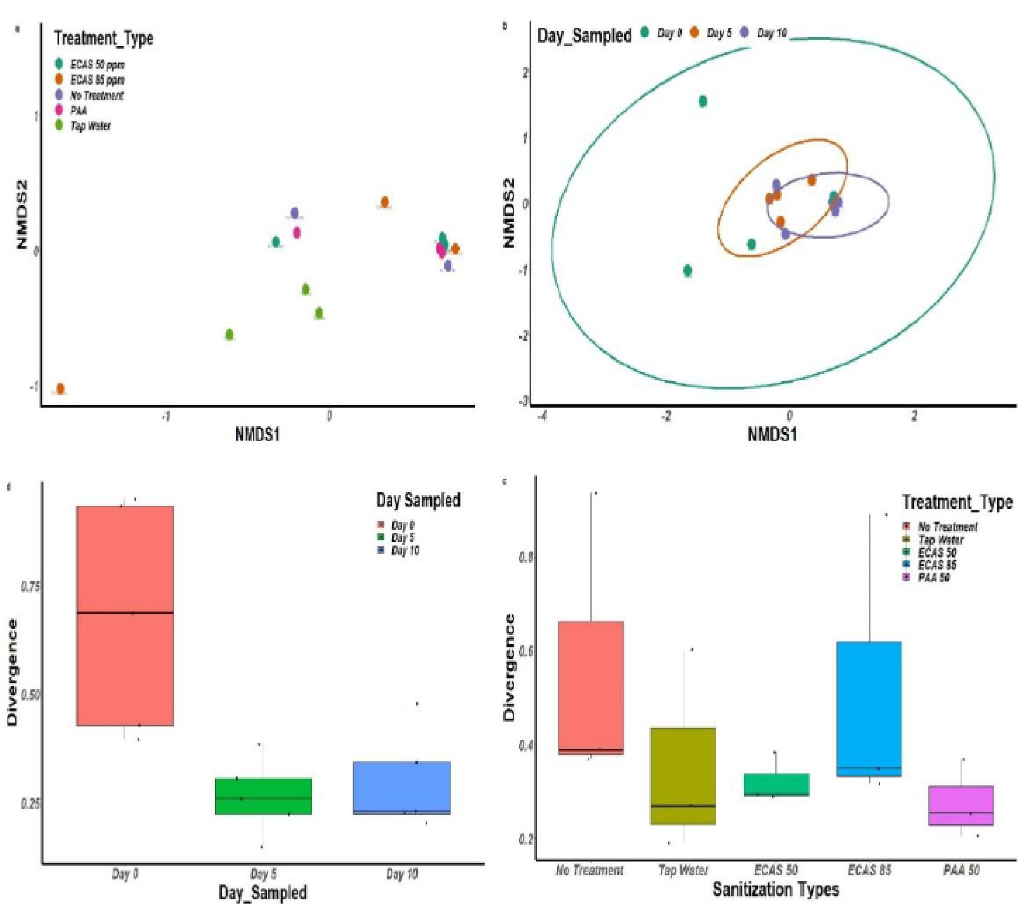


Figure 2. Microbial community cluster analysis of assembled sequence variants (ASV) non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarity index for (**a**) electrochemically activated solution (ECAS), tap water, and PAA washing, and (**b**) sampling days. Dispersion of the beta diversity group based on Bray–Curtis dissimilarity index for (**c**) ECAS, tap water, and PAA washing, and (**d**) sampling days.

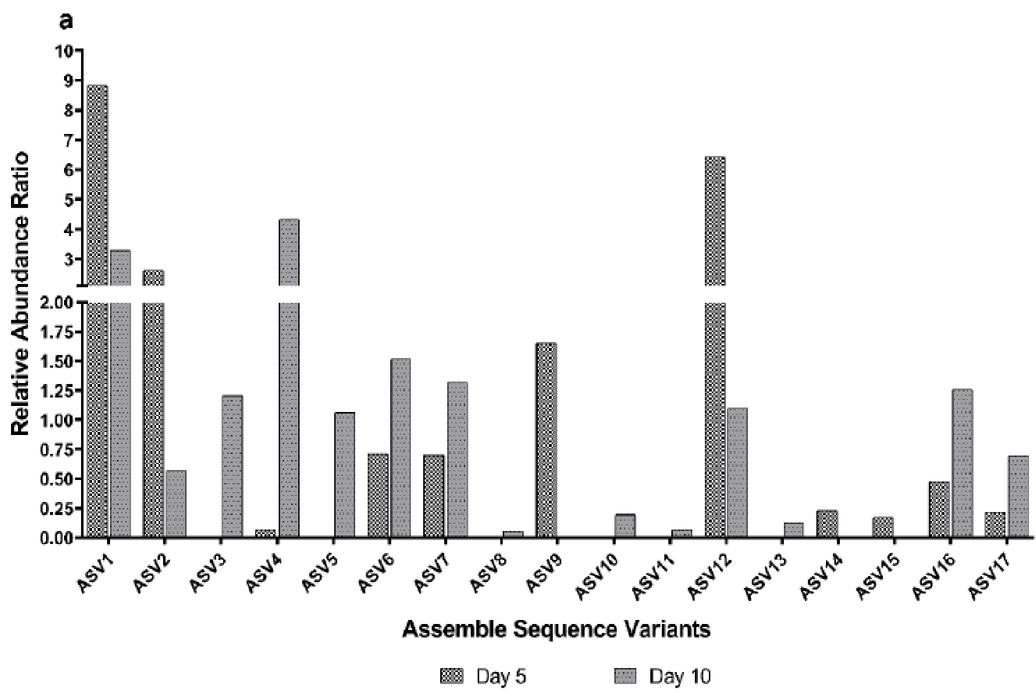
3.4. Taxa Differences Among the Different Sampling Days

ANCOM performed with a false discovery rate (FDR) of 0.05 identified 17 ASVs and four families with significantly different abundances (ANCOM W ≥ 5) among the different sampling days (Table 4). No significantly different ASVs and families were identified for the various washing treatments. The relative abundance ratios of ASVs and taxa rank family were calculated using day 0 as the basis for displaying the relative abundance in Figure 3a,b, respectively. Out of 17 ASVs identified as significantly different, 4 ASVs on day 5, and 8 ASVs on day 10 had an increase in relative abundance. An ASV identified as belonging to the *Pseudomonadaceae* family (unclassified genus) had the highest relative abundance (RA ratio of 8.82), followed by an ASV belonging to the *Moraxellaceae* family (*Alkanindiges illinoisensis*—RA ratio of 6.41) on day 5. ASVs identified as belonging to the *Flavobacteriaceae* (unclassified genus) and *Pseudomonadaceae* (unclassified genus) families had RAs of 4.3 and 3.2, respectively. ANCOM family-level analysis revealed that *Pseudomonadaceae* had the highest relative abundance (2.9) on day 5. The relative abundance of three additional families (*Spingobacteriaceae*, *Flavobacteriaceae* and *Xanthomonadaceae*) on day 5 and day 10 were lower than on day 0 (Table 4).

Table 4. Taxa (17 ASVs) and genera (5 genera) identified as significantly different in abundance on sampling days 0, 5 and 10 by analysis of composition of microbiomes (ANCOM) analysis at a false discovery rate (FDR) of 0.05. The higher the W value, the more significant are the differences in abundance levels between the sampling days.

ASVs *		Relative Abundance (RA)			Reject Hypothesis
Taxon [a]	W	Day 0	Day 5	Day 10	
f *Pseudomonadaceae*; g unclassified (ASV1)	37	0.0010	0.0087	0.0032	TRUE **
g *Alkanindiges*; s *illinoisensis* (ASV2)	14	0.0030	0.0079	0.0017	TRUE **
f *Flavobacteriaceae*; g unclassified (ASV3)	13	0.0016	0.0000	0.0019	TRUE **
f *Flavobacteriaceae*; g unclassified (ASV4)	12	0.0002	0.0000	0.0009	TRUE **
g *Herminiimonas*; s aquatilis (ASV5)	10	0.0011	0.0000	0.0012	TRUE **
f *Micrococcaceae*; g *Arthrobacter* (ASV6)	10	0.0107	0.0076	0.0162	TRUE**
f *Oxalobacteraceae*; g unclassified (ASV7)	10	0.0142	0.0099	0.0187	TRUE **
f Flavobacteriaceae; g *Persicivirga*(ASV8)	10	0.0028	0.0000	0.0001	TRUE **
o Bacillales; f unclassified (ASV9)	10	0.0010	0.0017	0.0000	TRUE **
g *Achromobacter*; s *xylosoxidans* (ASV10)	7	0.0009	0.0000	0.0002	TRUE **
g *Flavobacterium*; s *frigidarium* (ASV11)	7	0.0010	0.0000	0.0001	TRUE **
g *Alkanindiges*; s *illinoisensis* (ASV12)	6	0.0060	0.0388	0.0066	TRUE **
f *Flavobacteriaceae*; g unclassified (ASV13)	6	0.0013	0.0000	0.0002	TRUE **
f *Pseudomonadaceae*; g unclassified (ASV14)	6	0.0022	0.0005	0.0000	TRUE **
g *Flavobacterium*; s *frigidarium* (ASV15)	5	0.0013	0.0002	0.0000	TRUE **
f *Micrococcaceae*; g *Arthrobacter* (ASV16)	5	0.1405	0.0667	0.1766	TRUE **
f *Flavobacteriaceae*; g unclassified (ASV17)	5	0.0010	0.0002	0.0007	TRUE **
Family Taxon	**W**				
o Sphingobacteriales; f *Sphingobacteriaceae*	6	0.5810	0.1827	0.2363	TRUE **
o Pseudomonadales; f *Pseudomonadaceae*	5	0.2025	0.5894	0.2082	TRUE **
o Xanthomonadales; f *Xanthomonadaceae*	2	0.4953	0.1604	0.3443	TRUE **
o Flavobacteriales; f *Flavobacteriaceae*	2	0.4343	0.1861	0.3796	TRUE **

* Amplicon sequence variants, [a] Taxa are identified from Greengenes database. ** Indicate rejected null hypothesis. o-order, f-family, g-genus, s-species.



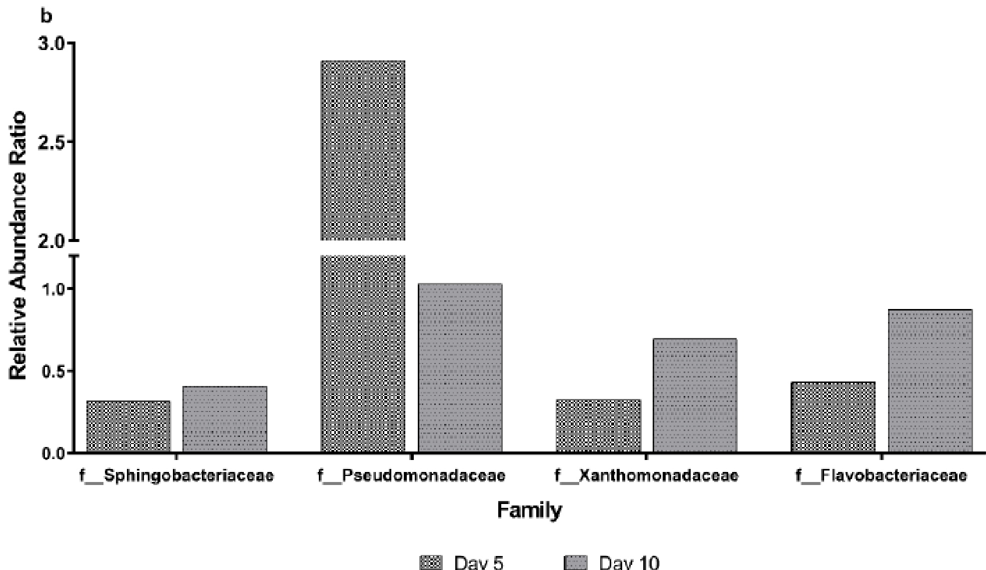


Figure 3. The relative abundance (RA) ratio of (**a**) ASVs (17 ASVs with taxa identification in Table 4) and (**b**) taxa rank family (4) identified as significantly different in abundance on sampling days 5 and 10 by ANCOM analysis at a false discovery rate (FDR) of 0.05. The RA ratio is calculated as RA on day 5 or day 10 divided by RA on day 0. f-family.

4. Discussion

This study investigated the microbiome profiles of RTE spinach leaves washed with different sanitizers (ECAS, PAA) and compared with leaves washed with tap water and not washed at all (control), at three time points over 10 days (day 0, day 5, and day 10). Although a higher proportion of ASVs was found compared to previous studies [26,27], their richness and evenness (alpha diversity) did not significantly differ among the types of sanitizer and the sampling points. We also found that the types of sanitizing washing, apart from a reduced heterogeneity over time, did not significantly

influence the community structure of the bacteria (beta diversity). ANCOM analyses identified that the composition of ASVs and families changed significantly over the sampling days.

The number of ASVs identified (>2000) in the present study was much higher than that observed in the spinach leaf microbiome profiling studies by Gu et al. [26] and Söderqvist et al. [27], who identified 673 and 190 operational taxonomic units respectively. In addition, 12 phyla were identified in this study compared to the four phyla observed by Söderqvist et al. [27] and 14 phyla detected by Gu et al. [26]; however, the number of predominant phyla (n = 4) and their relative proportions are similar in all three studies. In agreement with previous observations, the phylum Proteobacteria showed the highest total abundance on day 0, followed by phyla Bacteroidetes, Firmicutes, and Actinobacteria [3,6,26,34]. The basal bacterial microbiome of RTE spinach leaves is therefore very similar to that of other minimally processed fruits and vegetables [3,6,27,34,60].

Our analyses also showed that the Shannon and Inverse Simpson diversity indexes and richness (ACE and Chao1) measures did not differ significantly among all spinach samples. Furthermore, the community composition of bacteria (beta diversity) for all types of washing did not differ significantly, indicating that ECAS treatments did not affect bacterial microbial diversity. This could be seen as a good outcome, since it has been suggested that the microbiome on fresh produce is not responsible for spoilage but acts as a natural biological barrier against spoilage organisms and pathogens [13–15]. On the other hand, a significant grouping of spinach microbial community structures was observed for sampling days and reduction over time of the heterogeneity of bacterial composition. The reduction in heterogeneity can be attributed to the reduction in the relative abundance of phylum Proteobacteria on day 5 and day 10, in accordance with the reduction observed by Gu et al. [26] in RTE spinach leaves washed with chlorine and stored at 4 °C for a week. Moreover, the microbiome community on day 10 clustered distinctly due to a significant increase in the relative abundance of Bacteroidetes, similar to that observed by Gu et al. [26] when the spinach leaves were stored at 4 °C for a week.

ANCOM is a method based on compositional log-ratios to detect differences in relative abundance and has been used to detect taxa abundance in the spinach microbiome at ASV and family level. Taxa at ASV and family level for the different types of treatment were not significantly different, but differences in ASVs and family-related abundances were identified at different sampling days. ASVs identified as *Pseudomonadaceae* and *Moraxellaceae* families, and the order Bacillales (unclassified family) had a high relative abundance on day 5. The increase in the relative abundance of these families of bacteria (*Pseudomonadaceae* and *Moraxellaceae*) has been correlated strongly with the spoilage of leafy vegetables at cold storage temperatures [34]. Increases in the relative abundance of the order Bacillales were also observed by Söderqvist et al. [27] and have been positively correlated with the increase in the viable counts of bacteria causing food safety concerns (*Yersinia enterocolitica*, *Listeria monocytogenes* and *E. coli* O157:H7) [27]. A significant increase was observed for four ASVs (classified as *Pseudomonadaceae*, *Flavobacteriaceae*, *Micrococcaceae* and *Oxalobacteraceae*) on day 10 and it is interesting to note that the relative abundance of the order Flavobacteriales was negatively correlated with foodborne pathogens in a previous study [27]. The predominance of *Micrococcaceae* and *Oxalobacteraceae* may be explained by their ability to grow at extremely low temperatures [61]; they are considered putative protectors against *Rhizoctonia* (fungal) rot of root crops [62]. The abundance of the family *Xanthomonadaceae* was significantly reduced on day 10, as observed by Lopez-Velasco et al. [3] and Schwartz et al. [60]. Similarly, the abundance of *Spingobacteriaceae* was significantly reduced, and the order Sphingobacteriales was correlated positively to *Escherichia coli* O157:H7 counts and negatively to *L. monocytogenes* and *Y. enterocolitica* counts [27].

5. Conclusions

To our knowledge, this study represents the first documented profile of the bacterial microbiome present on minimally processed RTE Australian spinach treated with ECAS. We have shown that washing with a neutral ECAS did not significantly change the composition of the bacterial communities compared to washing with PAA (Tsunami® 100) and tap water. In addition, complete changes over

time in the community composition of bacterial species have been documented during storage at refrigeration temperature (4 ± 1 °C) on day 5 and day 10 after washing treatments, compared to day 0. The information that ECAS does not change the structure of the bacterial community could help select an environmentally friendly biocidal agent capable of meeting the aesthetic needs of current consumers and production industries.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/10/8/2986/s1, Figure S1. Relative Abundance ratio of bacterial taxonomy level order for sanitization wash types and sampling days.; Figure S2. Visualization of Shannon and Inverse–Simpson diversity (alpha-diversity) and Chao and ACE richness metrics of all samples.; Table S1. Total abundances and percentage abundances of most abundant taxa at family and genus level.; Table S2. Probability values of analysis of variance (ANOVA), Tukey's HSD test on ANOVA of Shannon diversity index, Kruskal–Wallis H test, and Wilcoxon pairwise rank-sum test of Chao1 richness comparing alpha diversity metrics among the types of sanitizing treatment (Treatment Types) and day post-sanitation treatment (Day Sampled). Alpha diversity was not significantly different among the types of treatment and the sampling days, as determined by ANOVA and Tukey's HSD test.

Author Contributions: Conceptualization, S.T. and A.D.O.; Data curation, S.T.; Formal analysis, S.T.; Methodology, S.T. and A.D.O.; Supervision, D.J.T. and P.D.; Visualization, S.T.; Writing, original draft, S.T.; Writing, review and editing, S.T., A.D.O, S.F., P.D., and D.J.T. All authors have read and agreed to the published version of the manuscript.

Funding: Ecas4 Australia Pty Ltd. funded the study, including the 16s rRNA pyrosequencing.

Acknowledgments: S.T. was supported by the Endeavour Postgraduate Scholarship. ANCOM analysis was performed with supercomputer resources provided by the Phoenix HPC service of the University of Adelaide. We would like to acknowledge Mark Van der Hoek (David Gunn Genomics Facility, South Australian Health and Medical Research Institute, Australia) for his generous technical guidance on preparing samples for genomic analysis.

Conflicts of Interest: Page: 12, Ecas4 Australia Pty Ltd. played no role in the study design, data collection and interpretation, and decision to submit the article for publication. The authors declare no conflict of interest.

References

1. Lindow, S.E.; Brandl, M.T. Microbiology of the Phyllosphere. *Appl. Environ. Microbiol.* **2003**, *69*, 1875–1883. [CrossRef] [PubMed]
2. Delmotte, N.; Knief, C.; Chaffron, S.; Innerebner, G.; Roschitzki, B.; Schlapbach, R.; Von Mering, C.; Vorholt, J.A. Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 16428–16433. [CrossRef] [PubMed]
3. Lopez-Velasco, G.; Welbaum, G.; Boyer, R.; Mane, S.; Ponder, M.A. Changes in spinach phylloepiphytic bacteria communities following minimal processing and refrigerated storage described using pyrosequencing of 16S rRNA amplicons. *J. Appl. Microbiol.* **2011**, *110*, 1203–1214. [CrossRef] [PubMed]
4. Rastogi, G.; Sbodio, A.; Tech, J.J.; Suslow, T.V.; Coaker, G.L.; Leveau, J.H.J. Leaf microbiota in an agroecosystem: Spatiotemporal variation in bacterial community composition on field-grown lettuce. *ISME J.* **2012**, *6*, 1812–1822. [CrossRef]
5. Whipps, J.; Hand, P.; Pink, D.; Bending, G. Phyllosphere microbiology with special reference to diversity and plant genotype. *J. Appl. Microbiol.* **2008**, *105*, 1744–1755. [CrossRef] [PubMed]
6. Leff, J.W.; Fierer, N. Bacterial Communities Associated with the Surfaces of Fresh Fruits and Vegetables. *PLoS ONE* **2013**, *8*, e59310. [CrossRef]
7. Cordano, A. Occurrence of Listeria monocytogenes in food in Chile. *Int. J. Food Microbiol.* **2001**, *70*, 175–178. [CrossRef]
8. De Oliveira, M.A.; De Souza, V.M.; Bergamini, A.M.M.; De Martinis, E.C.P. Microbiological quality of ready-to-eat minimally processed vegetables consumed in Brazil. *Food Control.* **2011**, *22*, 1400–1403. [CrossRef]
9. Klerks, M.M.; Franz, E.; Van Gent-Pelzer, M.; Zijlstra, C.; Van Bruggen, A.H.C. Differential interaction of Salmonella enterica serovars with lettuce cultivars and plant-microbe factors influencing the colonization efficiency. *ISME J.* **2007**, *1*, 620–631. [CrossRef]
10. Mitra, R.; Cuesta-Alonso, E.; Wayadande, A.; Talley, J.; Gilliland, S.; Fletcher, J. Effect of Route of Introduction and Host Cultivar on the Colonization, Internalization, and Movement of the Human Pathogen Escherichia coli O157:H7 in Spinach. *J. Food Prot.* **2009**, *72*, 1521–1530. [CrossRef]

11. Hackl, E.; Hölzl, C.; Konlechner, C.; Sessitsch, A. Food of plant origin: Production methods and microbiological hazards linked to food-borne disease. Reference: CFT/EFSA/BIOHAZ/2012/01 Lot 1 (Food of plant origin with high water content such as fruits, vegetables, juices and herbs). *EFSA Support. Publ.* **2013**, *10*, 402E. [CrossRef]
12. Gaglio, R.; Miceli, A.; Sardina, M.T.; Francesca, N.; Moschetti, G.; Settanni, L. Evaluation of microbiological and physico-chemical parameters of retail ready-to-eat mono-varietal salads. *J. Food Process. Preserv.* **2019**, *43*, e13955. [CrossRef]
13. Andrews, J.H.; Harris, R.F. The Ecology and Biogeography of Microorganisms on Plant Surfaces. *Annu. Rev. Phytopathol.* **2000**, *38*, 145–180. [CrossRef] [PubMed]
14. Janisiewicz, W.J.; Korsten, L. Biological Control of Postharvest Diseases of Fruits. *Annu. Rev. Phytopathol.* **2002**, *40*, 411–441. [CrossRef]
15. Barth, M.; Hankinson, T.R.; Hong, Z.; Breidt, F. Microbiological Spoilage of Fruits and Vegetables. In *Compendium of the Microbiological Spoilage of Foods and Beverages*; Springer: Berlin, Germany, 2009; pp. 135–183.
16. Schuenzel, K.M.; Harrison, M.A. Microbial Antagonists of Foodborne Pathogens on Fresh, Minimally Processed Vegetables. *J. Food Prot.* **2002**, *65*, 1909–1915. [CrossRef]
17. Scolari, G.; Vescovo, M. Microbial antagonism of Lactobacillus Casei added to fresh vegetables. *Ital. J. Food Sci.* **2004**, *16*, 465–475.
18. Johnston, M.A.; Harrison, M.A.; Morrow, R.A. Microbial Antagonists of Escherichia coli O157:H7 on Fresh-Cut Lettuce and Spinach. *J. Food Prot.* **2009**, *72*, 1569–1575. [CrossRef]
19. Cooley, M.B.; Miller, W.G.; Mandrell, R.E. Colonization of Arabidopsis thaliana with Salmonella enterica and Enterohemorrhagic Escherichia coli O157:H7 and Competition by Enterobacter asburiae. *Appl. Environ. Microbiol.* **2003**, *69*, 4915–4926. [CrossRef] [PubMed]
20. Jackson, C.R.; Randolph, K.C.; Osborn, S.L.; Tyler, H.L. Culture dependent and independent analysis of bacterial communities associated with commercial salad leaf vegetables. *BMC Microbiol.* **2013**, *13*, 274. [CrossRef] [PubMed]
21. Rudi, K.; Flateland, S.L.; Hanssen, J.F.; Bengtsson, G.; Nissen, H. Development and Evaluation of a 16S Ribosomal DNA Array-Based Approach for Describing Complex Microbial Communities in Ready-To-Eat Vegetable Salads Packed in a Modified Atmosphere. *Appl. Environ. Microbiol.* **2002**, *68*, 1146–1156. [CrossRef] [PubMed]
22. Handschur, M.; Piñar, G.; Gallist, B.; Lubitz, W.; Haslberger, A.G. Culture free DGGE and cloning based monitoring of changes in bacterial communities of salad due to processing. *Food Chem. Toxicol.* **2005**, *43*, 1595–1605. [CrossRef] [PubMed]
23. Truchado, P.; Gil, M.I.; Suslow, T.; Allende, A. Impact of chlorine dioxide disinfection of irrigation water on the epiphytic bacterial community of baby spinach and underlying soil. *PLoS ONE* 2018, *13*, e0199291. [CrossRef] [PubMed]
24. Lopez-Velasco, G. Molecular Characterization of Spinach (Spinacia Oleracea) Microbial Community Structure and Its Interaction with Escherichia Coli O157:H7 in Modified Atmosphere Conditions. Ph.D. Thesis, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA, 2010. Available online: https://vtechworks.lib.vt.edu/handle/10919/37601 (accessed on 19 September 2019).
25. Gu, L.; Bai, Z.; Jin, B.; Hu, Q.; Wang, H.; Zhuang, G.; Zhang, H. Assessing the impact of fungicide enostroburin application on bacterial community in wheat phyllosphere. *J. Environ. Sci.* **2010**, *22*, 134–141. [CrossRef]
26. Gu, G.; Ottesen, A.; Bolten, S.; Ramachandran, P.; Reed, E.; Rideout, S.; Luo, Y.; Patel, J.; Brown, E.; Nou, X. Shifts in spinach microbial communities after chlorine washing and storage at compliant and abusive temperatures. *Food Microbiol.* **2018**, *73*, 73–84. [CrossRef]
27. Söderqvist, K.; Osman, O.A.; Wolff, C.; Bertilsson, S.; Vågsholm, I.; Boqvist, S. Emerging microbiota during cold storage and temperature abuse of ready-to-eat salad. *Infect. Ecol. Epidemiol.* **2017**, *7*, 1328963. [CrossRef]
28. Izumi, H.; Kiba, T.; Hashimoto, S. Efficacy of electrolyzed water as a disinfectant for fresh-cut spinach. *ACIAR Proc.* **2000**, *100*, 216–221.
29. Rahman, S.; Ding, T.; Oh, D.-H. Inactivation effect of newly developed low concentration electrolyzed water and other sanitizers against microorganisms on spinach. *Food Control.* **2010**, *21*, 1383–1387. [CrossRef]

30. Gómez-López, V.M.; Marín, A.; Medina-Martínez, M.S.; Gil, M.I.; Allende, A. Generation of trihalomethanes with chlorine-based sanitizers and impact on microbial, nutritional and sensory quality of baby spinach. *Postharvest Boil. Technol.* **2013**, *85*, 210–217. [CrossRef]
31. Guentzel, J.L.; Lam, K.L.; Callan, M.A.; Emmons, S.A.; Dunham, V.L. Reduction of bacteria on spinach, lettuce, and surfaces in food service areas using neutral electrolyzed oxidizing water. *Food Microbiol.* **2008**, *25*, 36–41. [CrossRef]
32. Park, E.-J.; Alexander, E.; Taylor, G.; Costa, R.; Kang, D.-H. Effects of organic matter on acidic electrolysed water for reduction of foodborne pathogens on lettuce and spinach. *J. Appl. Microbiol.* **2008**, *105*, 1802–1809. [CrossRef]
33. Babic, I.; Watada, A.E.; Buta, J.G. Growth of Listeria monocytogenes Restricted by Native Microorganisms and Other Properties of Fresh-Cut Spinach. *J. Food Prot.* **1997**, *60*, 912–917. [CrossRef] [PubMed]
34. Tatsika, S.; Karamanoli, K.; Karayanni, H.; Genitsaris, S. Metagenomic Characterization of Bacterial Communities on Ready-to-Eat Vegetables and Effects of Household Washing on their Diversity and Composition. *Pathogens* **2019**, *8*, 37. [CrossRef] [PubMed]
35. Rivera-Garcia, A.; Santos-Ferro, L.; Ramirez-Orejel, J.C.; Agredano-Moreno, L.T.; Jimenez-Garcia, L.F.; Paez-Esquiliano, D.; Andrade-Esquivel, E.; Cano-Buendia, J.A. The effect of neutral electrolyzed water as a disinfectant of eggshells artificially contaminated with Listeria monocytogenes. *Food Sci. Nutr.* **2019**, *7*, 2252–2260. [CrossRef] [PubMed]
36. Premier, R. Evaluation of Vegetable Washing Chemicals. In Australia: Horticulture Australia Ltd. 2013. Available online: https://www.ausvegvic.com.au/pdf/r%26d_VG09086_vegetable_wash_water_extract.pdf (accessed on 12 September 2019).
37. Ogunniyi, A.D.; Tenzin, S.; Khazandi, M.; Ferro, S.; Venter, H.; Pi, H.; Amorico, T.; Deo, P.; Trott, D.J. A pH-neutral electrolyzed oxidizing water significantly reduces microbial contamination of fresh spinach leaves. *Food Microbiol.* **2020**. under review (Accessed on 25/02/2020).
38. Klindworth, A.; Pruesse, E.; Schweer, T.; Peplies, J.; Quast, C.; Horn, M.; Glöckner, F.O. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* **2012**, *41*, e1. [CrossRef]
39. Amplicon, P.; Clean-Up, P.; Index, P. 16s Metagenomic Sequencing Library Preparation. 2013. Available online: https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf (accessed on 17 November 2018).
40. Andrews, S. FastQC a Quality Control Tool for High Throughput Sequence Data. Available online: https://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (accessed on 22 January 2019).
41. Edgar, R.C.; Flyvbjerg, H. Error filtering, pair assembly and error correction for next-generation sequencing reads. *Bioinformatics* **2015**, *31*, 3476–3482. [CrossRef]
42. Core Team, R. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna. 2019. Available online: https://www.R-project.org/ (accessed on 14 February 2019).
43. Callahan, B.J.; McMurdie, P.; Rosen, M.J.; Han, A.W.; Johnson, A.J.; Holmes, S. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **2016**, *13*, 581–583. [CrossRef]
44. Wang, Q.; Garrity, G.M.; Tiedje, J.M.; Cole, J.R. Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. *Appl. Environ. Microbiol.* **2007**, *73*, 5261–5267. [CrossRef]
45. Quast, C.; Pruesse, E.; Yilmaz, P.; Gerken, J.; Schweer, T.; Yarza, P.; Peplies, J.; Glöckner, F.O. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Res.* **2012**, *41*, D590–D596. [CrossRef]
46. Callahan, B. Silva taxonomic training data formatted for DADA2 (Silva version 132). *Zenodo* **2018**. [CrossRef]
47. Wright, E.S. Using DECIPHER v2.0 to analyze big biological sequence data in R. *R Journal* **2016**, *8*, 352–359. Available online: https://journal.r-project.org/archive/2016-1/wright.pdf (accessed on 3 March 2019). [CrossRef]
48. Schliep, K. phangorn: Phylogenetic analysis in R. *Bioinformatics* **2010**, *27*, 592–593. [CrossRef] [PubMed]
49. McMurdie, P.; Holmes, S. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS ONE* **2013**, *8*, e61217. [CrossRef] [PubMed]
50. McMurdie, P.; Holmes, S. Shiny-phyloseq: Web application for interactive microbiome analysis with provenance tracking. *Bioinformatics* **2014**, *31*, 282–283. [CrossRef]

51. Lozupone, C.A.; Knight, R. Species divergence and the measurement of microbial diversity. *FEMS Microbiol. Rev.* **2008**, *32*, 557–578. [CrossRef]
52. Knight, R.; Vrbanac, A.; Taylor, B.C.; Aksenov, A.; Callewaert, C.; Debelius, J.; González, A.; Kosciolek, T.; McCall, L.-I.; McDonald, D.; et al. Best practices for analysing microbiomes. *Nat. Rev. Genet.* **2018**, *16*, 410–422. [CrossRef]
53. Oksanen, J.; Blanchet, F.; Friendly, M.; Kindt, R.; Legendre, P.; McGlinn, D.; Minchin, P.; O'Hara, R.; Simpson, G.; Solymos, P. Ordination methods, diversity analysis and other functions for community and vegetation ecologists. In Vegan: Community Ecology Package. 2019. Available online: https://www.researchgate.net/publication/319525938_vegan_Community_Ecology_Package_Ordination_methods_diversity_analysis_and_other_functions_for_community_and_vegetation_ecologists_Version_22-4_URL_httpsCRANR-projectorgpackagevegan/link/59b0e308a6fdcc3f888bf5da/download (accessed on 3 March 2019).
54. Bray, J.R.; Curtis, J.T. An Ordination of the Upland Forest Communities of Southern Wisconsin. *Ecol. Monogr.* **1957**, *27*, 325–349. [CrossRef]
55. Lozupone, C.; Knight, R. UniFrac: A New Phylogenetic Method for Comparing Microbial Communities. *Appl. Environ. Microbiol.* **2005**, *71*, 8228–8235. [CrossRef]
56. Anderson, M.J. Permutational Multivariate Analysis of Variance (PERMANOVA). In *Wiley StatsRef: Statistics Reference Online*; John Wiley & Sons, Ltd.: Hoboken, NJ, USA, 2014; pp. 1–15.
57. Lahti, L.; Shetty, S.; Blake, T.; Salojarvi, J. Microbiome R package. 2012–2019. [CrossRef]
58. Mandal, S.; Van Treuren, W.; White, R.A.; Eggesbø, M.; Knight, R.; Peddada, S.D. Analysis of composition of microbiomes: A novel method for studying microbial composition. *Microb. Ecol. Health Dis.* **2015**, *26*, 27663. [CrossRef]
59. Bolyen, E.; Rideout, J.R.; Dillon, M.R.; Bokulich, N.A.; Abnet, C.C.; Al-Ghalith, G.A.; Alexander, H.; Alm, E.J.; Arumugam, M.; Asnicar, F.; et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME. *Nat. Biotechnol.* **2019**, *37*, 852–857. [CrossRef]
60. Jarvis, K.; Daquigan, N.; White, J.R.; Morin, P.M.; Howard, L.M.; Manetas, J.E.; Ottesen, A.; Ramachandran, P.; Grim, C.J. Microbiomes Associated With Foods From Plant and Animal Sources. *Front. Microbiol.* **2018**, *9*, 2540. [CrossRef] [PubMed]
61. Schwartz, E.; Van Horn, D.J.; Buelow, H.N.; Okie, J.G.; Gooseff, M.; Barrett, J.E.; Takacs-Vesbach, C. Characterization of growing bacterial populations in McMurdo Dry Valley soils through stable isotope probing with (18) O-water. *FEMS Microbiol. Ecol.* **2014**, *89*, 415–425. [CrossRef] [PubMed]
62. Araujo, R.; Dunlap, C.; Barnett, S.; Franco, C. Decoding Wheat Endosphere-Rhizosphere Microbiomes in Rhizoctonia solani-Infested Soils Challenged by Streptomyces Biocontrol Agents. *Front. Plant Sci.* **2019**, *10*, 1038. [CrossRef] [PubMed]

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

Supplementary Information

Figure S1: Relative Abundance ratio of bacterial taxonomy level order for sanitization wash types and sampling days.

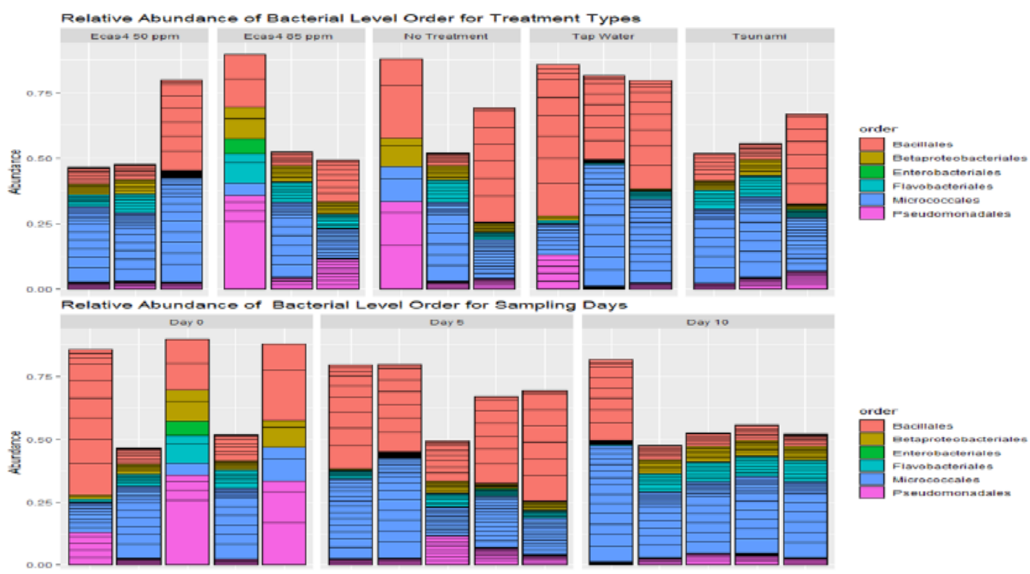
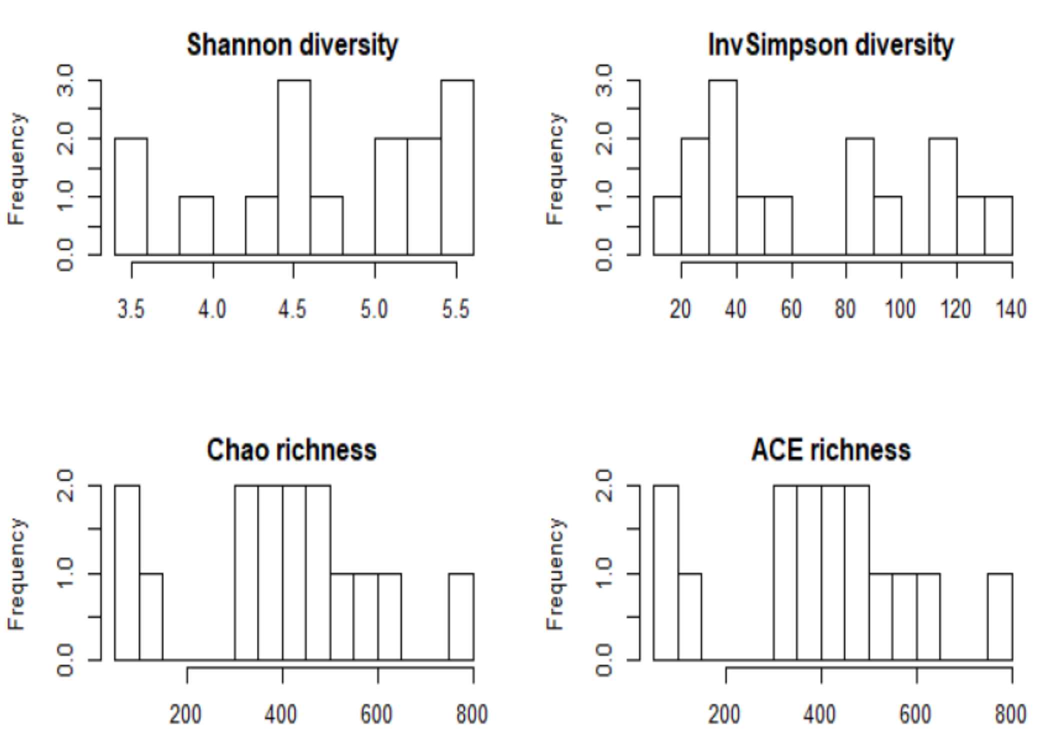


Figure S2: Visualization of Shannon and Inverse-Simpson diversity (alpha-diversity) and Chao and ACE richness metrics of all samples.



Supplementary Table 1: Percentage of most abundant family and genus

A. Family	Total Abundance	Percentage Abundance
Micrococcaceae	108098	28.20737633
Family_XII	75376	19.66881162
Flavobacteriaceae	68486	17.8709169
Pseudomonadaceae	48884	12.75591948
Burkholderiaceae	38733	10.10709086
Moraxellaceae	16523	4.311555062
Shewanellaceae	4897	1.277836055
Enterobacteriaceae	4197	1.095176215
Weeksellaceae	3992	1.041682976
Microbacteriaceae	3401	0.887465882
Paenibacillaceae	3038	0.792743707
Sphingobacteriaceae	3024	0.789090511
B. Genus		
Exiguobacterium	75376	19.66881162
Flavobacterium	67949	17.73079071
Arthrobacter	58908	15.3716084
Pseudomonas	48382	12.62492628
Paeniglutamicibacter	39378	10.27539885
Janthinobacterium	27242	7.108599103
Acinetobacter	14875	3.881521609
Pseudarthrobacter	7062	1.842776847
Duganella	6430	1.677861105
Shewanella	4897	1.277836055
Chryseobacterium	3894	1.016110598

Supplementary Table 2: Probability values of analysis of variance (ANOVA), Tukey's HSD test on ANOVA of Shannon diversity index, Kruskal-Wallis H test and Wilcoxon pairwise rank sum test of Chao1 richness comparing alpha diversity metrics among sanitizing treatment types (Treatment Types) and day post post storage at 4 ºC (Day Sampled). Alpha diversity was not significantly different among treatment types and sampling days as determined by ANOVA and Tukey's HSD test (with $p > 0.1$ for all the pairs).

	Shannon Diversity		**Chao1 richness**	
	ANOVA	**Tukey's HSD (ANOVA)**	**Kruskal-Wallis**	**Wilcoxon pairwise***
All treatment types	0.45		0.15	
ECAS 50 – ECAS 85		0.93		0.67
ECAS 50 – No Treatment		0.66		0.5
ECAS 50 – Tap Water		0.52		0.5
ECAS 50 – Tsunami® 100 (PAA)		0.99		0.67
ECAS 85 – No Treatment		0.97		0.88
ECAS 85 – Tap Water		0.91		0.88
ECAS 85 – Tsunami® 100 (PAA)		0.97		1
No Treatment – Tap Water		0.99		1
No Treatment – Tsunami® 100 (PAA)		0.76		0.5
Tap Water – Tsunami® 100 (PAA)		0.62		0.5
All sampling days	0.23		0.59	
Day 0 – Day 5		0.7		0.69
Day 0 – Day 10		0.21		0.69
Day 5 – Day 10		0.6		0.69

*false discovery rate (FDR) corrected p-value

Chapter 7: General Discussions

7.1 INTRODUCTION

Cleaning and decontamination in animal farming and agriculture production settings remain key strategies for disease prevention and control. Similarly, cleaning and disinfection to reduce and inactivate generic bacterial load and pathogens in food processing and packaging addresses a critical control point in a Hazard Analysis and Critical Control Points (HACCP) approach to ensuring food safety. For cleaning and disinfection, production and processing companies use chemical disinfectants that are cost-effective, easily usable and microbiologically active against a broad range of microorganisms. Chemical disinfectants with such qualities usually come with drawbacks such as health hazard due to chemical residue, environmental pollution and co-selection of antimicrobial resistance, are easily quenched by organic load and affect other quality parameters. The use of disinfectants is regulated to control residual impact on health, environment and bacterial antibiotic resistance. Moreover, increased understanding of the carry-over risks of traces of these chemicals from the food supply chain led consumer protection agencies to push for stringent regulation. The tightening rules mean that industries either pay a higher cost to comply with the regulation or altogether stop the use of some disinfectants. These situations present opportunities to explore non-hazardous and environmentally safe chemical sanitizers in livestock farming and food safety applications.

Electrochemically activated solution (ECAS) is one such biocidal agent that has broad-spectrum antimicrobial activity, with no reported toxicity, is environmentally compatible and produced from low cost readily available raw materials. ECAS has proven antimicrobial activity against a broad range of bacteria from animal houses, animal products and food commodities. ECAS has recently been used on a small scale in medical, food and veterinary decontamination processes. Therefore, this thesis focused on the evaluation of alternative disinfection ECAS application methods such as pressure spray and solution washing, and aerosol fog sanitizing that could be automated to suit different industrial settings.

A proof-of-concept neutral ECAS aerosol fog disinfection method for the swine barn air environment was developed to decontaminate aerosolized bacteria (Chapter 2). The EO water fog and spray washing effectiveness in disinfecting autochthonous bacteria and *S.* Enteritidis on the shelled eggs and, importantly, its effect on the eggshell cuticle layer was assessed. Neutral ECAS had disinfection efficiency similar to that of a commonly used disinfectant (NaOCl), without adversely affecting the eggshell cuticle layer (Chapter 3-4). In chapter 5, the efficacy of ECAS anolyte solution wash on total bacterial load and surrogate pathogens on post-harvest baby spinach leaves was assessed as well as its effect on sensory and nutritional attributes. The bacterial load reduction observed in this study was similar to that of PAA-based disinfectant, and it did not adversely affect the sensory attributes of leaves when stored at 4 ºC for 10 days. Chapter 6 evaluated the impact of ECAS anolyte solution wash and storage at 4 ºC on spinach leaf microbial diversity and structure. The amplicon sequences from V3 and V4 region of the 16S rRNA gene showed that ECAS and PAA did not alter the composition of the microbiota. However, bacterial community composition changes were observed on day 5 and day 10 after storing it at 4 ºC.

7.2 MAJOR FINDINGS

7.2.1 ECAS aerosol fogging decontaminates aerobic bacteria from the pig barn air environment and live/dead PMA-qPCR discerns viable and dead total bacterial load and *A. pleuropneumoniae*

SYBR Green dye-based qPCR was used to detect and quantify low levels of bacteria using universal bacterial primers for amplification of bacterial 16s rDNA and *A. pleuropneumoniae* primers for a repeat-in-toxin gene (*apxIVA*). The *A. pleuropneumoniae* qPCR could be used as a disease surveillance tool in specific pathogen-free herds. Since these qPCR methods amplify and quantify double-stranded DNA specific to these primers, we developed and optimized a propidium monoazide (PMA) sample treatment protocol to allow PMA qPCR to differentiate live/dead total bacteria load and *A. pleuropneumoniae*. The PMA qPCR for *A. pleuropneumoniae* is a novel quantification technique developed in Chapter 2 to detect low levels (0.05 pg/µL) of *apxIVA* genes and this could be used for assessment of viable but not culturable (VBNC) states in bacteria treated with antimicrobial compounds.

Moreover, to detect and quantify *A. pleuropneumoniae* and total bacterial load in the farm's environment, the cyclonic air sampler was optimized to collect air

samples in the pig barn. The optimized parameters were a collection of samples for 3 min in 10 mL of veterinary fastidious collection liquid and a pre-set collection air volume of 250 L/min using a cyclonic air sampler. These samplings allowed detection of *A. pleuropneumoniae* at 200 pg (5.1×10^6 GU) and total recoverable bacterial gDNA of 1,200 pg (1.5×10^8 GU) in 2 mL of VFM. The air sampling technique was used in conjunction with live/dead PMA qPCR to develop the ECAS aerosol disinfection method for the barn environment. The ECAS fog at a calculated concentration of 0.25 mg/m^3 FAC for 3 min every 30 mins was used to sanitize the air environment of a pig weaning room over five hours and reduced aerobic bacterial load by 5.12 $\log_{10}$ CFU/m^3 (i.e., 99.998% reduction). Figures 7 and 8 of Chapter 2 presents the hourly trend of bacterial inactivation. The high disinfection effectiveness on this empty weaning room could be due to high ORP of the solution (> 800 mV) and presence of a high concentration of HOCl (95%) (Cheng et al. 2012; Guentzel et al. 2008). Moreover, the efficacy may have been enhanced by applying the ECAS as aerosol fog, as the tiny mist particles containing aerosolized oxidative moieties bind more efficiently to the bacterial surface, causing rapid cell lysis. This science-based innovative proof-of-concept of neutral ECAS aerosol fogging could leverage the use of this environmentally friendly biocidal agent in farm biosecurity management and alleviate animal wellbeing and farmworkers health without the use of chemical disinfectants.

7.2.2 pH-neutral ECAS anolyte fog sanitization inactivates total bacterial load and *S.* Enteritidis cells from shelled eggs surfaces without affecting the cuticle layer

One of the significant components of safe egg production includes washing of the eggshell surface from organic materials and disinfection of bacteria to reduce the risk of egg-related foodborne illnesses and maintain consumer confidence in the microbiological safety of eggs. In egg disinfection, many biocidal agents are used to reduce general bacterial load to a safe level and eliminate pathogenic bacteria that are harmful to embryos and young chicks in case of hatching eggs and pathogens detrimental to humans in case of table eggs. Therefore, ECAS in the form of micron-sized aerosol fog and spray washing on disinfection of general bacteria and purposely inoculated *S.* Enteritidis on shelled eggs, and eggshell cuticle layer were evaluated.

Previous researches have employed ECAS either in the form of wash solution (Achiwa & Nishio, 2003; Surdu, Vătuiu, Jurcoane, Olteanu, & Vătuiu, 2017) or spray

sanitization (Fasenko, O'Dea Christopher, & McMullen, 2009; Rivera-Garcia et al., 2019). But, our research combined fog generation technology and neutral ECAS solution at 150 mg/L of FAC to assess its applicability in shelled egg sanitization. ECAS fogging completely inactivated bacterial cells in 120 s, whereas spray wash with same concentration took around 45 s. Since there are no comparable fogging studies, the comparison of sanitization efficiency was made to that of neutral ECAS immersion or spray washes. The total bacterial load reduction observed (2.21 Log) in this is higher than 0.4 log reduction by immersion washing for 15 min with 12 mg/ L (Surdu et al. 2017). The 4.32 Log reduction of *S.* Enteritidis observed in this study is significantly higher in comparison to 2.1 log reduction by immersing in acidic type ECAS at 70-80 mg/L FAC for 3 mins. The fogging data from our work confirm that effectiveness of disinfection depends on FAC and disinfection duration as demonstrated by other researches (Bialka et al., 2004; Fasenko, O'Dea Christopher, & McMullen, 2009; Ni et al., 2014, Park et al. 2004, Zang et al. 2019).

The cuticle layer of the egg is an *in utero* deposition on the egg surface (Hincke et al., 2000) and essential primary barrier to bacterial penetration of the egg contents (Wellman-Labadie, Picman, & Hincke, 2008). It consists of amino acids and traces of minerals (Baker & Balch, 1962; Board & Love, 1980) and covers the eggshell crystal layer (Board, 1982; Cooke & Balch, 1970). Some factors that affect cuticle parameters are hen housing types (Samiullah et al., 2013), hen age (Spark & Board 1984; Leleu et al. 2011), and sanitizing chemicals used for egg washing (Wang & Slavik, 1998). ECAS at 150 mg/L of FAC, both as spray and fog sanitization did not adversely affect the cuticle layer of shelled eggs, and this result confirms the finding of Rivera-Garcia et al. (2019) on neutral ECAS at 46 mg/L spray. However, ECAS at acidic pH (70-80 mg/L FAC) dip washing was reported to erode the cuticle layer (Bialka et al., 2004) attributable to the pH of the disinfectant. The total inactivation of bacterial load on unwashed visibly clean eggs and higher log reduction of *S.* Enteritidis, and its high concentration of available chlorine (HOCl) not corroding the cuticle layer when used as aerosol fog. This innovative application of ECAS as aerosol fog might augment its usage in shelled egg washing, reducing the use of chemical biocidal agents such as NaOCl and QACs.

7.2.3 Neutral ECAS significantly reduces total bacteria and surrogate pathogens, without affecting the quality parameters of RTE spinach leaves

A review of fresh leafy vegetables causing foodborne outbreaks identified lettuce, spinach, cilantro, watercress as the main implicated vegetables (Machado-Moreira et al. 2019). Pathogen and spoilage microorganisms in fresh vegetables are introduced through exposure to contamination sources during farming, processing, distribution and in kitchens. Spinach leaves are known to harbour high loads of aerobic bacteria as well as the presence of pathogens such as *E. coli, Salmonella* spp., *Listeria* spp. and *L. monocytogenes* (Ilic et al. 2008; Mritunjay & Kumar, 2017; Tango, Choi, Chung, & Oh, 2014; Valentin-Bon et al., 2008). *E. coli* O157: H7 in spinach leaves also caused a major foodborne illness outbreak in the US (Grant et al., 2008). Moreover, the physiology of spinach leaf favour bacterial adhesion (Zhang et al. 2013). Therefore, WHO (2008) listed spinach as one of the RTE vegetables that are a concern for foodborne hazards.

The effectiveness of neutral ECAS in sanitizing minimally processed spinach leaves of total bacteria, yeast and mould, and artificially contaminated surrogate pathogens (*E. coli, L. innocua* and *S.* Enteritidis) are presented in chapter 5. A pH-neutral ECAS anolyte at 50 and 85 mg/L of FAC were compared to a commonly used PAA-based disinfectant and tap water wash at day 0 (immediately after washing), at day 5 and day 10 after storing in 4 ºC. Shortly after treatment (on day 0), total bacterial reduction of all the wash types were not different from one another but significantly lower ($P < 0.01$) than that of the untreated spinach leaves. At day 5 after storage, ECAS at 85 mg/L FAC wash showed significantly ($P < 0.01$) higher sanitization effectiveness when compared with untreated, tap water and PAA washes (50 mg/L). ECAS (50 mg/L) had a significant reduction of total bacterial load in comparison to untreated ($P < 0.01$), whereas non-signification reduction was observed in tap water and PAA treatment. The differential effectiveness between ECAS at 50 and 85 mg/L at day 5, was probably because ECAS 85 mg/L had a longer residual antibacterial effect after treatment. On day 10, both the ECAS and PAA washes had a similar reduction, which was a significantly ($P < 0.01$) higher bacteria load reduction compared to tap water wash. In the case of total coliform reductions, both the ECAS and PAA disinfectant showed similar disinfection efficacy when compared with tap water washing on day 5 and day 10.

The effectiveness of disinfection against purposely inoculated bacterial isolates in these experiments varied with the bacterial species and disinfectant type. The reduction of *E. coli* on day 0 for ECAS at 85 mg/L FAC (P < 0.01) and PAA (P < 0.001) were significantly higher that the tap water. However, the reduction by ECAS 50 mg/L FAC was not significantly different from the tap water control. The *E. coli* counts on day 5, for both ECAS concentrations was similar to that of water wash, while PAA had significantly higher *E. coli* count. Interestingly, at day 10, ECAS at both concentrations causes a significant *E. coli* reduction, whereas PAA showed the lowest reduction. The rise in *E. coli* counts on day 5 and day 10 after PAA wash might be because of the induction of viable-but-nonculturable (VBNC) state. This induction of VBNC agrees with a recent paper (Teixeira et al. 2020) reporting PAA up to 50 mg/L PAA concentration induced VBNC in *E. coli*.

For *L. innocua,* on day 0 ECAS at 85 mg/L FAC caused a significant (P <0.001) reduction compared to tap water. However, PAA treatment (P <0.001) had the highest reduction on day 0 and was significantly higher (P <0.05) than both the ECAS treatments. On day 5 all treatment wash types did not show any significant difference in the *L. innocua* load reduction. However, on day 10 both the ECAS concentrations caused higher levels (P <0.05) of reduction, but PAA had the highest counts among all the treatment types, again indicating VBNC induction as reported in a few studies (Gu et al., 2020; Winkelströter & De Martinis, 2015).

All three disinfectant wash types were effective in reducing *S.* Enteritidis, and the reduction for all three sampling days was higher for PAA (Figure 6, Chapter 5). On day 0, the sanitization effectiveness of ECAS 50 mg/L was not different from tap water while both PAA and ECAS 85 mg/L caused a significant reduction (P <0.01). On day 5 and day 10, a similar trend of reduction (P <0.01) was observed for PAA and ECAS 85 mg/L, but ECAS 50 mg/L caused a significant reduction compared to tap water (P <0.05).

We also compared the quality parameters such as sensory attributes and vitamin C content among tap water, ECAS and PAA washed leaves treatment groups. The organoleptic assessment showed 85 mg/L FAC ECAS preserves sensory characteristics better than that of the PAA washing, confirming the observation of longer shelf life with ECAS treatment reported by Premier et al. (2013) and Bachelli et al. (2013). Moreover, both the ECAS washes retained vitamin C contents of the

leaves better than the PAA and tap water washes (Figure 3, Chapter 5). Overall, as ECAS at 85 mg/L significantly reduced total bacteria and pathogen loads in RTE spinach leaves, and as it performs better in conserving sensory parameters it could be used for sanitization in horticulture production industries.

7.2.4 ECAS washing do not affect bacterial community composition and structure

Some members of the bacterial microbiota of plants adversely affect pathogen survival (Cooley, Miller, & Mandrell, 2003) by competing for limited available nutrients and by producing growth inhibitors (Babic et al., 1997; Schuenzel & Harrison, 2002), whereas other members facilitate pathogenic organism growth through metabolism of different sources of carbon (Lopez-Velasco, 2010). Therefore, information on the effect of the disinfectants and storage at 4 ℃ on spinach bacterial microbiome community structure would facilitate the selection of disinfectant.

Among the four predominant phyla, phylum Proteobacteria showed the highest total abundance on day 0, followed by phyla Bacteroidetes, Firmicutes and Actinobacteria as observed in other studies (Gu et al., 2018; Leff and Fierer, 2013; Lopez-Velasco et al., 2011; Tatsika et al., 2019). The bacterial microbiome in terms of order, families and genera of RTE spinach leaves found here is similar to that found by other studies on spinach leaves (Leff and Fierer, 2013; Lopez-Velasco et al., 2011; Soderqvist et al., 2017; Tatsika et al., 2019). Bacteria orders observed are Bacillales, Betaproteobacteriales, Enterobacteriales, Flavobacteriales, Micrococcales and Pseudomonadales. Most abundant families identified were *Micrococcaceae* (28.2%), *Clostridiales Family XII* (19.7%), *Flavobacteriaceae* (17.9%), *Pseudomonadaceae* (12.8%), *Burkholderiaceae* (10.1%), *Moraxellaceae* (4.3%), *Shewanellaceae* (1.3%), *Enterobacteriaceae* (1.1%), *Weeksellaceae* (1.0%), *Microbacteriaceae* (0.9%), 0 *Paenibacillaceae* (0.8%) and *Sphingobacteriaceae* (0.8%).

The species richness (Shannon diversity and Inverse Simpson indexes) and species evenness (Chao1 and abundance-based coverage estimator, ACE) measures of bacterial community structure for the five treatment types and the three sampling days were not significantly different ($p > 0.05$). On non-metric multidimensional scaling (NMDS) cluster analysis, treatment groups did not cluster into distinct clusters or

groups (Fig. 2a, Chapter 6). In contrast, bacterial communities on day 5 and day 10 clustered distinctly (Fig. 2b, Chapter 6). PERMANOVA analysis confirmed significantly different community composition at days 5 and 10 from day 0 for Bray-Curtis (*P*=0.02) and weighted Unifrac distances (*P*=0.016), respectively. Beta diversity group dispersion based on Bray-Curtis dissimilarity index showed homogenous microbiota (Fig. 2d, Chapter 6) on day 5 and day 10 with a significant reduction in heterogeneity. The heterogeneity reduction can be attributed to the decrease in the relative abundances of phylum Proteobacteria on day 5 and day 10, in agreement with the reduction observed by Gu et al. (2018) in chlorine-washed RTE spinach leaves stored at 4 °C for a week.

ANCOM analysis identified differences in RA of SVs and families that contributed to the differentiation of day 5 and day 10 bacterial microbiota. SVs identified as families *Pseudomonadaceae* and *Moraxellaceae*, and the order *Bacillales* (unclassified family) had a high RA on day 5 post storage. At SVs level, a significant increase of 4 ASVs (classified as *Pseudomonadaceae, Flavobacteriaceae, Micrococcaceae* and *Oxalobacteraceae*) on day 10 was recorded, which could be explained by their ability to grow at freezing temperatures (Schwartz et al., 2014). Moreover, *Pseudomonas* spp. are known to dominate bacterial populations in RTE spinach at refrigeration temperatures (Gu et al., 2018; Lopez-Velasco, Davis, Boyer, Williams, & Ponder, 2010; Rudi, Flateland, Hanssen, Bengtsson, & Nissen, 2002; Soderqvist et al., 2017), and cause spoilage of leafy vegetables (Tatsika et al. 2019). *E. coli* counts on RTE salad was negatively correlated to Pseudomonadales RA (Soderqvist et al., 2017), whereas increases in the RA of the order Bacillales have been positively correlated with increased viable counts of bacteria that have food safety concerns (*Y. enterocolitica* and *L. monocytogenes*) (Soderqvist et al., 2017).

Overall, ECAS did not affect the bacterial community structure and did not shift bacterial microbiota composition at 4º C storage to spoilage and commensals species that promote spoilage and pathogens. This findings in addition to efficient biocidal action in inactivating pathogens and not adversely affecting the sensory qualities (shelf life) of spinach leaves, would enhance the use of neutral ECAS as an alternative RTE vegetable disinfectant.

7.3 IMPLICATIONS AND FUTURE DIRECTIONS

Disinfection protocols that have a minimal residual environmental effect that complies with occupation health and safety (OHS) guidelines and does not co-select for antimicrobial resistance are ideal for livestock farming, and production of agriculture commodities such as vegetables and fruits. ECAS, being a biologically safe disinfectant, has strong potential application in these industries.

A safe fogging sanitization process in the reduction of airborne bacteria developed in Chapter 2, will have practical applications in the commercial piggeries and reduce the use of toxic and hazardous chemical biocidal agents and prophylactic antibiotics that co-select for bacterial resistance. This decontamination technique could also be suitable for disinfection of animal (quarantine) sheds as part of the biosafety and biosecurity management strategies without issues related to occupation safety hazards. Moreover, use of ECAS could also enhance the health and welfare of the animals through the reduction of hazardous bioaerosol components in high-density animal farms.

The imminent future work should be focused on neutral ECAS fogging in APP endemic piggeries. Besides evaluating its disinfection effectiveness, its economic impact in terms of reduction of preventative vaccination, antibiotic prophylaxis and reduction in pleurisy levels should be assessed. We recommend APP disinfection studies because of the following reasons: i. PMA live/dead qPCR for *A. pleuropneumoniae* is available (Chapter 2), ii. fogging of QAC in weaner pigs (in APP endemic farm) delayed outbreaks of pleuropneumonia, resulting in one less water antibiotic prophylaxis and observed lower pleurisy scores (McKenzie, 2014), iii. APP has substantial economic costs due to use of serovar (18 serovars) specific vaccines, water medication and carcase condemnation (Hunneman, 1986). The additional work in evaluating the reduction of bioaerosol components, because of ECAS fogging, that cause occupational hazard and affect animal welfare, in intensive piggeries may have an impact on large-scale application of this environmentally friendly disinfectant.

In Australia, egg production from free-range farms has increased by 5 %, and caged farms decreased by 7 % as at June 2019, because of the shift in consumer sentiment regarding animal welfare and better capital returns (AEL, 2019). But free-range eggs have higher bacterial load and pathogen counts than conventional caged

eggs (De Reu et al., 2005; Parisi et al., 2015). The problem is further compounded by increased in the consumption of raw egg products (Kretser et al. 2014). Cleaning and sanitization of shelled eggs would remain an essential strategy for the foreseeable future in maintaining consumer confidence in egg safety. The fog and spray disinfection effectiveness of neutral ECAS at concentrations of 150 mg/L FAC, which was within the allowable limit of FAC for food surface sanitization (Veasey & Muriana, 2016) will promote chemical-free and ethically acceptable free-range poultry farming.

As this disinfectant reduced total bacteria on visibly clean unwashed shell eggs and *S.* Enteritidis seeded eggs below detection level by rinse and plating technique, and Ogunniyi et al. (2019) showed higher concentration of FAC resulted in effective bactericidal activity in the presence of organic matter. A further investigation of inactivating bacteria on unwashed organic matter laden eggs would shed light on the disinfection potential without use of pre-washing step before disinfection. The pH-neutral ECAS with a concentration of 150 mg/L FAC applied by immersion, spray and fog for various times on high organic material laden eggs without prior cleaning needs to be further studied. The other specific concerns that required investigation for commercial implementations are to determine effectiveness on multiple bacterial pathogen seeded shell eggs, affect on quality parameters and economic returns.

The use of disinfectant that reduces microorganisms that preserve RTE vegetable quality and public health safety is an essential step in the production chain. The high effectiveness of neutral ECAS in significantly reducing bacterial load and surrogate pathogens on minimally processed spinach leaves without affecting the shelf life, nutritional values and bacteria community structure make it a valuable eco-friendly alternative disinfectant. The evidence generated of the effectiveness of neutral ECAS sanitization of minimally processed spinach leaves encourages trials in washing other RTE vegetables and fruits, and assessing effect on sensory, nutritional and microbiota composition at higher concentrations of FAC. Moreover, to corroborate these experimental data to industrial application, a large scale sanitization trial is desirable.

7.4 CONCLUSION

Cleaning and disinfection methods across the horticulture and livestock sectors use disinfectants to ensure animal wellbeing and food safety through avoiding the

proliferation of bacteria, viruses and fungi. Besides disinfection effectiveness, other factors such as regulatory requirements, cost, environmental safety and easy availability influence the choice of biocidal agents in these sectors. Neutral ECAS is considered a cost-effective, environmentally friendly and non-hazardous biocidal agent (Al-Haq et al. 2005). This thesis has primarily contributed data on disinfection effectiveness of ECAC in various usable forms, against general bacteria and surrogate pathogens, relevant to each subsector of the primary production industries. This study also has contributed significant findings of ECASs effect on shelf-life, quality and nutritional parameters, and bacterial community composition. Thus, the information obtained in this thesis of disinfection effectiveness of neutral ECAS and its usage not adversely affecting the quality attributes, make it an alternative eco-friendly disinfectant. The widespread use of ECAS in agriculture and food production would ensure food safety and animal well being through sustainable and safe livestock farming and horticulture production. Moreover, it also would reduce the excessive use of chemical disinfectants and prophylactic antibiotics and minimize the agri-chemical hazard.

Bibliography

Abadias, M., Usall, J., Anguera, M., Solsona, C., & Viñas, I. (2008). Microbiological quality of fresh, minimally-processed fruit and vegetables, and sprouts from retail establishments. *International Journal of Food Microbiology, 123*(1-2), 121-129.

Abadias, M., Usall, J., Oliveira, M., Alegre, I., & Viñas, I. (2008). Efficacy of neutral electrolyzed water (NEW) for reducing microbial contamination on minimally-processed vegetables. *International Journal of Food Microbiology, 123*(1), 151-158.

Achiwa, N., & Nishio, T. (2003). The use of electrolyzed water for sanitation control of eggshells and GP center. *Food Science and Technology Research, 9*(1), 100-103.

Adams, M., & Moss, M. (2005). Food Microbiology. In. Cambridge: Published by the Royal Society of Chemistry.

Australian Eggs Limited (AEL). Annual Report 2018-2019. Australian Eggs Limited 2019. Retrieved from https://www.australianeggs.org.au/who-we-are/annual-reports/#item-881

Afari, G. K., Liu, H., & Hung, Y.-C. (2019). The effect of produce washing using electrolyzed water on the induction of the viable but non-culturable (VBNC) state in Listeria monocytogenes and Escherichia coli O157: H7. *LWT, 110*, 275-282.

Al-Ajeeli MN. *Development of Best Practices for Shell Egg Disinfection Based upon Efficacy and Egg Quality* (Masters Thesis, Texas A&M University).

Al-Haq, M. I., Seo, Y., Oshita, S., & Kawagoe, Y. (2005). Applications of electrolyzed water in agriculture & food industries. *Food Science and Technology Research, 11*(2), 135-150.

Alawneh, J. I., Parke, C. R., Lapuz, E. J., David, J. E., Basinang, V. G., Baluyut, A. S., . . . Meers, J. (2018). Prevalence and risk Factors associated with gross Pulmonary lesions in slaughtered Pigs in smallholder and commercial Farms in Two Provinces in the Philippines. *Frontiers in Veterinary Science, 5*, 7.

Alegbeleye, O. O., Singleton, I., & Sant'Ana, A. S. (2018). Sources and contamination routes of microbial pathogens to fresh produce during field cultivation: a review. *Food Microbiology, 73*, 177-208.

Allende, A., Aguayo, E., & Artés, F. (2004). Microbial and sensory quality of commercial fresh processed red lettuce throughout the production chain and shelf life. *International Journal of Food Microbiology, 91*(2), 109-117.

Allende, A., Selma, M. V., López-Gálvez, F., Villaescusa, R., & Gil, M. I. (2008). Role of commercial sanitizers and washing systems on epiphytic microorganisms and sensory quality of fresh-cut escarole and lettuce. *Postharvest Biology and Technology, 49*(1), 155-163.

Amass, S. F., Vyverberg, B. D., Ragland, D., Dowell, C. A., Anderson, C. D., Stover, J. H., & Beaudry, D. J. (2000). Evaluating the efficacy of boot baths in biosecurity protocols. *Journal of Swine Health and Production, 8*(4), 169-173.

Amy, G., Bull, R., Craun, G. F., Pegram, R., Siddiqui, M., & Organization, W. H. (2000). Disinfectants and disinfectant by-products.

Ayebah, B., & Hung, Y.-C. (2005). Electrolyzed water and its corrosiveness on various surface materials commonly found in food processing facilities. *Journal of Food Process Engineering, 28*(3), 247-264. doi:10.1111/j.1745-4530.2005.00424.x

Babic, I., Roy, S., Watada, A., & Wergin, W. (1996). Changes in microbial populations on fresh cut spinach. *International Journal of Food Microbiology, 31*(1-3), 107-119.

Babic, I., Watada, A., & Buta, J. (1997). Growth of Listeria monocytogenes restricted by native microorganisms and other properties of fresh-cut spinach. *Journal of Food Protection, 60*(8), 912-917.

Bach, S., Jones, S., Stanford, K., Ralston, B., Milligan, D., Wallins, G., . . . McAllister, T. (2006). Electrolyzed oxidizing anode water as a sanitizer for use in abattoirs. *Journal of Food Protection, 69*(7), 1616-1622.

Bachelli, M. L. B., Amaral, R. D. Á., & Benedetti, B. C. (2013). Alternative sanitization methods for minimally processed lettuce in comparison to sodium hypochlorite. *Brazilian Journal of Microbiology, 44*(3), 673-678.

Baker, J., & Balch, D. (1962). A study of the organic material of hen's-egg shell. *Biochemical Journal, 82*(2), 352.

Ball, R., Logan, V., & Hill, J. (1975). Factors affecting the cuticle of the egg as measured by intensity of staining. *Poultry science, 54*(5), 1479-1484.

Barrette Jr, W. C., Hannum, D. M., Wheeler, W. D., & Hurst, J. K. (1989). General mechanism for the bacterial toxicity of hypochlorous acid: abolition of ATP production. *Biochemistry, 28*(23), 9172-9178.

Beattie, G. A., & Lindow, S. E. (1999). Bacterial colonization of leaves: a spectrum of strategies. *Phytopathology, 89*(5), 353-359.

Becking, J. (1975). The ultrastructure of the avian eggshell. *Ibis, 117*(2), 143-151.

Beier, R. C., Bischoff, K. M., & Poole, T. L. (2004). Disinfectants (biocides) used in animal production: antimicrobial resistance considerations. *Preharvest and postharvest food safety: contemporary issues and future directions. Blackwell Publishing Professional, Ames*, 227-238.

Bernstein, N., Sela, S., & Neder-Lavon, S. (2007). Assessment of contamination potential of lettuce by Salmonella enterica serovar Newport added to the plant growing medium. *Journal of Food Protection, 70*(7), 1717-1722.

Bialka, K. L., Demirci, A., Knabel, S., Patterson, P., & Puri, V. (2004a). Efficacy of electrolyzed oxidizing water for the microbial safety and quality of eggs. *Poultry Science, 83*(12), 2071-2078.

Bilić, V., Habrun, B., Barač, I., & Humski, A. (2000). Distribution of airborne bacteria in swine housing facilities and their immediate environment. *Arhiv za higijenu rada i toksikologiju, 51*(2), 199-205.

Bing, S., Zang, Y. T., Li, Y. J., & Shu, D. Q. (2019). The synergistic effects of slightly acidic electrolyzed water and UV-C light on the inactivation of Salmonella enteritidis on contaminated eggshells. *Poultry science, 98*(12), 6914-6920. doi:10.3382/ps/pez454

Blackall, P. J., Klaasen, H. L. B. M., Van Den Bosch, H., Kuhnert, P., & Frey, J. (2002). Proposal of a new serovar of Actinobacillus pleuropneumoniae: serovar 15. *Veterinary Microbiology, 84*(1-2), 47-52. doi:10.1016/s0378-1135(01)00428-x

Blackwell, M., Kang, H., Thomas, A., & Infante, P. (1981). Formaldehyde: evidence of carcinogenicity. *American Industrial Hygiene Association journal, 42*(7), A34-A46.

Board, R. (1982). Properties of avian egg shells and their adaptive value. *Biological Reviews, 57*(1), 1-28.

Board, R., & Halls, N. (1973). The cuticle: a barrier to liquid and particle penetration of the shell of the hen's egg. *British Poultry Science, 14*(1), 69-97.

Board, R., Loseby, S., & Miles, V. (1979). A note on microbial growth on hen egg-shells.

Board, R., & Love, G. (1980). Magnesium distribution in avian eggshells. *Comparative Biochemistry and Physiology Part A: Physiology, 66*(4), 667-672.

Board, R., & Scott, V. (1980). Porosity of the avian eggshell. *American Zoologist, 20*(2), 339-349.

Bochev, I. (2007). Porcine respiratory disease complex (PRDC): A review. I. Etiology, epidemiology, clinical forms and pathoanatomical features. *Bulgarian Journal of Veterinary Medicine, 10*(3), 131-146.

Bohnstedt R, inventor; Ecas Srl, assignee. Membrane electrolytic reactors system with four chambers. United States patent application US 12/159,392. 2010 Mar 18.

Bonifait, L., Veillette, M., Létourneau, V., Grenier, D., & Duchaine, C. (2014). Detection of Streptococcus suis in bioaerosols of swine confinement buildings. *Applied Environmental Microbiology, 80*(11), 3296-3304.

Bore, E., & Langsrud, S. (2005). Characterization of micro-organisms isolated from dairy industry after cleaning and fogging disinfection with alkyl amine and peracetic acid. *Journal of Applied Microbiology, 98*(1), 96-105. doi:10.1111/j.1365-2672.2004.02436.x

Borkenhagen, L. K., Mallinson, K. A., Tsao, R. W., Ha, S.-J., Lim, W.-H., Toh, T.-H., . . . Chong, K.-S. (2018). Surveillance for respiratory and diarrheal pathogens at the human-pig interface in Sarawak, Malaysia. *PloS One, 13*(7).

Bossé, J. T., Li, Y., Sárközi, R., Fodor, L., Lacouture, S., Gottschalk, M., . . . Holden, M. T. (2018). Proposal of serovars 17 and 18 of Actinobacillus pleuropneumoniae based on serological and genotypic analysis. *Veterinary Microbiology, 217*, 1-6.

Brockmeier, S. L., Halbur, P. G., & Thacker, E. L. (2002). Porcine respiratory disease complex. In *Polymicrobial Diseases* (pp. 231-258): American Society of Microbiology.

Bruce, J., & Drysdale, E. (1994). Trans-shell transmission. In *Microbiology of the avian egg* (pp. 63-91): Springer.

Bügener, E., Kump, A. W.-S., Casteel, M., & Klein, G. (2014). Benefits of neutral electrolyzed oxidizing water as a drinking water additive for broiler chickens. *Poultry science, 93*(9), 2320-2326.

Cain, C. J., & Heyn, A. (1964). X-ray diffraction studies of the crystalline structure of the avian egg shell. *Biophysical Journal, 4*(1), 23-39.

Calughton, D. (2019, 2 September, 2019). Spinach production grows 20pc in past year with rise in popularity of fresh leafy greens. *Australian Broadcasting Corporation.* Retrieved from https://www.abc.net.au/news/rural/2019-09-02/spinach-growers-increase-production-and-eye-asian-markets/11463974

Cao, W., Zhu, Z. W., Shi, Z. X., Wang, C. Y., & Li, B. M. (2009). Efficiency of slightly acidic electrolyzed water for inactivation of Salmonella enteritidis and its

contaminated shell eggs. *International Journal of Food Microbiology, 130*(2), 88-93.

Centre for Disease Control and Prevention (CDC). (2016). *Center for Disease Control and Prevention*. Retrieved from https://www.cdc.gov/foodborneburden/2011foodborne-estimates.html.

Chang, C., Chung, H., Huang, C.-F., & Su, H.-J. J. (2001). Exposure of workers to airborne microorganisms in open-air swine houses. *Applied Environmental Microbiolopgy, 67*(1), 155-161.

Charatan, F. (2006). FDA warns US consumers not to eat spinach after E coli outbreak. *BMJ: British Medical Journal, 333*(7570), 673.

Cheng, K.-C., Dev, S. R., Bialka, K. L., & Demirci, A. (2012). Electrolyzed oxidizing water for microbial decontamination of food. In *Microbial Decontamination in the Food Industry* (pp. 563-591): Elsevier.

Cloete, T. E., Thantsha, M., Maluleke, M., & Kirkpatrick, R. (2009). The antimicrobial mechanism of electrochemically activated water against Pseudomonas aeruginosa and Escherichia coli as determined by SDS-PAGE analysis. *Journal of Applied Microbiology, 107*(2), 379-384.

Condell, O., Iversen, C., Cooney, S., Power, K. A., Walsh, C., Burgess, C., & Fanning, S. (2012). Efficacy of biocides used in the modern food industry to control Salmonella enterica, and links between biocide tolerance and resistance to clinically relevant antimicrobial compounds. *Applied Environmental Microbiology, 78*(9), 3087-3097.

Cooke, A., & Balch, D. (1970). Studies of membrane, mammilary cores and cuticle of the hen egg shell. *British Poultry Science, 11*(3), 345-352.

Cooley, M. B., Miller, W. G., & Mandrell, R. E. (2003). Colonization of Arabidopsis thaliana with Salmonella enterica and enterohemorrhagic Escherichia coli O157: H7 and competition by Enterobacter asburiae. *Applied Environmental Microbiology, 69*(8), 4915-4926.

Costa, A., Colosio, C., Gusmara, C., Guarino, M., & Sala, V. (2014). Effects of disinfectant fogging procedure on dust, ammonia concentration, aerobic bacteria

and fungal spores in a farrowing-weaning room. *Annals of Agricultural and Environmental Medicine, 21*(3), 494-499. doi:10.5604/12321966.1120589

Cruz, A. T., Cazacu, A. C., & Allen, C. H. (2007). Pantoea agglomerans, a plant pathogen causing human disease. *Journal of Clinical Microbiology, 45*(6), 1989-1992.

Cui, X., Shang, Y., Shi, Z., Xin, H., & Cao, W. (2009). Physicochemical properties and bactericidal efficiency of neutral and acidic electrolyzed water under different storage conditions. *Journal of Food Engineering, 91*(4), 582-586.

D'Alba, L., Jones, D. N., Badawy, H. T., Eliason, C. M., & Shawkey, M. D. (2014). Antimicrobial properties of a nanostructured eggshell from a compost-nesting bird. *Journal of Experimental Biology, 217*(7), 1116-1121.

Daniel, C., Poiret, S., Goudercourt, D., Dennin, V., Leyer, G., & Pot, B. (2006). Selecting lactic acid bacteria for their safety and functionality by use of a mouse colitis model. *Applied Environmental Microbiology, 72*(9), 5799-5805.

Darlison, J., Mogren, L., Rosberg, A. K., Grudén, M., Minet, A., Liné, C., . . . Uhlig, E. (2019). Leaf mineral content govern microbial community structure in the phyllosphere of spinach (Spinacia oleracea) and rocket (Diplotaxis tenuifolia). *Science of The Total Environment, 675*, 501-512.

De Reu, K., Grijspeerdt, K., Messens, W., Heyndrickx, M., Uyttendaele, M., Debevere, J., & Herman, L. (2006). Eggshell factors influencing eggshell penetration and whole egg contamination by different bacteria, including Salmonella enteritidis. *International Journal of Food Microbiology, 112*(3), 253-260.

Delaquis, P., Stewart, S., Toivonen, P., & Moyls, A. (1999). Effect of warm, chlorinated water on the microbial flora of shredded iceberg lettuce. *Food Research International, 32*(1), 7-14.

Delétoile, A., Decré, D., Courant, S., Passet, V., Audo, J., Grimont, P., . . . Brisse, S. (2009). Phylogeny and identification of Pantoea species and typing of Pantoea agglomerans strains by multilocus gene sequencing. *Journal of Clinical Microbiology, 47*(2), 300-310.

Dennis, J. E., Xiao, S. Q., Agarwal, M., Fink, D. J., Heuer, A. H., & Caplan, A. I. (1996). Microstructure of matrix and mineral components of eggshells from white leghorn chickens (Gallus gallus). *Journal of Morphology, 228*(3), 287-306.

Deza, M., Araujo, M., & Garrido, M. (2005). Inactivation of Escherichia coli, Listeria monocytogenes, Pseudomonas aeruginosa and Staphylococcus aureus on stainless steel and glass surfaces by neutral electrolysed water. *Letters in Applied Microbiology, 40*(5), 341-346.

Donaghy, J. A., Jagadeesan, B., Goodburn, K., Grunwald, L., Jensen, O. N., Jespers, A., . . . Quentin, M.-C. (2019). Relationship of Sanitizers, Disinfectants, and Cleaning Agents with Antimicrobial Resistance. *Journal of Food Protection, 82*(5), 889-902.

Dreyfus, A., Schaller, A., Nivollet, S., Segers, R. P. A. M., Kobisch, M., Mieli, L., . . . Frey, J. (2004). Use of recombinant ApxIV in serodiagnosis of Actinobacillus pleuropneumoniae infections, development and prevalidation of the ApxIV ELISA. *Veterinary Microbiology, 99*(3-4), 227-238. doi:10.1016/j.vetmic.2004.01.004

Du, J. (2013). *Chicken eggshell membrane and cuticle: insight from bioinformatics and proteomics* (Masters Thesis, University of Ottawa, Canada).

Dunn, I. C., Woolliams, J. A., Wilson, P. W., Icken, W., Cavero, D., Jones, A. C., . . . Bain, M. M. (2019). Genetic variation and potential for genetic improvement of cuticle deposition on chicken eggs. *Genetics Selection Evolution, 51*(1), 25.

ECDC, E. (2007). European food safety authority, European centre for disease prevention and control: the European union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2006. *EFSA Journal, 5*(12), 130r. doi:https://doi.org/10.2903/j.efsa.2012.2597

ECDC, E. (2012). European food safety authority, European centre for disease prevention and control: the European union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2010. *EFSA Journal, 10*(3), 2579. doi:https://doi.org/10.2903/j.efsa.2012.2597

ECDC, E. (2017). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. *EFSA Journal, 15*(12), e05077.

Ercolani, G. (1991). Distribution of epiphytic bacteria on olive leaves and the influence of leaf age and sampling time. *Microbial Ecology, 21*(1), 35-48.

European Commission Health and Consumer Protection, D.G. (2002). *Risk profile on the microbiological contamination of fruits and vegetables eaten raw*. Retrieved from Brussels: http://europa.eu.int/comm/food/fs/sc/scf/out125_en.pdf

Fabrizio, K., Sharma, R., Demirci, A., & Cutter, C. (2002). Comparison of electrolyzed oxidizing water with various antimicrobial interventions to reduce Salmonella species on poultry. *Poultry Science, 81*(10), 1598-1605.

Fasenko, G. M., O'Dea Christopher, E. E., & McMullen, L. M. (2009). Spraying hatching eggs with electrolyzed oxidizing water reduces eggshell microbial load without compromising broiler production parameters. *Poultry Science, 88*(5), 1121-1127. doi:10.3382/ps.2008-00359

Feliciano, L., Lee, J., & Pascall, M. A. (2012). Transmission electron microscopic analysis showing structural changes to bacterial cells treated with electrolyzed water and an acidic sanitizer. *Journal of Food Science, 77*(4), M182-M187.

Fernandez Marquez, M. L., Burgos, M. J. G., Pulido, R. P., Gálvez, A., & López, R. L. (2017). Biocide tolerance and antibiotic resistance in Salmonella isolates from hen eggshells. *Foodborne Pathogens and Disease, 14*(2), 89-95.

Fodor, L., Varga, J., Molnar, E., & Hajtos, I. (1989). Biochemical and serological properties of Actinobacillus pleuropneumoniae biotype 2 strains isolated from swine. *Veterinary Microbiology, 20*(2), 173-180.

Folkes, L. K., Candeias, L. P., & Wardman, P. (1995). Kinetics and mechanisms of hypochlorous acid reactions. *Archives of Biochemistry and iophysics, 323*(1), 120-126.

Fukuzaki, S. (2006). Mechanisms of actions of sodium hypochlorite in cleaning and disinfection processes. *Biocontrol Science, 11*(4), 147-157.

Gardner, I. A., Willeberg, P., & Mousing, J. (2002). Empirical and theoretical evidence for herd size as a risk factor for swine diseases. *Animal Health Research Reviews, 3*(1), 43-55. doi:10.1079/ahrr200239

Gilbert, A. (1971). The egg: its physical and chemical aspects. *Physiology and Biochemistry of the Domestic Fowl, 3*, 1379-1399.

Gole, V. C., Chousalkar, K. K., Roberts, J. R., Sexton, M., May, D., Tan, J., & Kiermeier, A. (2014). Effect of egg washing and correlation between eggshell characteristics and egg penetration by various Salmonella Typhimurium strains. *PloS one, 9*(3), e90987. doi:10.1371/journal.pone.0090987

Gómez-López, V. M., Marín, A., Medina-Martínez, M. S., Gil, M. I., & Allende, A. (2013). Generation of trihalomethanes with chlorine-based sanitizers and impact on microbial, nutritional and sensory quality of baby spinach. *Postharvest Biology and Technology, 85*, 210-217.

Goodburn, C., & Wallace, C. A. (2013). The microbiological efficacy of decontamination methodologies for fresh produce: a review. *Food control, 32*(2), 418-427.

Gosling, R. J. (2018). A review of cleaning and disinfection studies in farming environments. *Livestock, 23*(5), 232-237.

Gosling, R. J., Breslin, M., Fenner, J., Vaughan, K., West, E., Mawhinney, I., . . . Davies, R. (2016). An in-vitro investigation into the efficacy of disinfectants used in the duck industry against Salmonella. *Avian Pathology, 45*(5), 576-581.

Gosling, R. J., Mawhinney, I., Vaughan, K., Davies, R. H., & Smith, R. P. (2017). Efficacy of disinfectants and detergents intended for a pig farm environment where Salmonella is present. *Veterinary Microbiology, 204*, 46-53.

Graça, A., Abadias, M., Salazar, M., & Nunes, C. (2011). The use of electrolyzed water as a disinfectant for minimally processed apples. *Postharvest Biology and Technology, 61*(2-3), 172-177.

Grant, J., Wendelboe, A. M., Wendel, A., Jepson, B., Torres, P., Smelser, C., & Rolfs, R. T. (2008). Spinach-associated Escherichia coli O157: H7 outbreak, Utah and New Mexico, 2006. *Emerging Infectious Diseases, 14*(10), 1633.

Gu, G., Ottesen, A., Bolten, S., Luo, Y., Rideout, S., & Nou, X. (2020). Microbiome convergence following sanitizer treatment and identification of sanitizer resistant species from spinach and lettuce rinse water. *International Journal of Food Microbiology, 318*, 108458.

Gu, G., Ottesen, A., Bolten, S., Ramachandran, P., Reed, E., Rideout, S., . . . Nou, X. (2018). Shifts in spinach microbial communities after chlorine washing and storage at compliant and abusive temperatures. *Food Microbiology, 73*, 73-84.

Gu, L., Bai, Z., Jin, B., Hu, Q., Wang, H., Zhuang, G., & Zhang, H. (2010). Assessing the impact of fungicide enostroburin application on bacterial community in wheat phyllosphere. *Journal of Environmental Sciences, 22*(1), 134-141.

Guentzel, J. L., Lam, K. L., Callan, M. A., Emmons, S. A., & Dunham, V. L. (2008). Reduction of bacteria on spinach, lettuce, and surfaces in food service areas using neutral electrolyzed oxidizing water. *Food Microbiology, 25*(1), 36-41.

Guérin-Méchin, L., Dubois-Brissonnet, F., Heyd, B., & Leveau, J. (1999). Specific variations of fatty acid composition of Pseudomonas aeruginosa ATCC 15442 induced by quaternary ammonium compounds and relation with resistance to bactericidal activity. *Journal of Applied Microbiology, 87*(5), 735-742.

Gustafsson, G. (1997). Investigations of factors affecting air pollutants in animal houses. *Annals of Agricultural and Environmental Medicine, 4*(2), 203-215.

Gutiérrez, A. A. (2006). The science behind stable, super-oxidized water. *Wounds, 18*(1), 7.

Han, D., Hung, Y.-C., Bratcher, C. L., Monu, E. A., Wang, Y., & Wang, L. (2018). Formation of sublethally injured Yersinia enterocolitica, Escherichia coli O157: H7, and Salmonella enterica serovar enteritidis cells after neutral electrolyzed oxidizing water treatments. *Applied Environmental Microbiology, 84*(17), e01066-01018.

Han, D., Hung, Y.-C., & Wang, L. (2018). Evaluation of the antimicrobial efficacy of neutral electrolyzed water on pork products and the formation of viable but nonculturable (VBNC) pathogens. *Food Microbiology, 73*, 227-236.

Han, Y., Linton, R., Nielsen, S., & Nelson, P. (2001). Reduction of Listeria monocytogenes on green peppers (Capsicum annuum L.) by gaseous and aqueous chlorine dioxide and water washing and its growth at 7 C. *Journal of Food Protection, 64*(11), 1730-1738.

Handojo, A., Lee, J., Hipp, J., & Pascall, M. A. (2009). Efficacy of electrolyzed water and an acidic formulation compared with regularly used chemical sanitizers for tableware sanitization during mechanical and manual ware-washing protocols. *Journal of Food Protection, 72*(6), 1315-1320.

Handschur, M., Pinar, G., Gallist, B., Lubitz, W., & Haslberger, A. (2005). Culture free DGGE and cloning based monitoring of changes in bacterial communities of salad due to processing. *Food and Chemical Toxicology, 43*(11), 1595-1605.

Hao, J., Li, H., Wan, Y., & Liu, H. (2015). Effect of Electrolyzed Oxidizing Water Treatment on the Reduction of Nitrite Levels in Fresh Spinach during Storage. *Journal of Food Protection, 78*(3), 549-553.

Hao, J., Liu, H., Liu, R., Dalai, W., Zhao, R., Chen, T., & Li, L. (2011). Efficacy of Slightly Acidic Electrolyzed Water (Saew) for Reducing Microbial Contamination on Fresh-Cut Cilantro. *Journal of Food Safety, 31*(1), 28-34.

Hao, J., Wu, T., Li, H., & Liu, H. (2017). Differences of bactericidal efficacy on Escherichia coli, Staphylococcus aureus, and Bacillus subtilis of slightly and strongly acidic electrolyzed water. *Food and Bioprocess Technology, 10*(1), 155-164.

Hao, X., Li, B., Wang, C., Zhang, Q., & Cao, W. (2013). Application of slightly acidic electrolyzed water for inactivating microbes in a layer breeding house. *Poultry Science, 92*(10), 2560-2566.

Hao, X., Li, B., Zhang, Q., Lin, B. Z., Ge, L., Wang, C., & Cao, W. (2013). Disinfection effectiveness of slightly acidic electrolysed water in swine barns. *Journal of Applied Microbiology, 115*(3), 703-710.

Harris, L., Farber, J., Beuchat, L., Parish, M., Suslow, T., Garrett, E., & Busta, F. (2003). Outbreaks associated with fresh produce: incidence, growth, and survival

of pathogens in fresh and fresh-cut produce. *Comprehensive Reviews in Food Science and Food Safety, 2*, 78-141.

Hendriksen, R. S., Vieira, A. R., Karlsmose, S., Lo Fo Wong, D. M., Jensen, A. B., Wegener, H. C., & Aarestrup, F. M. (2011). Global monitoring of Salmonella serovar distribution from the World Health Organization Global Foodborne Infections Network Country Data Bank: results of quality assured laboratories from 2001 to 2007. *Foodborne Pathogens and Disease, 8*(8), 887-900.

Hibbing, M. E., Fuqua, C., Parsek, M. R., & Peterson, S. B. (2010). Bacterial competition: surviving and thriving in the microbial jungle. *Nature Reviews Microbiology, 8*(1), 15.

Hincke, M., Gautron, J., Panheleux, M., Garcia-Ruiz, J., McKee, M., & Nys, Y. (2000). Identification and localization of lysozyme as a component of eggshell membranes and eggshell matrix. *Matrix Biology, 19*(5), 443-453.

Hricova, D., Stephan, R., & Zweifel, C. (2008). Electrolyzed water and its application in the food industry. *Journal of Food Protection, 71*(9), 1934-1947.

Hsu, S. (2003). Effects of water flow rate, salt concentration and water temperature on efficiency of an electrolyzed oxidizing water generator. *Journal of Food Engineering, 60*(4), 469-473.

Hsu, S. (2005). Effects of flow rate, temperature and salt concentration on chemical and physical properties of electrolyzed oxidizing water. *Journal of Food Engineering, 66*(2), 171-176.

Hsu, W., Simonne, A., Jitareerat, P., & Marshall Jr, M. (2010). Low-dose irradiation improves microbial quality and shelf life of fresh mint (Mentha piperita L.) without compromising visual quality. *Journal of Food Science, 75*(4), M222-M230.

Huang, Y.-R., Hung, Y.-C., Hsu, S.-Y., Huang, Y.-W., & Hwang, D.-F. (2008). Application of electrolyzed water in the food industry. *Food Control, 19*(4), 329-345.

Hugenholtz, P., Tyson, G. W., Webb, R. I., Wagner, A. M., & Blackall, L. L. (2001). Investigation of candidate division TM7, a recently recognized major lineage of

the domain Bacteria with no known pure-culture representatives. *Applied Environmental Microbiology, 67*(1), 411-419.

Hunneman, W. (1986). Incidence, economic effects, and control of Haemophilus pleuropneumoniae infections in pigs. *Veterinary Quarterly, 8*(1), 83-87.

Hurst, J. K., Barrett Jr, W. C., Michel, B. R., & Rosen, H. (1991). Hypochlorous acid and myeloperoxidase-catalyzed oxidation of iron-slfur clusters in bacterial respiratory dehydrogenases. *European Journal of Biochemistry, 202*(3), 1275-1282.

Ilic, S., Odomeru, J., & Lejeune, J. T. (2008). Coliforms and prevalence of Escherichia coli and foodborne pathogens on minimally processed spinach in two packing plants. *Journal of Food Protection, 71*(12), 2398-2403.

Izumi, H. (1999). Electrolyzed water as a disinfectant for fresh-cut vegetables. *Journal of Food Science, 64*(3), 536-539.

Izumi, H., Kiba, T., & Hashimoto, S. (1999). Efficacy of electrolyzed water as a disinfectant for fresh-cut spinach. *Australian Centre for International Agricultural Research (ACIAR) Postharvest Newsl*(50).

, C., Stone, B., & Tyler, H. (2015). Emerging Perspectives on the Natural Microbiome of Fresh Produce Vegetables. *Agriculture, 5*(2), 170-187. doi:10.3390/agriculture5020170

Jackson, C. R., Randolph, K. C., Osborn, S. L., & Tyler, H. L. (2013). Culture dependent and independent analysis of bacterial communities associated with commercial salad leaf vegetables. *BMC Microbiology, 13*(1), 274.

Jacobs, J., Carroll, T., & Sundin, G. (2005). The role of pigmentation, ultraviolet radiation tolerance, and leaf colonization strategies in the epiphytic survival of phyllosphere bacteria. *Microbial Ecology, 49*(1), 104-113.

Jeong, J., Kim, C., & Yoon, J. (2009). The effect of electrode material on the generation of oxidants and microbial inactivation in the electrochemical disinfection processes. *Water Research, 43*(4), 895-901.

Jeong, J., Kim, J. Y., Cho, M., Choi, W., & Yoon, J. (2007). Inactivation of Escherichia coli in the electrochemical disinfection process using a Pt anode. *Chemosphere, 67*(4), 652-659.

Johnston, M. A., Harrison, M. A., & Morrow, R. A. (2009). Microbial antagonists of Escherichia coli O157: H7 on fresh-cut lettuce and spinach. *Journal of Food Protection, 72*(7), 1569-1575.

Junqueira-Gonçalves, M. P., Galotto, M. J., Valenzuela, X., Dinten, C. M., Aguirre, P., & Miltz, J. (2011). Perception and view of consumers on food irradiation and the Radura symbol. *Radiation Physics and Chemistry, 80*(1), 119-122.

Kader, A. A. (2002). Quality parameters of fresh-cut fruit and vegetable products. In *Fresh-cut fruits and vegetables* (pp. 20-29): CRC Press.

Kalit, S., Kos, T., Kalit, M. T., & Kos, I. (2015). The efficacy of electrolysed oxidizing water as a disinfectant in the dairy industry. *Journal of Hygienic Engineering and Design, 12*, 28-32.

Kawai, K., Shinozuka, Y., Uchida, I., Hirose, K., Mitamura, T., Watanabe, A., . . . Onda, K. (2017). Control of Pseudomonas mastitis on a large dairy farm by using slightly acidic electrolyzed water. *Animal Science Journal, 88*(10), 1601-1605.

Khazandi, M., Deo, P., Ferro, S., Venter, H., Pi, H., Crabb, S., . . . Trott, D. J. (2017). Efficacy evaluation of a new water sanitizer for increasing the shelf life of Southern Australian King George Whiting and Tasmanian Atlantic Salmon fillets. *Food Microbiology, 68*, 51-60.

Kim, C., Hung, Y.-C., & Brackett, R. E. (2000a). Efficacy of electrolyzed oxidizing (EO) and chemically modified water on different types of foodborne pathogens. *International Journal of Food Microbiology, 61*(2-3), 199-207.

Kim, C., Hung, Y.-C., & Brackett, R. E. (2000b). Roles of Oxidation–Reduction Potential in Electrolyzed Oxidizing and Chemically Modified Water for the Inactivation of Food-Related Pathogens. *Journal of Food Protection, 63*(1), 19-24. doi:10.4315/0362-028x-63.1.19

Kim, K., & Ko, H. (2019). Indoor distribution characteristics of airborne bacteria in pig buildings as influenced by season and housing type. *Asian-Australasian Journal of Animal Sciences, 32*(5), 742.

Kirk, M., Ford, L., Glass, K., & Hall, G. (2014). Foodborne Illness, Australia, Circa 2000 and Circa 2010. *Emerging Infectious Diseases, 20*(11), 1857-1864. doi:10.3201/eid2011.131315

Kristiansen, A., Saunders, A. M., Hansen, A. A., Nielsen, P. H., & Nielsen, J. L. (2012). Community structure of bacteria and fungi in aerosols of a pig confinement building. *FEMS Microbiology Ecology, 80*(2), 390-401.

Kulichevskaya, I. S., Ivanova, A. O., Belova, S. E., Baulina, O. I., Bodelier, P. L., Rijpstra, W. I. C., . . . Dedysh, S. N. (2007). Schlesneria paludicola gen. nov., sp. nov., the first acidophilic member of the order Planctomycetales, from Sphagnum-dominated boreal wetlands. *International Journal of Systematic And Evolutionary Microbiology, 57*(11), 2680-2687.

Kusuda, S., Iwasawa, A., Doi, O., Ohya, Y., & Yoshizaki, N. (2011). Diversity of the cuticle layer of avian eggshells. *The Journal of Poultry Science*, 1102220100-1102220100.

Lang, M. M., Harris, L. J., & Beuchat, L. R. (2004). Survival and recovery of Escherichia coli O157: H7, Salmonella, and Listeria monocytogenes on lettuce and parsley as affected by method of inoculation, time between inoculation and analysis, and treatment with chlorinated water. *Journal of Food Protection, 67*(6), 1092-1103.

Lata, S., Mohanty, S. K., Pradhan, P. K., Patri, G., Sinha, S. P., & Agrawal, P. (2016). Anti bacterial Effectiveness of Electro-Chemically Activated (ECA) Water as a Root Canal Irrigant-An In-vitro Comparative Study. *Journal of Clinical and Diagnostic Research: JCDR, 10*(10), ZC138.

Leff, J. W., & Fierer, N. (2013). Bacterial communities associated with the surfaces of fresh fruits and vegetables. *PloS One, 8*(3), e59310.

Len, S.V., Hung, Y.C., Chung, D., Anderson, J. L., Erickson, M. C., & Morita, K. (2002). Effects of storage conditions and pH on chlorine loss in electrolyzed

oxidizing (EO) water. *Journal of Agricultural and Food Chemistry, 50*(1), 209-212.

Len, S.V., Hung, Y.C., Erickson, M., & Kim, C. (2000). Ultraviolet spectrophotometric characterization and bactericidal properties of electrolyzed oxidizing water as influenced by amperage and pH. *Journal of Food Protection, 63*(11), 1534-1537.

Liao, L. B., Chen, W. M., & Xiao, X. M. (2007). The generation and inactivation mechanism of oxidation–reduction potential of electrolyzed oxidizing water. *Journal of Food Engineering, 78*(4), 1326-1332.

Lin, C. S., Wu, C., Yeh, J. Y., & Saalia, F. K. (2005). The evaluation of electrolysed water as an agent for reducing micro-organisms on vegetables. *International Journal of Food Science & Technology, 40*(5), 495-500.

Lindow, S. E., & Brandl, M. T. (2003). Microbiology of the phyllosphere. *Applied Environmental Microbiology, 69*(4), 1875-1883.

López-Gálvez, F., Allende, A., Selma, M. V., & Gil, M. I. (2009). Prevention of Escherichia coli cross-contamination by different commercial sanitizers during washing of fresh-cut lettuce. *International Journal of Food Microbiology, 133*(1-2), 167-171.

Lopez-Velasco, G. (2010). *Molecular characterization of spinach (Spinacia oleracea) microbial community structure and its interaction with Escherichia coli O157: H7 in modified atmosphere conditions.* (Doctoral Dissertation, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, United States).

Lopez-Velasco, G., Carder, P. A., Welbaum, G. E., & Ponder, M. A. (2013). Diversity of the spinach (Spinacia oleracea) spermosphere and phyllosphere bacterial communities. *FEMS Microbiology Letters, 346*(2), 146-154.

Lopez-Velasco, G., Davis, M., Boyer, R. R., Williams, R. C., & Ponder, M. A. (2010). Alterations of the phylloepiphytic bacterial community associated with interactions of Escherichia coli O157: H7 during storage of packaged spinach at refrigeration temperatures. *Food Microbiology, 27*(4), 476-486.

Lopez-Velasco, G., Welbaum, G., Boyer, R., Mane, S., & Ponder, M. (2011). Changes in spinach phylloepiphytic bacteria communities following minimal processing and refrigerated storage described using pyrosequencing of 16S rRNA amplicons. *Journal of Applied Microbiology, 110*(5), 1203-1214.

Machado-Moreira, B., Richards, K., Brennan, F., Abram, F., & Burgess, C. M. (2019). Microbial Contamination of Fresh Produce: What, Where, and How? *Comprehensive Reviews in Food Science and Food Safety*.

Maillard, J. Y. (2002). Bacterial target sites for biocide action. *Journal of Applied Microbiology, 92*, 16S-27S.

Majd, A. M. S., Ashtari, A. K., Riskowski, G. L., Mukhtar, S., Zhao, L., & Fang, W. (2015). Electrolyzed water spray scrubber for removing ammonia from air. *Transactions of the ASABE, 58*(4), 1069-1078.

Marais, J. (2002). Biocompatibility of electrochemically activated aqueous solutions: an animal study. *SADJ: Journal of the South African Dental Association= tydskrif van die Suid-Afrikaanse Tandheelkundige Vereniging, 57*(1), 12-16.

Maris, P. (1995). Modes of action of disinfectants. *Revue Scientifique et Technique-Office International des Epizooties, 14*, 47-47.

Marritto, N., & Gravani, R. (2006). Principles of food sanitation. In: Springer Science Business Media. Inc.

Martin, D. J., Wesgate, R., Denyer, S. P., McDonnell, G., & Maillard, J. Y. (2015). Bacillus subtilis vegetative isolate surviving chlorine dioxide exposure: an elusive mechanism of resistance. *Journal of Applied Microbiology, 119*(6), 1541-1551.

Martínez-de Jesüs, F., Remes-troche, J., Armstrong, D. W. S., & Beneit-montesinos, J. (2007). Efficacy and safety of neutral ph superoxidised solution in severe diabetic foot infections. *International Wound Journal, 4*(4), 330-332.

Martínez-Hernández, G. B., Navarro-Rico, J., Gómez, P. A., Otón, M., Artés, F., & Artés-Hernández, F. (2015). Combined sustainable sanitising treatments to reduce Escherichia coli and Salmonella Enteritidis growth on fresh-cut kailan-hybrid broccoli. *Food control, 47*, 312-317.

McDonnell, G., & Russell, A. D. (1999). Antiseptics and disinfectants: activity, action, and resistance. *Clinical Microbiology Reviews, 12*(1), 147-179.

McKenzie, P. (2010). *Evaluation Of Grower Pig Aerosol And Sucker Pigs APP Vaccination Programmes*. Retrieved from http://apri.com.au/2C-103_Final_Research_Report_100819.pdf

McKenzie, P. (2014). *Investigating the use of aerosol disinfection during the weaner period on APP prevalence in fogged grower/finisher pigs*. Retrieved from http://porkcrc.com.au/wp-content/uploads/2017/05/2C-117-Final-Report.pdf

McLaren, I., Wales, A., Breslin, M., & Davies, R. (2011). Evaluation of commonly-used farm disinfectants in wet and dry models of Salmonella farm contamination. *Avian Pathology, 40*(1), 33-42.

McPherson, L. (1993). Understanding ORP's role in the disinfection process. *Water Engineering and Management, 140*, 29-31.

Meakin, N. S., Bowman, C., Lewis, M. R., & Dancer, S. J. (2012). Comparison of cleaning efficacy between in-use disinfectant and electrolysed water in an English residential care home. *Journal of Hospital Infection, 80*(2), 122-127. doi:10.1016/j.jhin.2011.10.015

Mereghetti, L., Quentin, R., Marquet-Van Der Mee, N., & Audurier, A. (2000). Low sensitivity of Listeria monocytogenes to quaternary ammonium compounds. *Applied Environmental Microbiology, 66*(11), 5083-5086.

Migliarina, F., & Ferro, S. (2014). *A Modern Approach to Disinfection, as Old as the Evolution of Vertebrates.* Paper presented at the Healthcare.

Mikšík, I., Charvátová, J., Eckhardt, A., & Deyl, Z. (2003). Insoluble eggshell matrix proteins–their peptide mapping and partial characterization by capillary electrophoresis and high-performance liquid chromatography. *Electrophoresis, 24*(5), 843-852.

Miyamoto, T., Horie, T., Baba, E., Sasai, K., Fukata, T., & Arakawa, A. (1998). Salmonella penetration through eggshell associated with freshness of laid eggs and refrigeration. *Journal of Food Protection, 61*(3), 350-353.

Morente, E. O., Fernández-Fuentes, M. A., Burgos, M. J. G., Abriouel, H., Pulido, R. P., & Gálvez, A. (2013). Biocide tolerance in bacteria. *International Journal of Food Microbiology, 162*(1), 13-25.

Morita, C., Nishida, T., & Ito, K. (2011). Biological toxicity of acid electrolyzed functional water: Effect of oral administration on mouse digestive tract and changes in body weight. *Archives of Oral Biology, 56*(4), 359-366.

Mritunjay, S. K., & Kumar, V. (2017). A study on prevalence of microbial contamination on the surface of raw salad vegetables. *3 Biotechnology, 7*(1), 13. doi:10.1007/s13205-016-0585-5

Nagahata, H., Yuga, K., Abe, Y., Toskar, A., Higuchi, H., Mitamura, T., & Matsuyama, K. (2011). *Effectiveness of slightly acidic electrolyzed water for improvement of hygienic conditions of teat liners of automatic milking system (AMS). Animal hygiene and sustainable livestock production.* Paper presented at the Proceedings of the XVth International Congress of the International Society for Animal Hygiene. Vienna. Austria.

Nan, S., Wang, C., Cao, W., Li, B., Ma, Q., & Wang, S. (2010). *Disinfection effect and application of slightly acidic electrolyzed water in a dairy cattle farm.* Paper presented at the 2010 Pittsburgh, Pennsylvania, June 20-June 23, 2010.

Nascimento, V., Cranstoun, S., & Solomon, S. (1992). Relationship between shell structure and movement of Salmonella enteritidis across the eggshell wall. *British Ppoultry Science, 33*(1), 37-48.

Ni, L., Cao, W., Zheng, W.C., Chen, H., & Li, B.M. (2014). Efficacy of slightly acidic electrolyzed water for reduction of foodborne pathogens and natural microflora on shell eggs. *Food Science and Technology Research, 20*(1), 93-100.

Ni, L., Cao, W., Zheng, W., Zhang, Q., & Li, B. (2015). Reduction of microbial contamination on the surfaces of layer houses using slightly acidic electrolyzed water. *Poultry Science, 94*(11), 2838-2848.

Nicklas, W., Böhm, K., & Richter, B. (1981). Studies on the corrosive action of some disinfectants suitable for aerosol-disinfection (author's transl). *Zentralblatt fur*

Bakteriologie, Mikrobiologie und Hygiene. 1. Abt. Originale B, Hygiene, 173(5), 374-381.

Nielsen, R. (1986a). Serological characterization of Actinobacillus pleuropneumoniae strains and proposal of a new serotype: serotype 12. *Acta Veterinaria Scandinavica (Denmark).*

Nielsen, R. (1986b). Serology of Haemophilus (Actinobacillus) pleuropneumoniae serotype 5 strains: establishment of subtypes a and b. *Acta Veterinaria Scandinavica, 27*(1), 49.

Nielsen, R., Andresen, L. O., Plambeck, T., Nielsen, J. P., Krarup, L. T., & Jorsal, S. E. (1997). Serological characterization of Actinobacillus pleuropneumoniae biotype 2 strains isolated from pigs in two Danish herds. *Veterinary Microbiology, 54*(1), 35-46.

Niemira, B. A., & Fan, X. (2006). Low-dose irradiation of fresh and fresh-cut produce: safety, sensory and shelf life. In *Food Irradiation Research and Technology* (pp. 169-181): Wiley Online Library.

National Notifiable Diseases Surveillance System (NNDSS). *National notifiable diseases: Australia's notifiable diseases status 2015: Annual report of the National Notifiable Diseases Surveillance System*. Department of Health: Canberra, Australia 2015.

Noel J Riggs, & Scott, S. B. (2019). The Top 5 Spinach Producing Countries. *Top5ofAnything*. Retrieved from https://top5ofanything.com/list/02728b18/Spinach-Producing-Countries

Nys, Y., Gautron, J., Garcia-Ruiz, J. M., & Hincke, M. T. (2004). Avian eggshell mineralization: biochemical and functional characterization of matrix proteins. *Comptes Rendus Palevol, 3*(6-7), 549-562.

Nys, Y., Hincke, M., Arias, J., Garcia-Ruiz, J., & Solomon, S. (1999). Avian eggshell mineralization. *Poultry and Avian Biology Reviews, 10*(3), 143-166.

Nys, Y., Zawadzki, J., Gautron, J., & Mills, A. (1991). Whitening of brown-shelled eggs: mineral composition of uterine fluid and rate of protoporphyrin deposition. *Poultry Science, 70*(5), 1236-1245.

Ogunniyi, A. D., Dandie, C. E., Ferro, S., Hall, B., Drigo, B., Brunetti, G., . . . Donner, E. (2019). Comparative antibacterial activities of neutral electrolyzed oxidizing water and other chlorine-based sanitizers. *Scientific Reports, 9*(1), 1-13.

Oliveira, M., Usall, J., Vinas, I., Anguera, M., Gatius, F., & Abadias, M. (2010). Microbiological quality of fresh lettuce from organic and conventional production. *Food Microbiology, 27*(5), 679-684.

Olsen, R., Kudirkiene, E., Thøfner, I., Pors, S., Karlskov-Mortensen, P., Li, L., . . . Christensen, J. (2017). Impact of egg disinfection of hatching eggs on the eggshell microbiome and bacterial load. *Poultry Science, 96*(11), 3901-3911. doi:10.3382/ps/pex182

Oomori, T., Oka, T., Inuta, T., & ARATA, Y. (2000). The efficiency of disinfection of acidic electrolyzed water in the presence of organic materials. *Analytical Sciences, 16*(4), 365-369.

Ovissipour, M., Al-Qadiri, H. M., Sablani, S. S., Govindan, B. N., Al-Alami, N., & Rasco, B. (2015). Efficacy of acidic and alkaline electrolyzed water for inactivating Escherichia coli O104: H4, Listeria monocytogenes, Campylobacter jejuni, Aeromonas hydrophila, and Vibrio parahaemolyticus in cell suspensions. *Food Control, 53*, 117-123.

Ozer, N. P., & Demirci, A. (2006). Electrolyzed oxidizing water treatment for decontamination of raw salmon inoculated with Escherichia coli O157: H7 and Listeria monocytogenes Scott A and response surface modeling. *Journal of Food Engineering, 72*(3), 234-241.

OzFoodNet Working Group (2007). Monitoring the incidence and causes of diseases potentially transmitted by food in Australia: annual report of the Ozfoodnet Network, 2006. *Communicable Diseases Intelligence Quarterly Report, 31*(4), 345.

OzFoodNet Working Group. (2018). Monitoring the incidence and causes of diseases potentially transmitted by food in Australia: annual report of the OzFoodNet network, 2012. *Communicable Diseases Intelligence Quarterly Report, 42*, 43.

Park, C.M., Hung, Y.C., Lin, C.S., & Brackett, R. E. (2005). Efficacy of electrolyzed water in inactivating Salmonella enteritidis and Listeria monocytogenes on shell eggs. *Journal of Food Protection, 68*(5), 986-990.

Park, E. J., Alexander, E., Taylor, G., Costa, R., & Kang, D. H. (2008). Effect of electrolyzed water for reduction of foodborne pathogens on lettuce and spinach. *Journal of Food Science, 73*(6), M268-M272.

Park, H., Hung, Y.-C., & Chung, D. (2004). Effects of chlorine and pH on efficacy of electrolyzed water for inactivating Escherichia coli O157: H7 and Listeria monocytogenes. *International Journal of Food Microbiology, 91*(1), 13-18.

Parsons, A. (1982). Structure of the eggshell. *Poultry Science, 61*(10), 2013-2021.

Paulsen, I., Littlejohn, T., Rådström, P., Sundström, L., Sköld, O., Swedberg, G., & Skurray, R. (1993). The 3'conserved segment of integrons contains a gene associated with multidrug resistance to antiseptics and disinfectants. *Antimicrobial Agents and Chemotherapy, 37*(4), 761-768.

Pinkerton, L., Hein, M., & Stayner, L. (2004). Mortality among a cohort of garment workers exposed to formaldehyde: an update. *Occupational and Environmental Medicine, 61*(3), 193-200.

Poole, K. (2002). Mechanisms of bacterial biocide and antibiotic resistance. *Journal of Applied Microbiology, 92*, 55S-64S.

Popescu, S., Borda, C., Diugan, E. A., & Oros, D. (2014). Microbial air contamination in indoor and outdoor environment of pig farms. *Scientific Papers Animal Science and Biotechnologies, 47*(1), 182-187.

Premier, R. (2013). Evaluation of vegetable washing chemicals. *Australia: Horticulture Australia Ltd*. Retrieved from: https://ausveg.com.au/app/data/technical-insights/docs/130028_VG09086%20Final%20Report%20Complete.pdf

Prilutsky, V., & Bakhir, V. (1997). Electrochemically activated water: anomalous properties, mechanism of biological action. *M., VNIIIMT*.

Rahman, S., Ding, T., & Oh, D.-H. (2010). Inactivation effect of newly developed low concentration electrolyzed water and other sanitizers against microorganisms on spinach. *Food Control, 21*(10), 1383-1387.

Rahman, S., Jin, Y. G., & Oh, D. H. (2010). Combined effects of alkaline electrolyzed water and citric acid with mild heat to control microorganisms on cabbage. *Journal of Food Science, 75*(2), M111-M115.

Rasschaert, G., Piessens, V., Scheldeman, P., Leleu, S., Stals, A., Herman, L., . . . Messens, W. (2013). Efficacy of electrolyzed oxidizing water and lactic acid on the reduction of Campylobacter on naturally contaminated broiler carcasses during processing. *Poultry Science, 92*(4), 1077-1084.

Ren, T., & Su, Y.-C. (2006). Effects of electrolyzed oxidizing water treatment on reducing Vibrio parahaemolyticus and Vibrio vulnificus in raw oysters. *Journal of Food Protection, 69*(8), 1829-1834.

Rezzonico, F., Smits, T. H., Montesinos, E., Frey, J. E., & Duffy, B. (2009). Genotypic comparison of Pantoea agglomerans plant and clinical strains. *BMC Microbiology, 9*(1), 204.

Rico, D., Martin-Diana, A. B., Barat, J., & Barry-Ryan, C. (2007). Extending and measuring the quality of fresh-cut fruit and vegetables: a review. *Trends in Food Science & Technology, 18*(7), 373-386.

Rivera-Garcia, A., Santos-Ferro, L., Ramirez-Orejel, J. C., Agredano-Moreno, L. T., Jimenez-Garcia, L. F., Paez-Esquiliano, D., . . . Cano-Buendia, J. A. (2019). The effect of neutral electrolyzed water as a disinfectant of eggshells artificially contaminated with Listeria monocytogenes. *Food Science & Nutrition*.

Roberts, J., & Chousalkar, K. (2013). Egg quality and age of laying hens: implications for product safety. *Animal Production Science, 53*(12), 1291-1297.

Robinson, G., Thorn, R., & Reynolds, D. (2013). The effect of long-term storage on the physiochemical and bactericidal properties of electrochemically activated solutions. *International Journal of Molecular Sciences*, 14(1), 457-469.

Rodríguez-Navarro, A. B., Domínguez-Gasca, N., Muñoz, A., & Ortega-Huertas, M. (2013). Change in the chicken eggshell cuticle with hen age and egg freshness. *Poultry Science, 92*(11), 3026-3035.

Rodriguez Romo LA. *Control of Salmonella enterica serovar enteritidis in shell eggs by ozone, ultraviolet radiation, and heat* (Doctoral dissertation, The Ohio State University).

Roque, K., Lim, G.-D., Jo, J.-H., Shin, K.-M., Song, E.-S., Gautam, R., . . . Yoo, H.-S. (2016). Epizootiological characteristics of viable bacteria and fungi in indoor air from porcine, chicken, or bovine husbandry confinement buildings. *Journal of Veterinary Science, 17*(4), 531-538.

Rose-Martel, M., Du, J., & Hincke, M. T. (2012). Proteomic analysis provides new insight into the chicken eggshell cuticle. *Journal of Proteomics, 75*(9), 2697-2706.

Rouch, D., Cram, D., Di Berardino, D., Littlejohn, T., & Skurray, R. (1990). Efflux-mediated antiseptic resistance gene qacA from Staphylococcus aureus: common ancestry with tetracycline-and sugar-transport proteins. *Molecular Microbiology, 4*(12), 2051-2062.

Rubatzky, V. E., & Yamaguchi, M. (1997). Spinach, table beets, and other vegetable chenopods. In *World Vegetables* (pp. 457-473): Springer.

Rudi, K., Flateland, S. L., Hanssen, J. F., Bengtsson, G., & Nissen, H. (2002). Development and evaluation of a 16S ribosomal DNA array-based approach for describing complex microbial communities in ready-to-eat vegetable salads packed in a modified atmosphere. *Applied Environmental Microbiology, 68*(3), 1146-1156.

Russell, A. D. (1992). Factors influencing the efficacy of antimicrobial agents. *Principles and Practice of Disinfection, Preservation and Sterilization*, 89-113.

Russell, A. (1999). Bacterial resistance to disinfectants: present knowledge and future problems. *Journal of Hospital Infection, 43*, S57-S68.

Russell, A. (2000). Do biocides select for antibiotic resistance? *Journal of Pharmacy and Pharmacology, 52*(2), 227-233.

Samiullah, S., Chousalkar, K., Roberts, J., Sexton, M., May, D., & Kiermeier, A. (2013). Effects of eggshell quality and washing on Salmonella Infantis penetration. *International Journal of Food Microbiology,* 2013; 165(2):77-83.

Sárközi, R., Makrai, L., & Fodor, L. (2015). Identification of a proposed new serovar of Actinobacillus pleuropneumoniae: serovar 16. *Acta Veterinaria Hungarica, 63*(4), 444-450.

Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR). Assessment of the antibiotic resistance effects of biocides. *European Commission, Health & Consumer Protection*. Scientific Committee on Emerging and Newly Identified Health Risks 2009. Retrieved from: https://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_021.pdf

Schuenzel, K. M., & Harrison, M. A. (2002). Microbial antagonists of foodborne pathogens on fresh, minimally processed vegetables. *Journal of Food Protection, 65*(12), 1909-1915.

Seier-Petersen, M. A., Ussery, D., Aarestrup, F. M., & Agersø, Y. (2013). Development of bacterial resistance to biocides and antimicrobial agents as a consequence of biocide usage.

Selkon, J., Babbt, J., & Morris, R. (1999). Evaluation of the antimicrobial activity of a new super-oxidized water, Sterilox®, for the disinfection of endoscopes. *Journal of Hospital Infection, 41*(1), 59-70.

Sergio Ferro, T. A. a. E. D. (2017). First results of an electrochemical water management system in Australia. *The Australian Hospital Healthcare Bulletin, Winter, 2017*, 56-57.

Shiroodi, S. G., & Ovissipour, M. (2018). Electrolyzed Water Application in Fresh Produce Sanitation. In *Postharvest Disinfection of Fruits and Vegetables* (pp. 67-89): Elsevier.

Sidhu, M. S., Sørum, H., & Holck, A. (2002). Resistance to Quaternary Ammonium Compounds in Food-Related Bacteria. *Microbial Drug Resistance, 8*(4), 393-399. doi:10.1089/10766290260469679

Simons, P. C. M. (1971). *Ultrastructure of the Hen Eggshell and its Physiological Interpretation*: Centre for Agricultural Publishing and Documentation Wageningen.

Slifierz, M. J., Friendship, R. M., & Weese, J. S. (2015). Methicillin-resistant Staphylococcus aureus in commercial swine herds is associated with disinfectant and zinc usage. *Applied Environmental Microbiology, 81*(8), 2690-2695.

Soderqvist, K., Ahmed Osman, O., Wolff, C., Bertilsson, S., Vagsholm, I., & Boqvist, S. (2017). Emerging microbiota during cold storage and temperature abuse of ready-to-eat salad. *Infectious Ecology and Epidemiology, 7*(1), 1328963. doi:10.1080/20008686.2017.1328963

Solomon, S. (1991). Egg and Eggshell Quality, Wolfe Publ. *Ltd, London*.

Sparks, N., & Board, R. (1984). Cuticle, shell porosity and water uptake through hens' eggshells. *British Poultry Science, 25*(2), 267-276.

Stoner, G., Cahen, G., Sachyani, M., & Gileadi, E. (1982). 457—The mechanism of low frequency ac electrochemical disinfection. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry, 141*(3), 229-243.

Sundheim, G., Langsrud, S., Heir, E., & Holck, A. (1998). Bacterial resistance to disinfectants containing quaternary ammonium compounds. *International Biodeterioration & Biodegradation, 41*(3-4), 235-239.

Surdu, I., Vătuiu, D., Jurcoane, Ş., Olteanu, M., & Vătuiu, I. (2017). The antimicrobial activity of neutral electrolyzed water against germs and fungi from feedstuffs, eggshells and laying hen house.

Suslow, T. (2000). Chlorination in the production and postharvest handling of fresh fruits and vegetables. *Fruit and Vegetable Processing*, 2-15.

Taban, B. M., & Halkman, A. K. (2011). Do leafy green vegetables and their ready-to-eat [RTE] salads carry a risk of foodborne pathogens? *Anaerobe, 17*(6), 286-287.

Tanaka, Fujisawa, Daimon, Fujiwara, Yamamoto, & Abe, T. (1999). The Cleaning and Disinfecting of Hemodialysis Equipment Using Electrolyzed Strong Acid

Aqueous Solution. *Artificial Organs, 23*(4), 303-309. doi:10.1046/j.1525-1594.1999.6223r.x

Tanaka, N., Fujisawa, T., Daimon, T., Fujiwara, K., Tanaka, N., Yamamoto, M., & Abe, T. (1999). The effect of electrolyzed strong acid aqueous solution on hemodialysis equipment. *Artificial Organs, 23*(12), 1055-1062.

Tang, W., Zeng, X., Zhao, Y., Ye, G., Gui, W., & Ni, Y. (2011). Disinfection effect and its mechanism of electrolyzed oxidizing water on spores of Bacillus subtilis var. niger. *Food Science and Biotechnology, 20*(4), 889.

Tango, C. N., Choi, N.-J., Chung, M.-S., & Oh, D. H. (2014). Bacteriological quality of vegetables from organic and conventional production in different areas of Korea. *Journal of food protection, 77*(8), 1411-1417.

Tatsika, S., Karamanoli, K., Karayanni, H., & Genitsaris, S. (2019). Metagenomic Characterization of Bacterial Communities on Ready-to-Eat Vegetables and Effects of Household Washing on their Diversity and Composition. *Pathogens, 8*(1), 37.

Teixeira, P., Fernandes, B., Silva, A. M., Dias, N., & Azeredo, J. (2020). Evaluation by Flow Cytometry of Escherichia coli Viability in Lettuce after Disinfection. *Antibiotics, 9*(1), 14.

Thacker, E. L., & Thanawongnuwech, R. (2002). Porcine respiratory disease complex (PRDC). *Thai Journal of Veterinary Medicine, 32*, 125-134.

Til, H., Woutersen, R., Feron, V., Hollanders, V., Falke, H., & Clary, J. (1989). Two-year drinking-water study of formaldehyde in rats. *Food and Chemical Toxicology, 27*(2), 77-87.

Tournas, V. (2005). Moulds and yeasts in fresh and minimally processed vegetables, and sprouts. *International Journal of Food Microbiology, 99*(1), 71-77.

Truchado, P., Gil, M. I., Suslow, T., & Allende, A. (2018). Impact of chlorine dioxide disinfection of irrigation water on the epiphytic bacterial community of baby spinach and underlying soil. *PloS One, 13*(7), e0199291.

Tsuji, Kawano, Oshita, Ohmae, Shinomura, Miyazaki, . . . Maeda. (1999). Endoscope Disinfection Using Acidic Electrolytic Water. *Endoscopy, 31*(7), 528-535. doi:10.1055/s-1999-55

Turantaş, F., Ersus-Bilek, S., Sömek, Ö., & Kuşçu, A. (2018). Decontamination Effect of Electrolyzed Water Washing on Fruits and Vegetables. *The Journal of Microbiology, Biotechnology and Food Sciences, 7*(4), 337.

Turni, C. (2014). Antibiotic sensitivity of Haemophilus parasuis plus Actinobacillus pleuropneumoniae and other respiratory pathogens. Retrieved from http://porkcrc.com.au/wp-content/uploads/2015/07/2A-107-Final-report.pdf

Turni, C., Singh, R., Schembri, M. A., & Blackall, P. J. (2014). Evaluation of a multiplex PCR to identify and serotypeActinobacillus pleuropneumoniaeserovars 1, 5, 7, 12 and 15. *Letters in applied microbiology, 59*(4), 362-369. doi:10.1111/lam.12287

Umimoto, K., Kawanishi, H., Kobayashi, K., & Yanagida, J. (2008). *Development of a Device to Provide Electrolyzed Water for Home Care.* Paper presented at the 4th Kuala Lumpur International Conference on Biomedical Engineering 2008.

Umimoto, K., Morimoto, N., Kamada, A., Nagata, S., & Yanagida, J. (2015). *A simple device producing electrolyzed water for home care.* Paper presented at the World Congress on Medical Physics and Biomedical Engineering, June 7-12, 2015, Toronto, Canada.

Valentin-Bon, I., Jacobson, A., Monday, S. R., & Feng, P. C. H. (2008). Microbiological Quality of Bagged Cut Spinach and Lettuce Mixes. *Applied and Environmental Microbiology, 74*(4), 1240-1242. doi:10.1128/aem.02258-07

VanderWaal, K., & Deen, J. (2018). Global trends in infectious diseases of swine. *Proceedings of the National Academy of Sciences, 115*(45), 11495-11500. doi:10.1073/pnas.1806068115

Veasey, S., & Muriana, P. (2016). Evaluation of Electrolytically-Generated Hypochlorous Acid ('Electrolyzed Water') for Sanitation of Meat and Meat-Contact Surfaces. *Foods, 5*(4), 42. doi:10.3390/foods5020042

Venkitanarayanan, K. S., Ezeike, G. O., Hung, Y.-C., & Doyle, M. P. (1999). Efficacy of electrolyzed oxidizing water for inactivating Escherichia coli O157: H7, Salmonella enteritidis, and Listeria monocytogenes. *Appl. Environ. Microbiol., 65*(9), 4276-4279.

Venturini, M. (2013). Acqua elettrolizzata: tecnologia emergente del settore ortofrutticolo. *Alim Bev, 4*, 50-55.

Vestergaard, D. V., Holst, G. J., Basinas, I., Elholm, G., Schlünssen, V., Linneberg, A., . . . Marshall, I. P. (2018). Pig farmers' homes harbor more diverse airborne bacterial communities than pig stables or suburban homes. *Frontiers in Microbiology, 9*, 870.

Wang, H., Duan, D., Wu, Z., Xue, S., Xu, X., & Zhou, G. (2019). Primary concerns regarding the application of electrolyzed water in the meat industry. *Food Control, 95*, 50-56.

Wang, H., & Slavik, M. E. (1998). Bacterial penetration into eggs washed with various chemicals and stored at different temperatures and times. *Journal of Food Protection, 61*(3), 276-279.

Wangensteen, O. D., Wilson, D., & Rahn, H. (1970). Diffusion of gases across the shell of the hen's egg. *Respiration physiology, 11*(1), 16-30.

Wedral, E. M., Vadehra, D., & Baker, R. (1974). Chemical composition of the cuticle, and the inner and outer shell membranes from eggs of Gallus gallus. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, 47*(3), 631-640.

Wellman-Labadie, O., Picman, J., & Hincke, M. (2008). Antimicrobial activity of cuticle and outer eggshell protein extracts from three species of domestic birds. *British Poultry Science, 49*(2), 133-143.

White, D. G., & McDermott, P. F. (2001). Biocides, drug resistance and microbial evolution. *Current Opinion in mMicrobiology, 4*(3), 313-317.

World Health Organization (WHO). *WHO estimates of the global burden of foodborne diseases: foodborne disease burden epidemiology reference group 2007-2015*. World Health Organization 2015.

Williams, J., Dillard, L., & Hall, G. O. (1968). The penetration patterns of Salmonella typhimurium through the outer structures of chicken eggs. *Avian Diseases, 12*(3), 445-466.

Wingender, J., & Flemming, H.-C. (2011). Biofilms in drinking water and their role as reservoir for pathogens. *International Journal of Hygiene and Environmental Health, 214*(6), 417-423.

Winkelströter, L. K., & De Martinis, E. C. (2015). Different methods to quantify Listeria monocytogenesbiofilms cells showed different profile in their viability. *Brazilian Journal of Microbiology, 46*(1), 231-235.

Wong, H., Townsend, K., Fenwick, S., Trengove, R., & O'handley, R. (2010). Comparative susceptibility of planktonic and 3-day-old Salmonella Typhimurium biofilms to disinfectants. *Journal of Applied Microbiology, 108*(6), 2222-2228.

Yang, H., Swem, B., & Li, Y. (2003). The effect of pH on inactivation of pathogenic bacteria on fresh-cut lettuce by dipping treatment with electrolyzed water. *Journal of Food Science, 68*(3), 1013-1017.

Yang, Y., Bing, S., Li, Y., & Shu, D. (2019). Application of slightly acidic electrolyzed water and ultraviolet light for Salmonella enteritidis decontamination of cell suspensions and surfaces of artificially inoculated plastic poultry transport coops and other facility surfaces. *Poultry Science*.

Zang, Y. T., Bing, S., Li, Y. J., Shu, D. Q., Huang, A. M., Wu, H. X., . . . Wu, H. D. (2019). Efficacy of slightly acidic electrolyzed water on the microbial safety and shelf life of shelled eggs. *Poultry Science, 98*(11), 5932-5939. doi:10.3382/ps/pez373

Zeng, X., Tang, W., Ye, G., Ouyang, T., Tian, L., Ni, Y., & Li, P. (2010). Studies on disinfection mechanism of electrolyzed oxidizing water on E. coli and Staphylococcus aureus. *Journal of Food Science, 75*(5), M253-M260.

Zhang, C., Chen, X., Xia, X., Li, B., & Hung, Y.-C. (2018). Viability assay of E. coli O157: H7 treated with electrolyzed oxidizing water using flow cytometry. *Food Control, 88*, 47-53.

Zhang, M., Oh, J. K., Cisneros-Zevallos, L., & Akbulut, M. (2013). Bactericidal effects of nonthermal low-pressure oxygen plasma on S. typhimurium LT2 attached to fresh produce surfaces. *Journal of Food Engineering, 119*(3), 425-432.

Zhang, S., & Farber, J. (1996). The effects of various disinfectants against Listeria monocytogeneson fresh-cut vegetables. *Food Microbiology, 13*(4), 311-321.

Zheng, W., Li, B., Cao, W., Zhang, G., & Yang, Z. (2012). Application of neutral electrolyzed water spray for reducing dust levels in a layer breeding house. *Journal of the Air & Waste Management Association, 62*(11), 1329-1334.

Zheng, W., Zhao, Y., Xin, H., Gates, R. S., Li, B., Zhang, Y., & Soupir, M. L. (2014). Airborne particulate matter and culturable bacteria reduction from spraying slightly acidic electrolyzed water in an experimental aviary laying-hen housing chamber. *Transactions of the ASABE, 57*(1), 229-236.

Zinkevich, V., Beech, I., Tapper, R., & Bogdarina, I. (2000). The effect of super-oxidized water on Escherichia coli. *Journal of Hospital Infection, 46*(2), 153-156.