EFFICACY AND SUITABILITY OF COLD PLASMA GENERATED NOVEL SANITIZER IN EGG WASHING

By

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ABSTRACT OF THE THESIS

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Salmonella Enteritidis has been recognized as the cause of over 60% of reported salmonellosis cases, associated with shell eggs and egg products (CDC, 2013). In order to reduce occurrence of salmonellosis, egg washing on an industrial scale was mandated in some countries such as the United States of America and Japan (Hutchinson et al., 2003). Chemical sanitizers like quaternary ammonia (QA), used in commercial egg washing have been seen to cause degradation of the egg cuticle, which is a natural protectant against bacterial penetration (Bialka et al., 2004). So as to preserve the cuticle, and not compromise on microbial safety, plasma activated water (PAW) was investigated as a possible sanitizer on the basis of microbial reduction on artificially contaminated eggs, as well as its effect on the quality of cuticle and egg shell strength.

PAW was generated by exposing distilled water to atmospheric pressure plasma jet from filtered dry air. PAW and QA were then used to wash eggs artificially contaminated with *Enterobacter aerogenes* (non-pathogenic *Salmonella* surrogate), to compare their effectiveness in microbial reduction on egg surface as well as in wash water. Colorimetric analysis was performed on eggs washed with sanitizers

and stained with a cuticle sensitive dye, to evaluate loss of cuticle. Additionally, damage to cuticle was studied using microscopic analysis of egg shells. Damage to shell integrity was studied by means of texture analysis of sanitized eggs, to ensure that the acidic nature of PAW did not affect egg shell strength.

Preliminary planktonic-cell experiments with distilled water (DW) which served as control, caused bacterial reduction of 0.2±0.1 log CFU/ml from initial concentration of 9.2±0.1 log CFU/ml. On the other hand, treatment with equal volumes of PAW or QA resulted in bacterial concentration below detection limit (3.2 log CFU/ml). This proved that microbial reduction was not due to osmotic pressure generated by water. This was further enforced by analysis of artificially contaminated shell egg surface, with an initial bacterial concentration of 7.9±0.3 log CFU/egg, sanitized with PAW, QA and DW. Upon exposure of eggs to sanitizers by massaging of egg surface, it was noted that bacterial survival after treatment with both PAW and QA was below detection limit (2.3 log CFU/egg). Thus, the microbial reduction efficiencies of PAW and QA were comparable, at over 5.3 log CFU/egg. Conversely, treatment with DW showed survival of 3.7±0.1 log CFU/egg on the surface of egg. Colorimetric analysis showed statistically significant difference between un-sanitized farm eggs, and farm-eggs treated with PAW and, QA and store-bought eggs suggesting that eggs treated with PAW retained their cuticle layer better.

Texture analysis of treated whole eggs revealed that the acidic nature of PAW did not affect the peak force needed to crack eggs. Eggs sanitized with both QA and PAW showed highest peak strength at the small-end up position and lowest peak force at the equatorial positions. A significant difference was observed between the

orientations of eggs; however, the difference in strength within a given orientation, among different sanitizing treatments was not significant.

This study showed that PAW possessed both comparable anti-microbial efficacy and effect on egg shell strength as QA, while being better at cuticle retention on shell egg surface. QA was observed to erode the cuticle layer, while similar treatment with PAW showed that the cuticle coverage was relatively intact and comparable to unsanitized eggs. Thus, PAW appeared to show promise for use in egg washing process to replace current chemical sanitizers (QA) with minimal cuticle loss.

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1. INTRODUCTION

Serotype Enteritidis, of *Salmonella*, has been associated most frequently with eggs and poultry (Braden, 2006; Jackson et al., 2013). Although both egg and poultry are bracketed as root cause for Salmonellosis, eggs alone contribute to 65% of outbreaks caused by *Salmonella* Enteritidis (EFSA, 2009; CDC, 2013; Jackson et al., 2013). With the continuous rise in both egg production and consumption, there is now a higher risk of outbreaks associated with eggs and egg products (Choi et al., 2015; http://faostat.fao.org).

1.1. Egg Structure

Eggs have long since been identified as a source of nourishment for humans, and the nutrients from eggs are obtained not only from the albumen and yolk, but also from the shell (Mine, 2008). The egg is composed of an outer, calciferous shell, which is surrounded by a cuticle layer. The egg shell houses albumen or egg white which surrounds the egg yolk, as shown in Fig. 1 (Hincke et al., 2012). In *Gallus gallus* (domestic chicken), formation of eggs has been studied to show that the yolk is formed first, followed by the albumen (Nys et al., 1999; Nys et al., 2004; Hincke et al., 2012). Even though all eggs have similar structures, their compositions vary depending upon species, feed provided to hens, and also the age of the hen (Sugino et al., 1997b; Li-Chan and Kim et al., 2008).

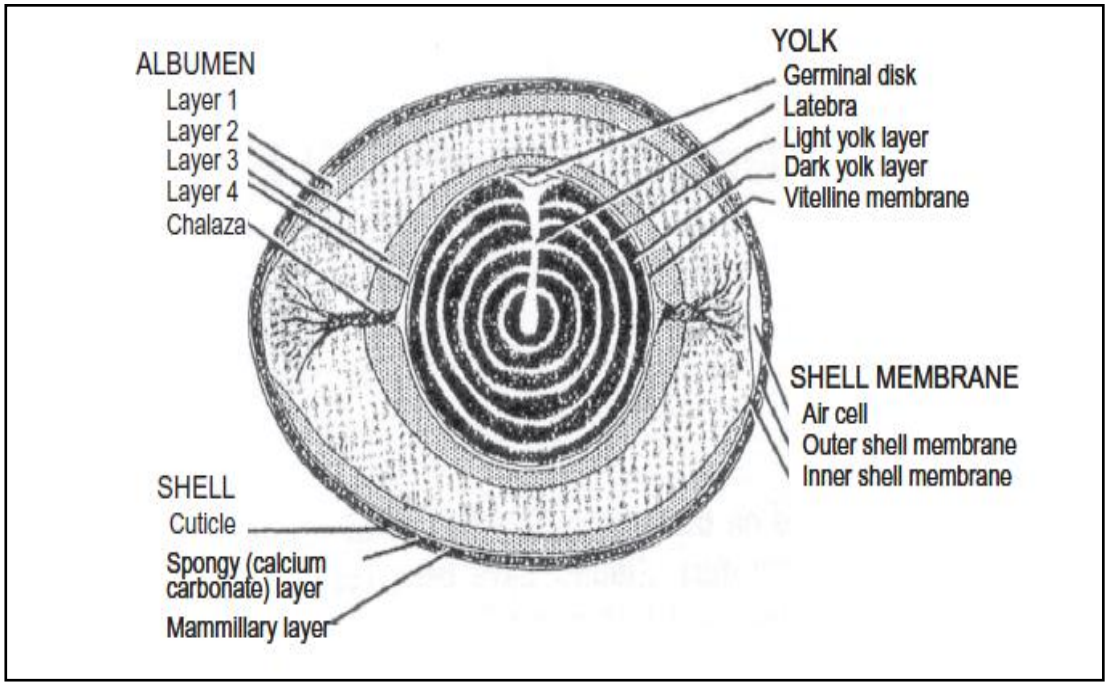


Figure 1: Structural anatomy of a chicken egg (Source: USDA, 2000 egg grading manual)

1.1.1. Egg shell, Outer and Inner Membranes

The egg shell is a strong, calcite layer that develops in chicken in a span of approximately a day. This makes it one of the fastest occurring calcification processes known (Hincke et al., 2012). The egg shell is a bio-ceramic material, composed principally of calcium carbonate, also known as polycrystalline calcite (Nys et al., 2004; Jonchere et al., 2010). The egg shell contains a deposition of minerals, which occurs in a process within the hen's oviduct, known as mineralization (Nys et al., 1999; Hincke et al., 2012). The formation of shell over yolk and albumen, starts with mammillary cone or mammillary nodes. These nodes are deposited over the albumen as organic masses, which when passed through the oviduct, turn into nucleation sites for mineralization and calcium deposition (Nys et al., 2004; Hincke et

al., 2012). This is followed immediately by development of vertical calcite crystals or the palisade layer. This layer ends in the vertical crystal layer, which forms part of the hard exterior of shell eggs (Hincke et al., 2012).

The mammillary zone is responsible for egg shell strength, since it contains nodes made of calcite, like the egg shell exterior. However, this calcite is mobilized into the embryo for nourishment, unlike the vertical crystal layer (egg shell exterior), where calcite is mobilized to contribute in protection of egg constituents (Nys et al., 2004; Hincke et al., 2012). Thus, there is an internal conflict between crystal growth stacks (Grigoriev, 1965; Rodriguez-Navarro and Garcia-Ruiz, 2000; Jonchere et al., 2010) causing a higher concentration of mammillary nodes, leading to weaker egg shells (Toledo et al., 1982; Riley et al., 2014). Underneath mammillary and palisade zones, are the outer and inner shell membranes (Jonchere et al.,2010; Hincke et al., 2012). Fig. 2 highlights each layer of the egg shell, starting with the cuticle, until the inner membrane (Adopted from Hincke et al., 2012).

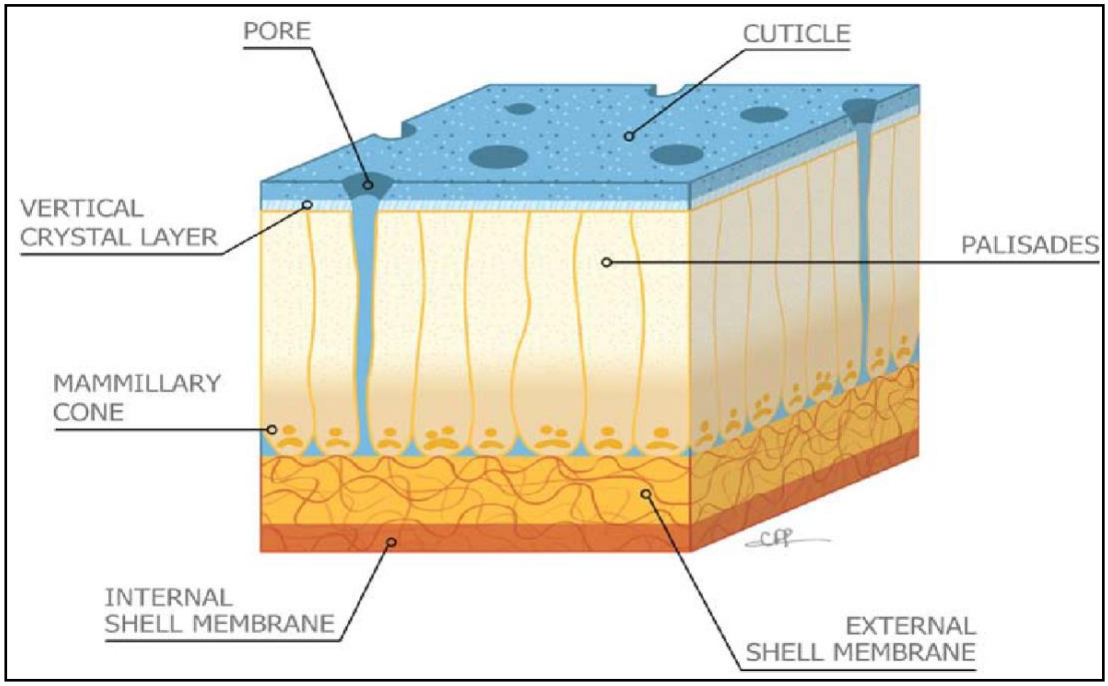


Figure 2: Cross-section of egg shell of *Gallus gallus* (Source: Hincke 2012) (Diagram not to scale)

The inner and outer membranes of egg shell are a complex organic medium composed of proteins (Gautron and Nys, 2007; Jonchere et al., 2010). The shell membranes form a base for mineralization and calcium deposition (Krampitz and Graser, 1988; Nys et al.,1999). The membranes were originally thought to be composed of elastin, but were proven to be composed of collagen instead (Chowdhury, 1990).

1.1.2. Pores

Pores are orifices on the calcite outer egg shell, that allow for exchange of gases as well as water vapor (Board, 1980) between the atmosphere and shell membranes. The typical size of a pore on a hen egg shell is between 1.4 µm and 5.6 µm, as determined by mercury porosimetry (Scala et al., 2000). But, some pores could be as large as 65 µm, which is significantly larger than most microorganisms (Hutchinson et al., 2003). Analysis of egg shell samples presented that the diameter of pores on the smaller or sharper end is lower than pores on the larger or blunter end (Tullett, 1978; Riley et al., 2014). This fact is also seen to resonate in pore size distribution, such that pore frequency on the larger end is higher than that on the smaller end (Riley et al., 2014). In fact, the small end of the egg has only approximately half as many pores per unit area (Sollomon et al., 1994). This showed that the distribution of pores on an egg shell is not uniform (Tyler, 1953). Pore size and frequency distribution is seen to be consistent with the presence of air sac at the blunt end of eggs, which has been known to be primary site of gas exchange (Visschedijk et al.,

1968, Riley et al., 2014). Additionally, pore size is also seen to vary between species of birds and it reduces with acclimatization to increased altitude conditions (Rahn et al., 1977).

Initially, it was assumed that the pores extend to the last layer of the egg shell, however, electron microscopy images showed that pore canals seemed to extend until the mammillary layer, but did not always contribute to gas exchange (Sylin-Roberts, 1982; Solomon, 2010). The average diameter of pores is larger than oxygen and water molecules, thus it is believed that pores play a role in intake of water (Board, 1980). This intake of water occurs through capillary action in the pore canals, and is not influenced by osmotic pressure. If the water surrounding egg surface were to be contaminated, then pores along with water, may also permit entry of microorganisms (Sparks and Board, 1983). Thus, pores are covered or plugged by the first layer of naturally occurring physical defense known as the cuticle (Sparks, 1994; Hutchinson et al., 2003).

1.1.3. Cuticle

1.1.3.1. Structure and Composition of Cuticle

The cuticle is a non-calcified, organic layer that surrounds the egg shell (Rodriguez-Navarro et al., 2013; Hincke et al., 2012). As mentioned in section 1.1.2, this protein rich layer plugs close to 10,000 pores on the avian egg shell (Nys and Guyot, 2011). As the outermost layer of the mineralized egg shell, the cuticle acts as the first layer of defense of the egg against microorganisms (De Reu et al., 2004;Wellman-Labadie et al., 2008). The thickness of this layer ranges between 0.5 µm to about 12 µm, but the cuticle surface is irregular throughout the eggshell (Simons and Wiertz 1963;

Board and Halls., 1973).The irregularity is due to the process of formation of the egg shell that starts at the last phase of oviposition of the egg, within the body of the hen (Nys et al., 1999) and the liquid sheen-like cuticle solidifies within minutes of the egg being laid, causing it to develop cracks (Sparks and Board, 1985). This causes the cuticle to appear flaky and patchy when viewed under an electron microscope (Parsons, 1982; Samiullah and Roberts, 2014).

Apart from having high protein content, the cuticle also contains polysaccharides (approximately 4%), lipids (approximately 3%) and porphyrin pigment in the case of brown eggs (Baker and Balch, 1961; Rodriguez-Navarro et al., 2013). The proteins present in the cuticular layer differ from those present in the egg shell matrix. The matrix has less than half the amount of lysine and glycine, but twice the amount of cysteine compared to the egg shell matrix (Baker and Balch, 1961). Majority (85% - 87%) of the cuticle is composed of proteins that are neither water-soluble, nor soluble in KCl. Lipid content of the cuticle layer although low, is observed to be higher than the outer shell membrane of eggs, which had an average of 2.3% lipids (Wedral et al., 1973). Sugar analysis of the layer showed that some of the polysaccharides present in the cuticle are mannose, fructose and galactose, with trace amounts of hexosamine nitrogen (Baker and Balch, 1961). Apart for amino acids, lipids and neutral sugars, mineralization of eggs also causes deposition of phosphorus in the cuticle (Wedral et al., 1974; Dennis et al., 1996; Miksik et al., 2003). The role of phosphorus alongside hydroxyapatite has been concluded as contributing to termination of calcite accumulation on shell, during the developmental stages (Simkiss, 1964; Fraser et al., 1999).

The precise structure of cuticle has been misinterpreted in the past as homogenous, vesicular and granulated (Simons, 1971). However, transmission electron microscopy images brought forth the two-layered nature of avian cuticle, as shown in Fig. 3 (Fraser et al., 1999). The outer layer or non-vesicular cuticle layer (NVC) was revealed to be the thinner and more homogenous of the two layers. The inner layer, otherwise known as vesicular cuticle (VC) layer comprised of vesicles of varied sizes. Each vesicle has a distinct core and mantle, such that the core was transparent to electrons (electron lucent) while the mantle was concentrated with electrons (electron dense) (Fraser et al., 1999; Samiullah and Roberts, 2014).

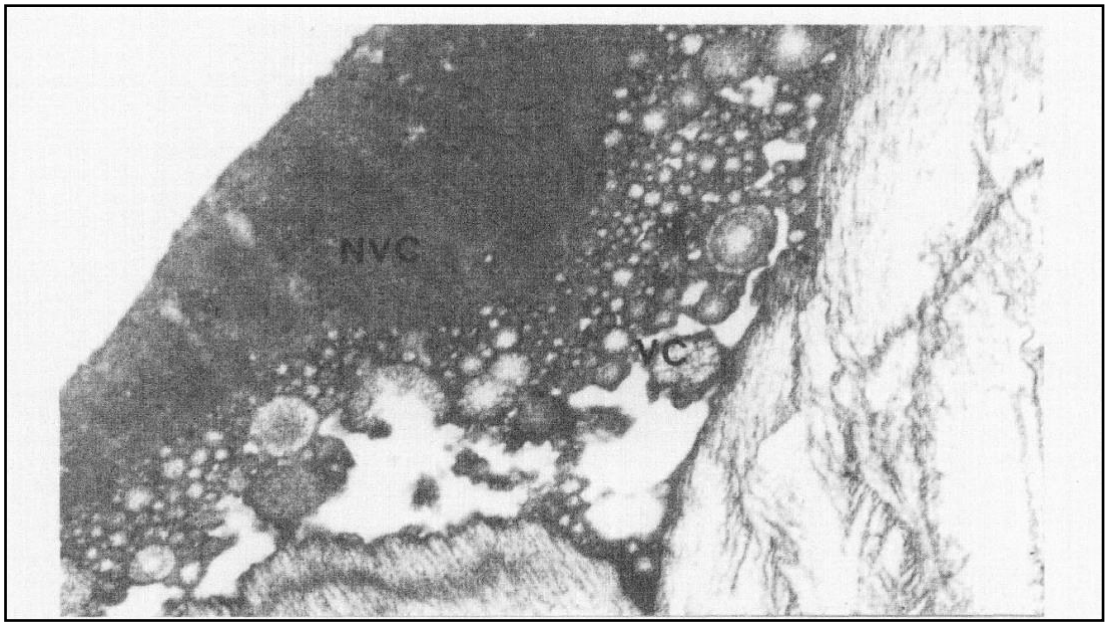


Figure 3: Scanning electron microscopy image depicting non-vesicular cuticle layer (NVC) and vesicular cuticle layer (VC) (Source: Fraser et al., 1999)

1.1.3.2. Function of Cuticle Layer

Cuticle has long since been known to play a major role in the innate defense mechanism of eggs (Romanoff 1931; Sparks and Board 1984). However, the cuticle is not as effective when undeveloped and moist, as it is when the outer layer is not entirely mature. The efficacy of cuticle as a barrier to microorganisms is directly proportional to its maturity, and to some extent the hardness of the layer (Miyamoto et al., 1998). The cuticle takes a few hours, after exposure to the outside environment, to completely stabilize and mature. Until this occurs, pores on the surface of eggs are left unprotected, making freshly laid eggs accessible to bacteria like *Salmonella* (Sparks and Board, 1984). A negative pressure develops in eggs, when they are rapidly exposed to the outside environment, since the temperature within the hen is higher than the room temperature. Although unconfirmed, it has been hypothesized that this temperature difference, in conjunction with an immature cuticle layer may lead to ease of entry of *Salmonella* (Board, 1966; Bruce and Drysdale, 1994; Miyamoto et al., 1998). Studies have shown that as little as three hours of maturation of the integumental cuticle layer caused a significant difference in penetration of *Salmonella* Enteritidis and *Salmonella typhimurium*, through the egg shell (Miyamoto et al., 1998). Upon attaining maturity, the cuticle covers pores to prevent entry of microorganisms, thereby functioning as a primary protective layer (Board and Fuller, 1974; Samiullah and Roberts, 2013).

There could be two ways in which the cuticle may plug pores (Cooke and Balche, 1970; Samilullah and Roberts, 2013) -

- The cuticle may extend over the egg shell surface, covering the outer opening of pores (Board, 1982)

- The cuticle may also extend through the pore canal, thus plugging pores from within (Cooke and Balch, 1970)

When the cuticle extends over the orifice of pores, the permeability of gases in pores may be reduced. But the cracks on a mature cuticle may aid oxygen and carbon dioxide transmission (through pores), which is integral to the development of embryo in eggs (Wangensteen, 1970; Tullett et al., 1975). The egg shell prevents gas exchange in eggs while the inner and the outer membranes of eggs do not seem to offer much resistance to gas permeation (Wangensteen, 1970). On the other hand, cuticle extending down through the pore canal may aid in diffusion of necessary gases (Board and Scott, 1980; Samiullah and Roberts, 2013).

Plugging of most pores by the cuticle aids not only in prevention of entry of microorganisms, but also obstructs entry of water into eggs (Sparks and Board, 1984). Deterrence of flooding of pores is a characteristic function of the water insoluble cuticle layer (Parsons, 1982; Sparks and Board, 1984; Thompson and Goldie, 1990). Sparks and Broad (1984) revealed with their study, that the complete loss of cuticle layer, caused more water entry into eggs, than eggs with a patchy cuticle. The tendency of eggs to take up water may increase due to the lower temperature of water, in comparison to inner egg temperature. This phenomenon could be explained by the fact that shell egg constituents shrink more than the shell itself, leading to development of a negative pressure within eggs, causing entry of water into eggs as explained earlier in this section (Haines and Moran, 1940).

Together with the cuticle, the pore canal is also seen to provide a certain resistance to flooding (Board and Halls, 1973; Board, 1980). Even though the resistance network provided by the cuticle was initially debated upon, the cuticle membrane

has been proven to effectively prevent penetration of foreign bodies into pores of egg shells (Board and Hall, 1973; Solomon, 2010).

Another study showed that spoilage of eggs by exposure to *Pseudomonas aeruginosa* was expedited in the absence of bloom (cuticle) in hen eggs. This study proved the importance of bloom in prevention of spoilage by microbial contamination (Vadhera et al., 1970; Sparks and Board, 1980). The inhibitory effect that cuticle possesses to microorganisms could be attributed to proteins with antimicrobial activity present in the cuticle matrix (Wellman-Labadie et al., 2008; Solomon, 2010). Antimicrobial lysozymes have been detected recently in protein extracts from egg shell cuticle. This protein extract was seen to generate an antimicrobial response against both gram-positive and gram-negative bacteria (Wellman-Labadie et al., 2008). It is believed that due to the above mentioned attributes if the cuticle, it would be advantageous in concentrating resources on improvement of cuticle quality eggs (Solomon, 2010).

1.1.3.3. Detection of Cuticle

The mere presence or absence thereof, of the cuticle could be detected by staining of egg shells with cuticle sensitive dyes (Messens et al., 2005; Solomon, 2010). Combination of Edicol pea green and tartrazine dyes has been used traditionally to stain egg shells and analyze the presence of cuticle layer (Board and Halls, 1973; DeReu et al., 2004; Messens et al., 2005; Solomon, 2010). Edicol supra pea-green dye has also been utilized to analyze cuticle presence on egg shells (Liu et al., 2016). The dye is allowed to stain the egg surface for 1 min and washed under tap water for a

few seconds, in order to remove excess stain. Stained eggs are then analyzed by colorimetry, to evaluate the (intensity?) of green color on eggs (Board and Halls, 1973; Ball et al., 1975; Messens et al., 2005; Leleu et al., 2011). Samiullah and Roberts (2013) validated the use of MST cuticle blue dye instead of a mixture of Edicol pea green and tartrazine. The staining procedure used was similar to that described by Board and Halls (1973). Colorimetric analysis was employed to detect the presence of cuticle (Samiullah and Roberts, 2013; Ragni et al., 2010; Rodriguez-Navarro et al., 2013).

X-ray microanalysis has been applied to analyze decalcified cuticle layer. This technique aided in investigation of chemical composition of the cuticle layer, concentrating especially on elements such as calcium, phosphorus, carbon and oxygen (Kusuda et al., 2011; Samiullah and Roberts, 2013). X-ray diffraction and electron microscopy have also been used to determine crystalline phase and concentration of elements in the cuticle (Kususda et al., 2011). Scanning electron microscopy has also been widely used to determine bacterial penetration (Sparks and Board, 1985) and thickness of the cuticle layer (Kususda et al., 2011). Transmission electron microscopy (TEM) is another form of microscopy which has been proven to be useful in determination of cuticle thickness as well as cuticle structure (Rodriguez-Navarro et al., 2013). Another potential use for electron microscopy imaging of shell eggs is to determine thickness of cuticle prior to and post washing with sanitizers (seen in Figure 4) (Kim and Slavik, 1996). Figure 4a shows the cuticle coverage of an unwashed egg surface, while Fig. 4b, Fig 4c and Fig. 4d represent eggs sprayed with sodium hypochlorite at increasing pressures, thus causing depletion of the cuticle.

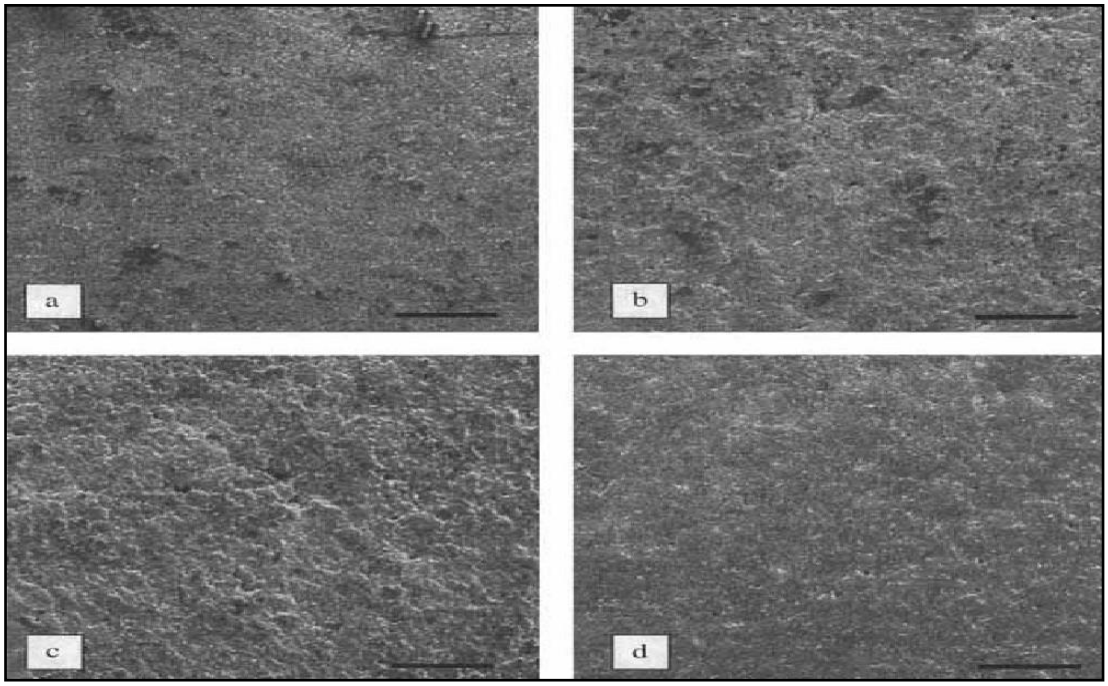


Figure 4: Scanning electron microscopy image Source: Kim and Slavik, 1996; taken from Hutchinson et al., 2003 Micrographs represent (a) the typical shell surface of an unwashed egg and (b - d) eggs that have been spray washed (with sodium hypochlorite). Bar marker at the corner of each image shows 100 µm.

Initially, presence of proteins in egg shell cuticle was examined Lowry's method of protein analysis (Lowry et al., 1951; Vadhera et al., 1970). Later, micro-Kjeldahl method (Markham, 1942) was used in analyzing proteins (Baker and Balch, 1961; Wedral et al., 1973) and performic acid method and TSM Dual column were used in determining amino acid concentration in eggs (Wedral, 1971; Wedral et al., 1973).

1.2. Modes of Contamination

Salmonella enterica has been assessed to be the leading cause of food borne illnesses, with over 1.2 million cases of it being reported between 1998 and 2008 (Scallan et al., 2011; Jackson et al., 2013). This bacterium, of serotype Enteritidis, has

been associated most frequently with eggs and poultry (Braden, 2006; Jackson et al., 2013). Although both egg and poultry are a bracketed as root cause for Salmonellosis, eggs alone contribute to 65% of outbreaks caused by *Salmonella* Enteritidis (EFSA, 2009; CDC, 2013; Jackson et al., 2013). With the continuous rise in both egg production and consumption, there is now a higher risk of outbreaks associated with eggs and egg products (Choi et al., 2015; http://faostat.fao.org/download/Q/QL/E)

Studies have confirmed that there are two possible routes of *Salmonella* contamination in eggs (Miyamoto et al., 1998) –

- Vertical transmission
- Horizontal transmission

Vertical transmission entails bacteria being transferred into the yolk or albumen of eggs, directly from the reproductive organs. This mode of transmission occurs before the egg has been completely formed (Hopper and Mawer, 1988; Bygrave et al., 1989; Miyamoto et al., 1998). Hence, this mode of transmission is also known as trans-ovarian transmission (Miyamoto et al., 1998). Ovarian infection usually occurs through contamination of eggs with *Salmonella*, in the course of formation of eggs, primarily in the vaginal tract of hen (Miyamoto et al., 1997). Also, ovarian contamination of eggs leads to infection in older hens (1 year or older), as compared to hens which were 20 weeks or lower in age (Humphrey et al., 1991).

On the other hand, it was also observed that the albumen is much more likely, to be contaminated than the yolk, which presents evidence for occurrence of horizontal transfer (Humphrey et al., 1991; Miyamoto et al., 1998). In horizontal transfer, the transfer of *Salmonella* occurs post completion of formation of egg shell, rather than

prior to it, as in vertical transfer (Miyamoto et al., 1998). Horizontal transmission was enhanced, and in some case initiated by the presence of water outside egg shell (Pardon, 1990). Although, the presence of water, in the form of stagnant, infected water or contaminated feces on egg shell, may be accounted for as major sources of contamination, but it is not mandatory for shell egg contamination. This is because *Salmonella* are motile bacteria, which could use their flagella for movement and thus entry into pores of eggs (Cantor and McFarlane, 1948; Padron, 1990; Hutchinson et al., 2003).

The most prevalent source of contamination in horizontal transfer is through the eggshell, immediately after oviposition (Fromm, 1959; Board, 1966; Miyamoto et al., 1998). Two factors seen to contribute to the contamination of eggs after oviposition (Hutchinson et al., 2003) are:

a) For a short time after oviposition cuticle is moist, and not completely formed.
b) Natural cooling of egg after being laid, causes generation of a negative pressure.

As described in Section 1.1.3.2, the cuticle does not attain maturity as soon as egg is laid, and this gap of a few hours, between the egg being laid and cuticle maturity, is a primary time for contamination of eggs, through uncovered pores (Sparks and Board et al., 1985; Miyamoto et al., 1998). After oviposition (process of laying of eggs), eggs are at approximately 42°C, which is higher than room temperature. This fall in temperature causes contents within the egg shell to shrink, causing a void to be formed between shell membranes and inner egg shell constituents, thus building a negative pressure within the egg (Miyamoto et al., 1998; Hutchinson et al., 2003). To

equalize this pressure difference, egg pulls in water or air from the medium surrounding egg, into the shell (Haines and Morgan, 1940). The influx of water may also cause inflow of bacteria, along with water. This contaminated water may cause pore canals to flood and pull in water until there is space in the egg (air sac) to accommodate water) (Board, 1980).

1.3. Salmonellosis Outbreaks Associated with Shell Eggs

Over 2,300 serotypes of *Salmonella* have been recognized, of which Enteritidis serotype has been identified as the causal organism for over one tenth of cases reported of food-borne illnesses (Cao et al., 2009). In shell eggs and egg products alone, *Salmonella* Enteritidis is recognized as one of the main causes of food-borne outbreaks. Over 60% of reported salmonellosis cases due to contaminated eggs reported by the Centers for Disease Control and Prevention (CDC) have been attributed to this microorganism (Cao et al., 2009; Vaninni et al., 2009; CDC, 2013). *Salmonella* Enteritidis finds its way to the egg shell from the oviduct because of *Salmonella* infection of the hen's reproductive organs or by exposure to a contaminated environment (Gole et al., 2014; Park et al., 2005). In 2010, a multistate outbreak of *Salmonella* Enteritidis was linked to shell eggs. This outbreak resulted in a total of 3,578 reported cases (CDC, 2010) and a nationwide recall of more than 500 million eggs (FDA, 2010). More recently, (March 2016), an outbreak attributed to *Salmonella* Enteritidis in Western Ohio (77 cases) triggered another recall of shell eggs (ODA, 2016).

1.4. Problems Associated with Egg Washing in the Industry

In the European Union, washing of Grade-A eggs (shell eggs sold to customers in a marketplace) is strictly prohibited (Hutchinson et al., 2003; Stolz et al., 2015). This is due to the fact that the EU has faced outbreaks of salmonellosis in the past, when cuticle of eggs was removed due to washing of eggs (Stolz et al., 2015). However, countries like United States of America, Japan, and Australia are willing to take this risk of cuticle removal by egg washing, and mandate egg washing prior being brought into the market place for sale. This was done as a preventive measure to eliminate cross-contamination by *Salmonella* (Hutchinson et al., 2003).

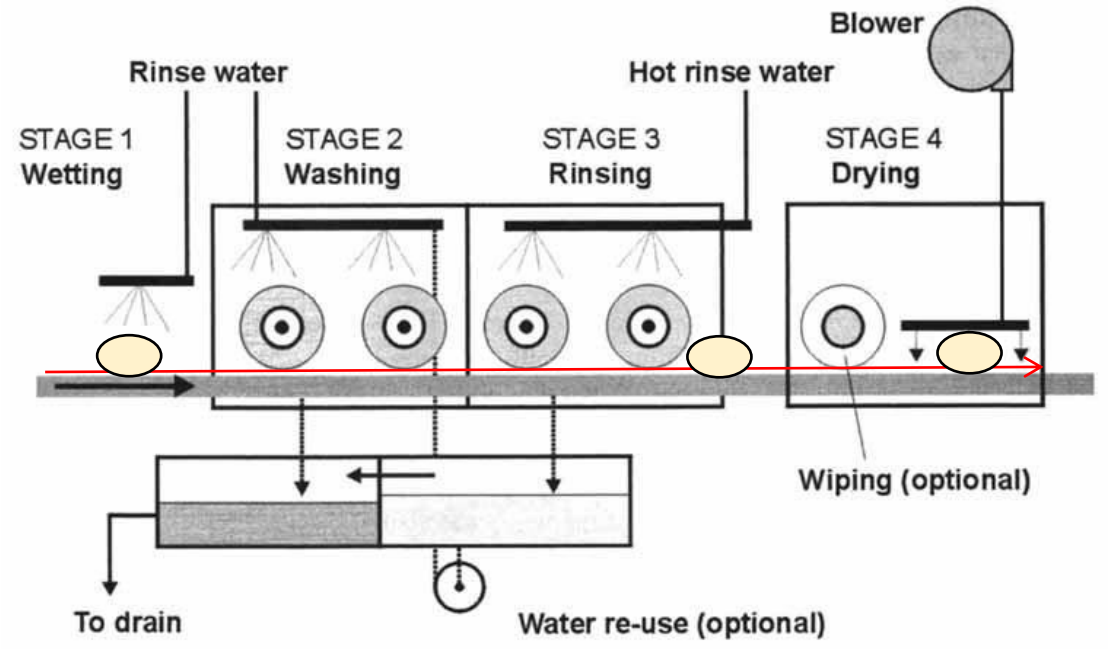


Figure 5: Typical egg washing set-up used in the industry (Source: Hutchinson et al., 2003) (the red arrow represents path followed by eggs in this washing set-up)

Figure 5 demonstrates a typical egg washing line approved by the USDA, involving 4 major steps (Hutchinson et al., 2003) –

a) Wetting of eggs with water and brushing to remove debris.

b) Washing of eggs with water and sanitizer (and/or detergent). This step may also involve mechanical brushing of eggs.
c) Rinsing of sanitizer (and/or detergent) off the surface of eggs.
d) Drying of eggs with a blower (this step may also on occasion contain a wiping step, to remove excess water before drying).

It has been determined that one of the major problems of the egg washing industry is the removal of cuticle layer during the washing process. One of the primary causes for cuticle removal has been associated with high chlorine in sanitizers and detergents used in egg washing (Bialka et al., 2004). Thus, research on non-chlorine based sanitizers is gaining importance (Davies and Breslin, 2002; Russell, 2003). Another concern with the use of sanitizers such as quaternary ammonium (QA), sodium hydroxide, phenol and formaldehyde is that they may deposit residues on the shell egg, further etching away the cuticle layer (Turtoi et al., 2014).
Ball et al. (1975) studied loss of cuticle in part, due to mechanical brushing of egg shells, and observed brush marks on shells. This proved that abrasion too has a role to play in cuticle damage. Additionally, sometimes the wash water used in the industry is re-used without effective treatment (as shown in Fig. 5), leading to cases of cross-contamination of a fresh batch of eggs washed with recycled batch of water (Board, 1980; Hutchinson et al., 2003).

1.5. Egg Washing Techniques Explored

Some techniques explored in the past include UV and ozone treatment of surface of eggs, to eliminate pathogenic microorganism. However, it was found that use of UV

alone, could not eliminate bacteria. Also, the use of ozone alone may not be effective, since there is reduction in ozone activity, with increase in organic load (Rodriguez-Romo and Yousef, 2005). Chlorine and other chlorine based sanitizers have been used effectively in the industry, but the use of these sanitizers is purely due to low cost and high efficacy. These sanitizers still pose a threat to the environment by causing chlorine toxicity in soil, as well as tend to leave residues on the surface (Cao et al., 2008).

More recently, acid electrolyzed water has been used in succession with basic electrolyzed water for treatment of egg surface (Park et al., 2005). Acid electrolyzed water and basic electrolyzed water constitute the acidic and basic components of water, after it is subjected to electrolysis. In a similar study by Bialka et al. (2004), the process of washing with basic electrolyzed water and acid electrolyzed water lasted up to 6 min for maximum reduction of *S. Enteritidis.* However, this process is longer than the processing time feasible by the industry, which is typically about 3 min. Also, in a typical egg washing line, reduction of ≥5 log of *Salmonella* Enteritidis is expected from the sanitizer (USDA, 2008), however, EO water produced a maximum reduction of only 2.98 log.

Techniques that do not use chemical sanitizers, but instead use mechanical and physical processes have also been studied. The use of pasteurization of eggs with infrared exposure and hot air and water have been proven to be effective against *Salmonella sp.*, but have been shown to cause damage to egg shells, such as causing cracks on shells (Himathongkham et al., 1999).

Thermal techniques such as those described above are uncommon when it comes to egg washing, since they disrupt rheological properties of the egg albumen, as well as

compromise the sensory characteristics of eggs (Kiosseoglou, and Paraskevopoulou, 2005). Thus, the use of non-thermal techniques is more popular in egg washing. Pulsed light is a non-thermal technique studied for decontamination of egg surface among, other techniques such as gas plasma and ionizing radiation. Although pulsed light is a non-thermal technique, the temperature of egg shells could be above the measured temperature of egg surface after light flash (4.2±0.2 °C). It was hypothesized that the temperature attained by the surface of egg shell at the exact point of light flash might cause structural alteration in proteins, and may cause undesired rheological changes to egg white (Lasagabaster et al., 2011).

Another non-thermal technique that had shown promise entailed exposure of eggs to atmospheric gaseous plasma, which caused reduction in pathogenic bacteria, without affecting shell strength and cuticle layer on the eggs shell (Ragni et al., 2010). However, the utilization of this technique would entail the industry to use processing times of up to 90 minutes, which would not be feasible at a large scale. Though chemical methods have shown to reduce microbial load on shell eggs, damage to cuticle increases chances of contamination during handling, storage and transport. Thus, it is essential to find novel methods to inactivate pathogenic bacteria while causing minimal damage to cuticle of egg after washing.

Table 1: Techniques explored for disinfection of eggs

Serial Number	**Technology explored**	**Microorganism was tested against**	**Maximum Reduction achieved**	**Reference**

1	Pulsed light technology	S. enterica subsp. enterica serovar Typhimurium CECT 4156	5 log CFU/ eggshell	Lasagabaster et al. (2011)
2	Radical Water Biocide	S. enteritidis PT4	(Information not provided)	Davies and Breslin (2002)
3	Protecta II (Natural herb extract)	S. enteritidis PT4	(Information not provided)	Davies and Breslin (2002)
4	Gas plasma	S. enteritidis PT4	(Information not provided)	Davies and Breslin (2002)
5	Ozone	S. enteritidis PT4	(Information not provided)	Davies and Breslin (2002)
6	Electrolyzed oxidative water (EO water)	*Salmonella* Enteritidis; *Escherichia coli*	2.6 log10 CFU/g; 2.6 log10 CFU/g	Bialka et al. (2004)
7	Electrolyzed oxidative water (EO water)	*Listeria monocytogenes; Staphylococcus Aureus Salmonella typhimurium*	>1 log10 CFU/ml; >3 log 10 CFU/ml; >4 log10 CFU/ml	Russell (2003)
8	Ionizing	(Not mentioned	(Not mentioned)	Pinto et al.

	Radiation	)		(2004)
9	Ultra violet light sanitation	*Salmonella typhimurium; Escherchia coli*	4 log 10 CFU/egg; 5 log 10 CFU/egg	Coufal et al. (2003)
10	Chlorine dioxide	*Salmonella enterica*	2.6 log 10 CFU/egg	Choi et al. (2015)
11	Ultraviolet light treatment followed by ozone	*Salmonella* Enteritidis	4.6 log CFU/g	Rodriues-Romo and Yousef, (2004)
12	Non-thermal atmospheric pressure plasma	*Salmonella* Enteritidis	2.4 log10 CFU/egg	Stolz et al., (2015)
13	Flumisol (antibiotic);	Coliform and aerobic plate count	<1 log CFU/egg	Kaudia, (1999)
14	Quaternary ammonia	Coliform and aerobic plate count	1.8 log CFU/egg	Kaudia, (1999)
15	Formaldehyde	Coliform and aerobic plate count	<1 log CFU/egg	Kaudia, (1999)
16	Ultraviolet light	*Escherichia coli*	4 log CFU/eggshell	De Reu et al.,

	treatment	and *Staphylococcus aureus*		(2006b)
17	Non-thermal atmospheric gas plasma	*Salmonella Enteritidis and Salmonella Typhimurium*	4.5 log CFU/eggshell 3.5 CFU/eggshell	Ragni et al., (2010)

1.6. Acid Electrolyzed Water (AEW)

1.6.1. Generation of AEW

Water with dissolved salts, both naturally occurring and dissolved artificially, when split by electrophoresis into an acidic component (pH 2-3) and a basic component (pH 10-13) is known as electrolysis of water. Amongst the dissociated solutions, the acidic component is known by the name acid electrolyzed water (AEW) and its basic counterpart is known as basic electrolyzed water (BEW) (Hricova et al., 2008).

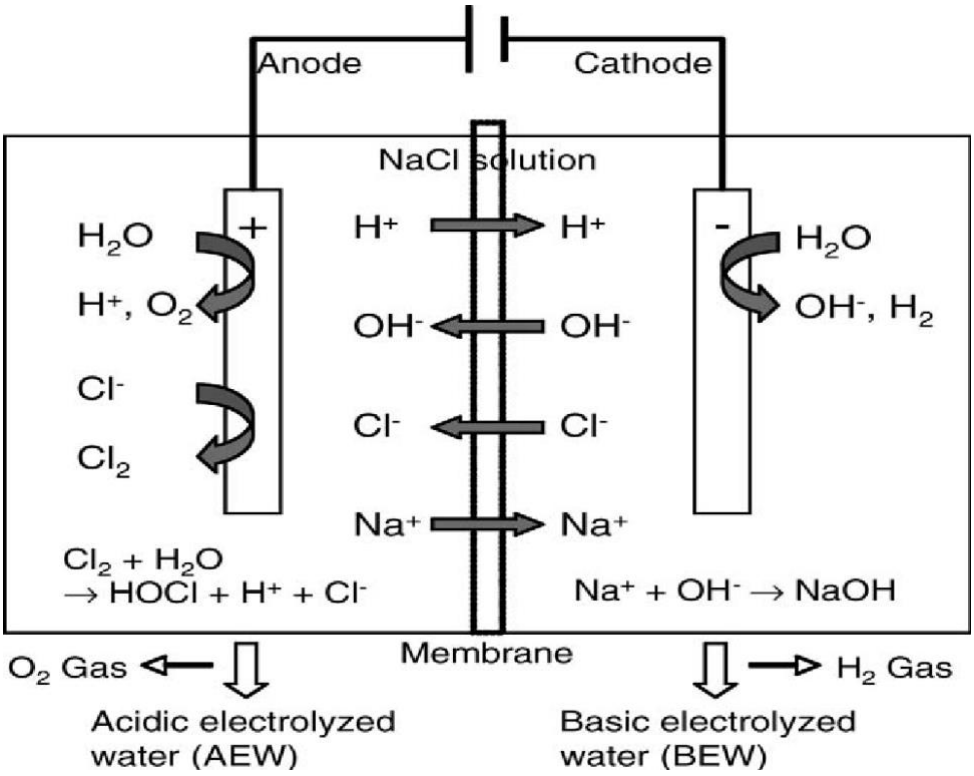


Figure 6: Schematic diagram explaining the splitting of water into anions and cations, to form AEW and BEW during the process of electrolysis (Source: Hricova et al., 2008)

As shown in the figure above (Fig. 6), water with dissolved salts (NaCl, KCl), when subjected to a voltage of 9-10 V, within the confine of an electrolytic cell, dissociates into positively and negatively charged ions (Al Haq et al., 2005).

The chemical reaction occurring at the cathode could be summarized as (Huang et al., 2008) -

$$2H_2O \rightarrow 4H^+ + O_2 \uparrow + 4e^-$$
$$2NaCl \rightarrow 2Na^+ + Cl_2 \uparrow + 2e^-$$
$$Cl_2 + H_2O \rightarrow HCl + HOCl$$

The chemical reaction occurring at the anode could be summarized as (Huang et al., 2008) -

$$2H_2O + 2e^- \rightarrow 2OH^- + H_2 \uparrow$$
$$NaCl + OH^- \rightarrow NaOH + Cl^-$$

The reactions occurring within the electrolysis cell show that the negative ions collect at the anode, and after releasing oxygen into the atmosphere, form an acidic solution (AEW) with pH 2-3. Conversely, on the cathode side of the membrane, cations collect to absorb electrons and form an alkaline solution (BEW), with the release of hydrogen gas (Hricova et al., 2008).

1.6.2. Mechanism of Inactivation of Microorganisms and Application of AEW

Yoshida et al. (2004) studied the rise in popularity in usage of AEW in the food industry in Japan. The mechanism of inactivation of microorganisms by AEW has been studied to reveal that multiple factors contribute to anti-microbial activity of the solution (Hricova et al., 2008). The most important factors that contribute towards antimicrobial property are low pH, presence of chlorine ions, and high oxidation reduction potential (ORP) (Al Haq et al., 2005). Although debatable, it has been hypothesized by Park et al. (2005) that acidic character of AEW makes bacteria susceptible to active chlorine compounds, thus easing the entry of hypochlorous acid (HOCl) into bacterial cells. Active chlorine may contribute by eroding cell membranes of bacteria (Koseki et al., 2000; Hricova et al., 2008). Additionally, the high ORP (~ 1000 mV) values of AEW add to the antimicrobial effect by increasing oxidation capability of the solution (Al Haq et al., 2005). These properties have been exploited in using AEW as a disinfectant (Hricova et al., 2008). Table 2 highlights some of the food and non-food applications of AEW.

Table 2: Application of AEW in food and non-food based products

Serial no.	Application	Reference
Water Sanitization		
1.	Water treatment	Krivobok et al., 1982
2.	Water decontamination to generate drinking water	Kunina, 1967
Medical procedures and institutions		
3.	Sanitization of hands prior to surgical procedure	Nikitin et al., 1965
4.	Disinfection of hospitals and other therapeutic institutions	Nikulin et al., 1977
Food contact surface sanitization		
5.	Sanitization of plastic cutting boards	Venkitanarayanan et al., 1999
6.	Sanitization of stainless steel surfaces and cutting boards	Chiu et al., 2006
Disinfection of food surfaces		
7.	Reduction of naturally occurring bacteria and fungi from surface of strawberries	Koseki et al., 2004
8.	Reduction of *V. parahaemolyticus* from skin of tilapia	Huang et al., 2006
9.	Spray washing of chicken to reduce total aerobic bacteria and coliform	Fabrizio et al., 2002

	bacteria	
10.	Reduction of pathogenic microorganisms from the surface of eggs	Russell, 2003

1.7. Cold Atmospheric Pressure Plasma

1.7.1. Plasma Definition and Types

When discovered, plasma was considered as a suspension of ions, electrons and neutrally charged species in fluid gaseous agent (Langmuir, 1928; Misra et al., 2011). Further research showed absence of a fluid gaseous medium, instead a neutral, but ionized gas, thus causing plasma to gain popularity as the 'fourth state of matter' following solid, liquid, and gaseous states (Moreau et al., 2008; Misra et al., 2011). Plasma is said to be partially or completely ionized mixture of gases, comprising photons, positive and negative ions, electrons, free radicals and atoms, such that all existing charges balance out to produce a neutral state of matter (Moreau et al., 2008; Kudra and Majumdar, 2009; Misra et al., 2011).

Plasma could be divided into two prominent categories, based on condition of generation -

a) Thermal plasma (TP) - Generation of TP is done at pressures maintained >1 atm, creating a discharge in which all species are in thermodynamic equilibrium. The temperature of ionized gases is uniform throughout and is of the order of 10^4 K. Some common examples of TP would be plasma torch and electric arc discharge plasma (Moreau et al., 2008).

b) Non-thermal plasma (NTP)- Plasma creation occurs at lower pressures (~ 1 atm), however, 'light' species (electrons and photons) and 'heavy' species (other components of plasma with the exception of electrons and photons, such as neutrals and ions) are not in thermodynamic equilibrium. Thus, there exists a range of temperatures in the discharge, such that heavy species are at temperatures close to room temperature and the light species have temperatures in the range of 10^4 K. Some common examples of NTP would be plasma jet, dielectric barrier discharge plasma (DBD plasma), corona discharge, and microwave discharge plasma (Misra et al., 2011; Surowsky et al., 2015). NTP is more economically and practically viable from an industrial stand point, since it could be generated at atmospheric pressure (Moreau et al., 2008).

1.7.2. Atmospheric Pressure Plasma Jets (APPJ)

This study used atmospheric pressure plasma jet system to generate NTP. APPJ is seen to produce jets of plasma, otherwise known by the term 'plasma flames' (Surowsky et al., 2015). These flames are produced in the RF (radio frequency) range of 13.56 MHz or 27.12 MHz by allowing gas to pass through closely placed electrodes (order of 10^{-3} m) (Ehlbeck et al., 2010; Surowsky et al., 2015).

Typical APPJ systems contain two electrodes (for example ring and needle electrodes), but single-electrode operations have also been identified, consisting of simulated grounded electrode (Surowsky et al., 2015). Figure 7 shows the various modes of operation of electrodes on APPJ. The most popular choices of gases are

noble gases such as helium and argon, although, a mixture of compressed atmospheric gases has also been observed to produce APPJ (Ehlbeck et al., 2010).

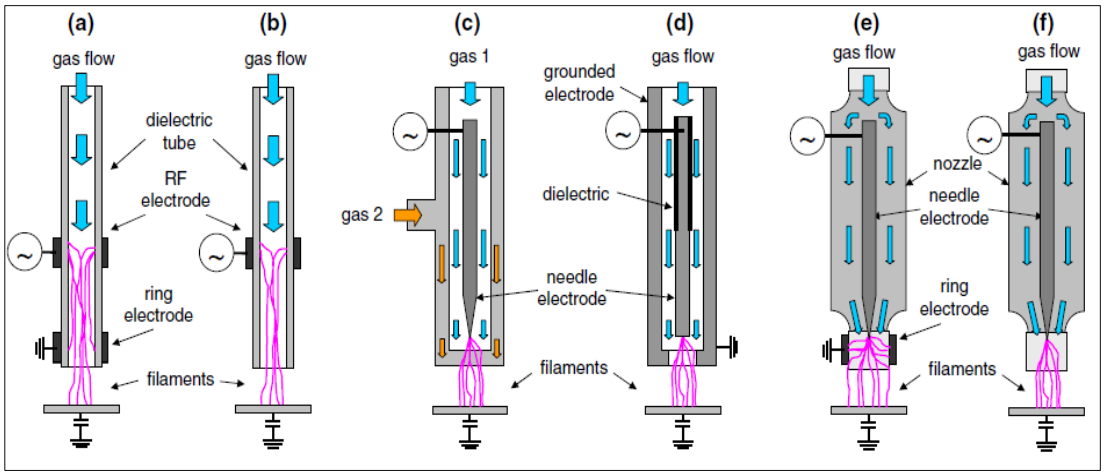


Figure 7: Schematic diagram showing various designs of electrode setup in APPJ equipment (Source: Ehlbeck et al., 2010).

1.8. Plasma Activated Water

1.8.1. Plasma-liquid interaction

Plasma, when in contact with water molecules, causes water to undergo dissociation or activation, to form plasma activated water or PAW. These dissociation reactions may follow 4 typical reaction types (Surowsky et al., 2015)–

a) Acid-base reactions
b) Oxidation reactions
c) Reduction reactions
d) Photochemical reactions

a) Acid-base reactions – These reactions could be caused by plasma secondary species such as nitrous and nitric acids, excited nitrogen species, and their products

(NO_x). Additionally, ionization of water, formation of hydrogen ions and hydrogen peroxide lead to decrease in pH of PAW (described in reaction given below) (Surowsky et al., 2015).

$$H_2O + e^- \rightarrow H_2O^+ + 2e^-$$

$$O^+ + 3H_2O \rightarrow H_3O^+ + 3OH$$

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

b) Oxidation reactions – Reactive oxygen and nitrogen species initiate one of the most significant reactions in plasma –liquid interactions, that is oxidation reactions. This reaction is responsible for the antimicrobial effect of PAW. The source of ROS in water is $OH^{\cdot}$, which may combine with other $OH^{\cdot}$ to form hydrogen peroxide. Hydrogen peroxide acts by entering the cell and disrupting DNA (Surowsky et al., 2015).

$$H^{\cdot} + OH^{\cdot} \rightarrow H_2O$$

$$OH^{\cdot} + OH^{\cdot} \rightarrow H_2O_2$$

Ozone also acts as a contributing factor to formation of ROS, and is generated by combination of molecular oxygen and atomic oxygen (Surowsky et al., 2015; Liu et al., 2016).

$$O + O_2 \leftrightarrow O_3$$

RNS or reactive nitrogen species contribute to formation of peroxynitrite radicals that also contribute to the low pH of PAW (Surowsky et al., 2015).

$$O_2^- + NO^{\cdot} \rightarrow (O = N - OO^{-\cdot})$$

$$NO_2^- + H_2O_2 \rightarrow (O = N - OO^{-\cdot}) + H_2O$$

$$OH^{\cdot} + NO_2 \rightarrow (O = N - OO^{-\cdot}) + H^+$$

c) Reduction reactions – They are initiated by reductive species such as $OH^{\cdot}$, $H^{\cdot}$ and (superoxide radicals) $O_2^{-\cdot}$, and occur simultaneously with oxidation reactions during plasma-liquid interaction. They undergo hydrogen addition and abstraction upon combination with organic compounds (Surowsky et al., 2015).

d) Photochemical reactions – These reactions occur due to the presence of photons in the UV-visible range. However, this reaction occurs in vacuum and not under atmospheric conditions (Zvereva, 2010; Surowsky et al., 2015).

$$H_2O + h\nu \rightarrow H^{\cdot} + OH^{\cdot}$$

$$O_2 + h\nu \rightarrow O^{\cdot} + O^{\cdot}$$

1.8.2. Modes of Generation of PAW

PAW could be generated by two means (as shown in Fig. 8) –

a) indirect exposure
b) direct exposure

Indirect exposure (PAW-A) entails the nozzle (outlet of plasma jet) to be placed away from surface of water, so as to establish no physical contact between plasma jet and water. Contrarily, direct exposure to create plasma activated water-B or PAW-B is formed by immersing plasma microjet nozzle into water, so as to establish direct contact with water which is being activated (Tian et al., 2015).

Figure 8: Modes of generation of PAW: PAW-A is formed by indirect exposure to plasma jet and PAW-B is formed by direct exposure to plasma (Source: Tian et al., 2015).

1.8.3. Mechanism of Inactivation by PAW

Tian et al. (2015) studied that direct exposure of bacterial suspension in water to plasma (PAW-B, as shown in Fig. 8) caused a higher amount of nucleic acids (DNA and RNA) to be leaked from *S. aureus* cells into water than indirect exposure to plasma jet. This study indicated that the mechanism of inactivation in PAW involves rupture of cell membranes, causing DNA/RNA to ooze out of the cell, in turn causing cell death. Further, Zhang et al. (2013) confirmed that the means of inactivation by PAW was by reactive oxygen species (ROS), which penetrated cell membrane of *S. aureus* and damaged the structure of the bacterial cell. Hence, ORP values could be used in order to determine microbial inactivation efficacy of PAW.

ROS could contribute to microbial inactivation by creating oxidative-stress in cells and causing degradation of sugars in macromolecules such as DNA. Lipid

peroxidation (as described in the series of reactions given below, where L denotes lipid molecule) may lead to depletion of phospholipids in the cell membrane. Lipid peroxidation results in shorter fatty acids, which cause a loss in structural integrity of the cell membrane. This is a chain reaction that occurs in the presence of $OH^{\cdot}$ (hydroxide radicals) in three steps, namely, initiation, propagation and termination (Gaunt et al., 2006).

$$L + OH^{\cdot} \rightarrow L^{\cdot} + H_2O$$

$$L^{\cdot} + O_2 \rightarrow L - OO^{\cdot}$$

$$L - OO^{\cdot} + L \rightarrow L^{\cdot} + LOOH$$

Chain reaction

$$LOOH \xrightarrow{Fe\ or\ Cu} L - O^{\cdot}$$

RNS and hydrogen peroxide have also been seen to contribute to microbial inactivation. Peroxynitrite ($O = N - OO^{-\cdot}$) dissociates into reactive hydroxide radicals ($OH^{\cdot}$) and reactive nitrogen dioxide radicals ($NO_2{}^{\cdot}$), that may in turn contribute to initiation of lipid peroxidation. Low pH could be attributed to the production of nitrous acid and nitric acid in PAW, thus allowing activation of ROS and RNS in water (Sarowsky et al., 2015).

1.8.4. Current Research in PAW

PAW was identified and studied by Kamgang and Youbi (2008), when they were analyzing the effects of a suspension of *Hafnia alvei* under plasma generated by gliding arc technology. It was seen that a maximum of 5 log CFU/ml reduction was observed upon exposure of the suspension for 20 minutes. A similar study was performed by the same group (Kamgang and Youbi, 2009) to verify their results with other

microorganisms, and a higher reduction was obtained in *Staphylococcus epidermis* and *Leuconstac mesenteroides.*

Comparable results were obtained by Taylor et al. (2011), where air was used a feed in a (dielectric barrier discharge) DBD setup. *Esterichia coli* suspension was exposed to PAW for 15 minutes, to obtain close to 5.6 log CFU/ml reduction. Joshi (2017) have explored the use of PAW in washing of fruits, with varying surface roughness. The study showed the maximum microbial inactivation efficacy of PAW to be 4.65 ± 1.34 log CFU/surface and that surface roughness did not play a role in microbial inactivation efficacy of PAW.

1.9. Rationale, Hypothesis and Objectives

Salmonella Enteritidis has been recognized as the primary cause of Salmonellosis associated with shell eggs and egg products, associated with 60% of reported cases (CDC, 2013). Chemical sanitizers currently used may have an undesirable environment impact and can cause degradation of the egg cuticle, a natural defense mechanism against bacterial penetration into eggs (Bialka et al., 2004).

PAW has been used in the past to treat fruit surfaces, and in suspension to obtain microbial reduction (Joshi, 2017; Kamgang and Youbi, 2008). In light of the current need for a non-chlorine based sanitizer to wash eggs with, PAW with its proven antimicrobial efficacy (as seen in Section 1.8.3), may possess the characteristics to be used in the egg washing industry. This may minimize loss of cuticle; maintain egg shell strength; all the while not compromising on microbial safety of eggs and wash water.

The overall aim of this study was to use plasma activated water as a novel sanitizer to replace currently used chemical sanitizers in egg washing, to inactivate bacteria on shell egg surface, with minimal loss to cuticle presence and egg shell integrity. The specific objectives were:

a) To assess the efficacy of PAW as a novel sanitizer on the inactivation of *Enterobacter aerogenes* on surface of eggs and compare it to conventional methods (quaternary ammonium sanitizer).
b) To evaluate the effect of PAW on cuticle of farm fresh shell eggs
c) To measure the effects of PAW on the structural integrity of washed eggs

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Shell Eggs

Farm fresh, white shell eggs were obtained from the poultry farm facility at the Department of Animal Sciences (Rutgers University, New Brunswick, NJ). Five leghorn chickens were raised and maintained on an identical diet, for the duration of this research study. The reasons behind maintaining a flock of hen for our study were - it is illegal to sell unwashed/unclean eggs in the marketplace, in the United States, and to maintain uniformity as much as possible, in eggs used for this study. The laying cycle of each leghorn chicken was typically 5 eggs per week. Unfertilized eggs from chicken were collected every day, and immediately transferred to a refrigerator (maintained at ~ 4°C), for storage. Eggs in storage were kept unwashed, until the time of experimentation, in order to maintain natural micro-flora on the shell, as well as to avoid damage to the cuticle layer.

2.1.2. Bacterial Culture

Nalidixic acid resistant *Enterobacter aerogenes* B 199A (VivolacCultures, Indianapolis, Indiana, USA) was used as a non-pathogenic surrogate for *Salmonella* spp. *E. aerogenes*, a gram negative bacterium (Liu and Schaffner, 2007), has been shown to possess similar attachment properties to *Salmonella* spp., (Zhao et al., 1998). The strain has been used in past studies to study cross-contamination, as a surrogate of *Salmonella* spp. (Chen et al., 2001). The presence of nalidixic acid resistant gene in *E. areogenes* enables the bacterium to be cultured and enumerated in a medium with

nalidixic acid, despite the presence of background microorganisms (Chen et al., 2001).

2.1.3. Culture Media And Solutions

2.1.3.1. Tryptic Soy Broth

Tryptic Soy Broth (TSB) was used in order to culture *E. aerogenes* overnight. It was prepared by adding 2.95 g of Bacto™ Tryptic Soy Broth (Soybean- Casein Digest Medium, Becton, Dickinson & Company, USA) to 100 ml of distilled water, and mixing at 200 °F for approximately 15 minutes. After mixing, the broth was autoclaved at 121 °C, for 15 minutes, and allowed to cool down to 55 °C. To this cooled broth, 0.1 ml of nalidixic acid, at a concentration of 50 µg/ml was added, and allowed to mix for 2 minutes, before being used or stored at 4°C.

2.1.3.2. Glycerol Stock Solution

Eighty per cent glycerol stock solution was prepared by adding 80 ml of glycerol to 20 ml of distilled water, which was then autoclaved at 121 °C, for 15 minutes and allowed to cool to room temperature. Equal proportions of bacterial culture grown in TSB (at 37 °C for 18 h) and 80% glycerol solution are added, and this mixture was stored in a sterile, screw-cap microcentrifuge tube, at -80 °C.

2.1.3.3. Nalidixic Acid

Half gram of Nalidixic acid (Fisher Bioreagents, USA) was added to a 10 ml solution made of 20% 10N Sodium hydroxide (Fisher Science Education, USA) and 80% distilled water, in a sterile 15 ml Falcon™ tubes. The Nalidixic acid was mixed

thoroughly to ensure complete dissolution, and filtered through a 0.22 µm sterile disc filter with MF-Millipore™ MCE Membrane (Millex® -GS) (Merck Millipore Ltd., Ireland).

2.1.3.4. Tryptic Soy Agar

Difco Tryptic Soy Agar (TSA), obtained from Becton, Dickinson & Company, USA, was prepared my adding 40 g of TSA to 1 liter of distilled water, and autoclaving at 121 °C for 15 min. This was then poured into 20 ml sterile plates and allowed to set overnight, before storing at 4 °C.

2.1.3.5. Peptone Water

In order to make peptone water, 1.5 g of Peptone (Becton, Dickinson & Company, USA) was dissolved in 1 liter of distilled water. This was then divided into 9 ml aliquots of the first part were poured into glass test tubes, and the rest was split into 100 ml aliquots, poured into 200 ml glass bottles. Aliquots, in both test tubes, as well as in bottles were autoclaved at 121 °C for 15 min.

2.1.4. Quaternary Ammonia Sanitizer

Quaternary ammonium (QA) sanitizing solution (Zep Food Division, Georgia, USA) at 100 ppm was used in this study, as an industrial standard; to compare the effects of plasma activated water on microbial quality, cuticle presence and strength of egg shell. QA is an industrially used sanitizer in egg washing, to remove organic debris from egg shells, as well as to inactivate bacteria that reside on surface of eggs.

2.1.5. Cuticle Blue Dye

Cuticle Blue (MS Technologies, Kettering, UK) is a cuticle sensitive dye, that attaches only to the cuticle on egg shells, that delivers a green color to portions of the egg shell with cuticle coverage. This solution was stored in a plastic container, at room temperature and re-used as desired.

2.1.6. Instruments Used in this Study

2.1.6.1. CAPP Equipment

Cold Atmospheric Pressure Plasma or CAPP (Plasma Treat Inc., IL, USA) jet, shown in Fig. 9, was used in this study. CAPP equipment operated on an Openair ™ Plasmajet technology, where plasma was released through a rotating nozzle (RD1004), into the discharge chamber. The plasma generator (FG5001) operated at 295 V at a frequency of 22.5 kHz and at an air pressure of 1990 mBar.

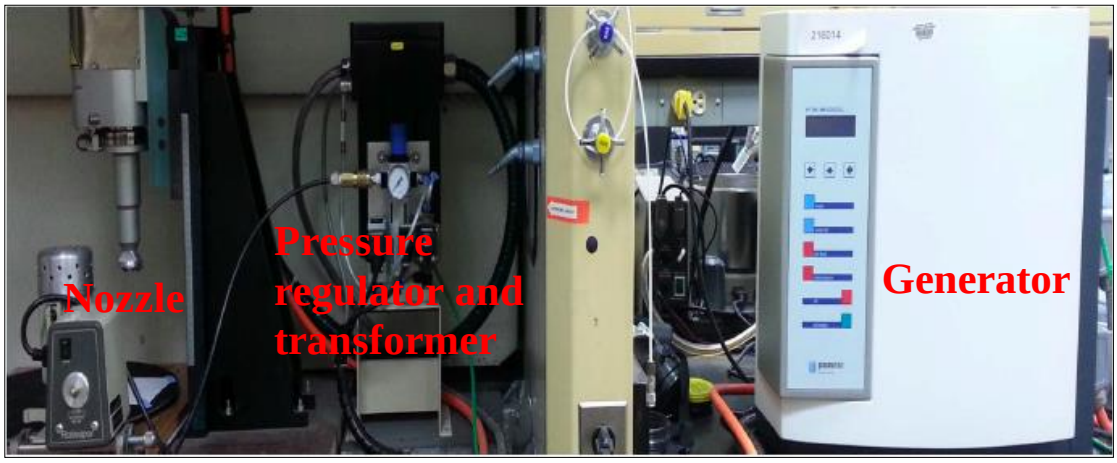


Figure 9: Cold Atmospheric Plasma Processing Unit (Plasma Treat Inc., Elgin, IL) for generation of PAW at the Department of Food Science, Rutgers University.

High voltage is maintained between the stator and rotor of the plasma jet motor, which enables plasma to be discharged through rotating nozzle, using working gas.

Rotating nozzle is equipped to ensure equal distribution of plasma throughout the exposed surface. The height of the nozzle could be adjusted in order to obtain desired temperature at the target surface; in this case, temperature of plasma activated water. Feed gas or working gas used in production of plasma in this study, was compressed, dry, filtered air.

2.1.6.2. Water Ionizer

Water ionizer was used to produce acid electrolyzed water, which was tested for its efficacy is microbial inactivation after exposure to plasma. Bawell Platinum Water Ionizer (Model 2195), shown in Fig. 10, utilized in this study, was equipped with a dual internal filtration system, which allowed the water to be filtered prior to ionization. The ionizer was fitted to a supply of tap water, which was filtered and subject to electrophoresis. The filtration system was fitted with Granular Activated Carbon (GAC), which aided in eliminating sand, dust, and some forms of molecular fungi that may be present in water. The second stage of filtration occurred through a column composed of Pre-Activated Carbon (PAC) which enabled removal of components such as chlorine, Volatile organic compounds and heavy metals. Filtered water was then electrolyzed to form alkaline and acid electrolyzed water.

Figure 10: Ionized Water System (Bawell Platinum Water Ionizer (Model 2195), Boca Raton, FL) for generation of AEW and BEW at the Department of Food Science, Rutgers University

This machine operated at 8 programmed levels, the levels being-

- Weak alkaline
- Medium alkaline
- Strong alkaline
- Ultra-strong alkaline
- Purified water
- Weak acidic
- Strong acidic

Each setting varied in pH and ORP, which ranged from 3.0 to 11.5 and +786 RmV to -800 RmV, respectively. Strong acidic water was used in this study as acid electrolyzed

water (AEW), which was then exposed to plasma jet, to make plasma activated acid electrolyzed water (PAEW) (explained in section 2.2.2).

2.1.6.3. Colorimeter

Konica-Minolta Colorimeter CR-410 (Fig. 11) was used to detect the color of egg shells, after staining with Cuticle Blue dye. The CIE lab scale was used to determine L*, a* and b* values, where L* values represented lightness/darkness of a substance, a* represented blue/green color while b* represented red/yellow color.

Figure 11: Konica Minolta CR-410 colorimeter

2.1.6.4. Texture Analyzer

The texture analyzer (CT3 Brookfield texture analyzer) used in this study was obtained from Brookfield AMTEK, USA. This analyzer was used in determining the effect of washing eggs with sanitizers on eggshell strength. The texture analyzer, shown in Fig. 12, was attached to an aluminum probe, and a curve depicting force needed to crack the egg shell was obtained against time needed to crack the egg shell.

Figure 12: CT3 Brookfield Texture Analyzer at the Department of Food Science, Rutgers University with custom designed aluminum probe and farm egg at the Small end up position.

2.1.6.5. Wide-field Microscope

Nikon® Wide-field Microscope of the TE-2000 series (Nikon Instruments Inc., NY, USA) was used to study the cuticle retention on eggshells. Stained egg shells both before and after treatment were studied under the inverted microscope, to obtain images which were later analyzed for the presence of cuticle layer. Nikon Elements ™ Version 4.50 (Nikon Instruments Inc., Melville, NY, USA) for obtaining magnified photographs, as well as for quantitative analysis of the images.

2.2. Methods

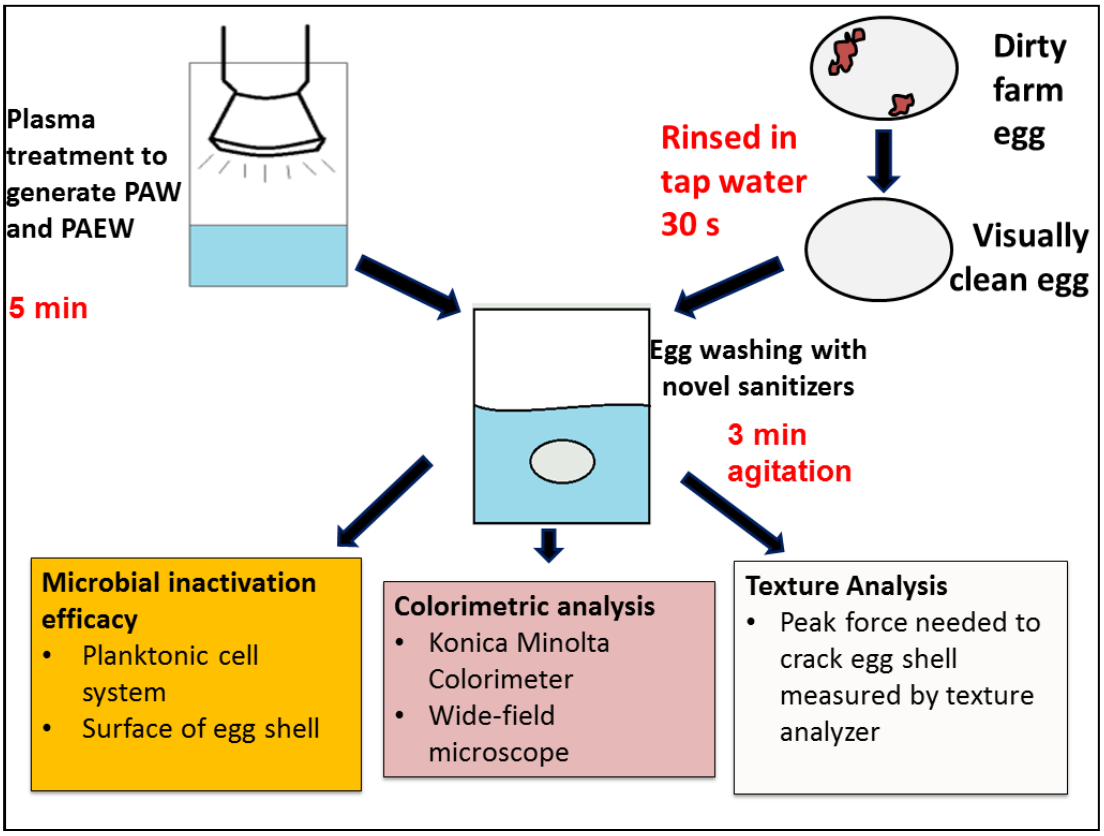


Figure 13: Flow chart of methodologies applied in this study

2.2.1. Optimization of Nozzle Height for Production of PAW and PAEW

USDA egg washing regulations require that the temperature of sanitizer or water used to wash eggs be maintained at least 20 °F (11.1 °C) above average temperature of the egg, but a difference of less than 40 °F (22.2 °C) must be maintained between egg and sanitizing solution (USDA, FSIS 2008). Additionally, temperature of the sanitizer was to be maintained higher than temperature of wash water. If the temperature of the sanitizer surrounding the egg was to be lower than the egg temperature, water and contaminants might enter the pores of eggs. This phenomenon is explained in Section 1.1.3.2. In case this sanitizer/water was contaminated, low temperature of sanitizers may directly lead to uptake of

contaminants such as bacteria, from this contaminated environment (Sparks and Board, 1984; Haines and Moran, 1941). In order to adhere to the USDA guidelines, the temperature of plasma activated water was maintained between 39 °C and 50 °C.

A narrow 1000 ml glass beaker was used in this study, and filled with 300 ml sterile distilled water. Height of plasma nozzle was determined to be at or above 7.7 cm above surface of target, in order to maintain the temperature of the sanitizer obtained, at or below 50 °C. Bhide (2016) studied the temperature of surfaces treated by plasma jet, at various distances of exposure. This was done by measuring the temperature of plasma treated surface with the help infrared thermometer. The study indicated that at a distance of 7.7 cm from the plasma jet nozzle, a maximum temperature of 50 °C was obtained.

In our study, various distances at and above 7.7 cm between were examined to obtain an optimum temperature of 50 °C, for plasma activated water. This study involved exposing 300 ml of water to plasma for 5 min (as shown in Fig. 13) and checking temperature of plasma activated water. Height of 7.7 cm above surface of water was used in further experiments to obtain the desired temperature.

2.2.2. Production of Plasma Activated Water (PAW) and Plasma Activated Acid Electrolyzed Water (PAEW)

Sterilized (autoclaved) distilled water (DW) was used in the production of PAW, in order to avoid contamination from water. Three hundred milliliters of sterile DW was taken in a 1000 ml sterile glass beaker, and exposed to plasma jet for 5 minutes. The final temperature of PAW was measured to be ~ 50 °C with the help of a

handheld laser thermometer. The pH of PAW was measured to be 2.95 ± 0.02 with a pH Benchtop Meter while the oxidation reduction potential was 575.8 RmV with the help of Metallic Combination Electrode.

pH measurements were performed with Orion Star™ A111 pH Benchtop Meter (Thermo Fisher Scientific, USA) equipped with Orion™ 9157BNMD Triode™ 3-in-1 pH/ATC Probe (Thermo Fisher Scientific, USA).

ORP or oxidation reduction potential was measured with the help of the same Orion Star™ A111 pH Benchtop Meter, connected to 9678BNWP Orion™ Metallic Combination Electrode (Redox/ ORP model with Epoxy body) probe. This probe was filled with 4 M KCl, with AgCl solution and calibrated to +220 RmV, using ORP standard solution (967901) (Thermo Fisher, USA).

Acid electrolyzed water was obtained from Bawell Platinum Water Ionizer (Model 2195) and the pH was noted to be 3.20 ± 0.1, with higher ORP as compared to PAW, at 786 RmV. AEW was then exposed to plasma jet for 5 minutes to make PAEW. The temperature of PAEW was recorded to be ~ 50 °C, similar to the temperature of PAW. The plasma jet nozzle was maintained at the same level as before (7.7 cm), in order to ensure consistency in temperature of product. However, the ORP value of AEW dropped to 576.1 RmV, upon exposure to plasma.

2.2.3. Microbiological Analysis

2.2.3.1. Bacterial Culture Preparation

Enterobacter aerogenes, stored at -80°C, was taken in a sterile plastic loop and was plated by streaking on to a sterile plate containing TSA with 50 µg/ml Nalidixic acid. This plate was stored at 37 °C for 21 h, to allow the bacterial growth. A single colony

of *E. aerogenes* was selected and inoculated in 30 ml TSB with 50 µg/ml Nalidixic acid (as discussed in Section 2.1.3.1 and 2.3.3.3), in a sterile Corning™ Falcon™ 50 mL Conical Centrifuge Tube. The inoculated culture was incubated at 37°C for 21 h. This culture was then centrifuged Sorvall™ 46 Legend™ X1 Centrifuge (Thermo Fisher Scientific, USA) at 4 °C, 5000G, for 10 min. The supernatant obtained after centrifugation was discarded and the pellet was washed with 30 ml peptone water (prepared as discussed in Section (2.1.3.5)). The pellet was re-suspended in peptone water by vortexing for about 10-15 s. This process of centrifugation, washing and re-suspension was repeated thrice, and the final re-suspension was in 10 ml of peptone, to obtain 10 ml stock solution.

The concentration of bacteria in the stock solution was enumerated by serial dilution and spread plating on TSA plates with 50 µg/ml Nalidixic acid. Initial bacterial concentration was determined to be (9.2±0.1) log CFU/ml.

2.2.3.2. Planktonic System

From the above mentioned re-suspended and washed culture, 100 µl was added to 150 ml of autoclaved Distilled water (DW), in a sterile Nasco™ Whirl-Pak® bag (18 oz). Autoclaved DW was used as a control in all experiments, since PAW was made from plasma exposure of autoclaved DW. Similarly, 100 µl of bacterial culture was added to 150 ml of sanitizing solutions, namely, PAW, AEW, PAEW, and Quaternary ammonia (QA), kept in sterile bags.

These bags were then agitated on a rotary shaker for 3 min, at 90 RPM after being placed in a 1 L Pyrex ®Griffin beakers. After treatment for 3 min, 1 ml of each

sample was serially diluted and plated in duplicates on TSA plates with 50 µg/ml Nalidixic acid. These plates were then incubated at 37 °C for 21 h and enumerated to determine survival of bacteria after treatment with sanitizers.

2.2.3.3. Egg System

2.2.3.3.1. Preliminary Study

2.2.3.3.1a. Native Background Micro Flora

Farm eggs were collected from the Rutgers New Jersey Agricultural Experiment Station (NJAES) Animal Care Program, at the Department of Animal Sciences, Rutgers University, New Brunswick, NJ, USA. The facility at Rutgers housed 5 leg horn chickens, whose laying cycle is described in Section 2.1.1. Unfertilized eggs were collected and stored at 4 °C as explained previously.

Prior to experimentation and analysis, eggs were removed from storage at 4 °C and washed in 1 L of tap water (kept in a tub) at room temperature, for about 20 s - 30 s. This was done in order to remove debris and visible dirt from the surface of eggs. Washing also caused for removal of background microbes, to some extent, in order to prevent contamination and interference in microbiological analysis of efficacy of sanitizers. Physically damaged eggs as well as excessively dirty eggs were discarded. Literature suggested sequential washing of eggs in tap water and in 70 % ethanol solution or with chlorine-based sanitizers, to completely eliminate background micro flora (Rodriguez-Romo and Yousef, 2004; Russell, 2003). The protocol followed in this study did not involve sanitizing eggs prior to experimentation, in order to mimic industrial egg washing as closely as possible.

In order to validate and enumerate the presence of background microorganisms, total aerobic plate count was performed. Unwashed eggs or dirty eggs (DE) obtained from the farm were individually transferred to sterile Whirl-Pak® bags containing 20 ml of peptone water. Washed eggs (washed in tap water as described above) or 'visually clean' eggs (VC) were also individually transferred into sterile Whirl-Pak® bags containing 20 ml of peptone water. Eggs were massaged in peptone water for 1 min, and 0.1 ml of peptone water from each bag was spread plated in duplicates on TSA plates, but without Nalidixic acid. Plates were incubated at 37 °C for 21 h and enumerated to determine presence of background micro flora both prior to and post washing in tap water.

2.2.3.3.1b. Optimization of Drying Time

Eggs were washed as described in Section 2.2.3.3.1 and allowed to dry in a laminar hood for 30 min. Dried eggs were placed in a plastic egg carton sanitized with 70% ethanol, dried under a laminar flow hood. Eggs were then transferred to a bio-safety cabinet and placed on sterile petri plates and a section of the egg was marked for spot inoculation. Hundred microliters of overnight, centrifuged culture was spot inoculated on surface of 9 eggs. Amongst these eggs, one egg was chosen in random and used as 0 min control, to determine the concentration of bacteria inoculated on the surface of eggs.

For bacterial enumeration, egg was transferred into sterile Whirl-Pak® bag containing 20 ml of peptone water, and gently massaged in peptone water for 1

minute. One milliliter of peptone water from bag was serially diluted and plated in duplicates on TSA plates with 50 µg/ml Nalidixic acid.

After every hour of inoculation, an egg was selected at random, and the concentration of bacteria after each hour of drying was enumerated as described above. This process was repeated every hour for 8 h, in order to optimize the time required for bacterial culture to dry and attach to the surface of eggs without causing depletion to bacterial concentration, contributed by drying. One hour of drying was confirmed to be optimum for bacterial attachment to the surface of eggs, as described by Russel (2003) as well as Cao et al. (2008).

2.2.3.3.2. Treatment of Shell Egg Surface

Eggs were stored and washed as described previously. Washed eggs were allowed to dry in a laminar hood, and transferred to petri plates in a bio-safety cabinet for spot inoculation. Eggs were allowed to dry for 1 h to ensure bacterial attachment on to surface of eggs.

An egg selected at random was enumerated to determine the initial bacterial concentration on egg surface. Other inoculated eggs were transferred individually into sterile bags containing 150 ml DW or sanitizing solutions, to test the efficacy of sanitizers on inactivation of *E. aerogenes*. The sterile bags were gently shaken at 90 RPM on a rotary shaker for 3 min, to ensure that neither the egg nor sanitizing solution was kept still through the treatment procedure. This method was used to keep the egg submerged in sanitizer.

Industrial treatment of eggs with sanitizers is usually by spraying, brushing and submerging of eggs. However, due to lack of feasibility of industrial grade egg

washing equipment, treatment of eggs with sanitizers was carried forward by two methods – submerging egg in a sanitizer solution (as described above) and massaging of egg in a sanitizer solution. These methods were chosen to evaluate the two extremes in egg washing; the latter (massaging) being a harsher treatment, to allow maximum contact with sanitizer, as compared to the former, submerging, which is relatively mild and does not always allow maximum contact of inoculated surface with sanitizer.

Treatment of eggs by massaging was done by gently massaging the surface of eggs for 3 min, after eggs were transferred into sterile bags with 150 ml DW or PAW or QA. Post treatment (massaging and submerging) with sanitizer and DW control, eggs were transferred to 20 ml peptone water to enumerate bacterial survival on surface of eggs.

2.2.3.3.3. Analysis of Wash Water

Solutions used to treat inoculated eggs, both with submerging and massaging of eggs, were serially diluted and 0.1 ml of serially diluted wash water was spread plated in duplicates on TSA plates with 50 µg/ml Nalidixic acid. Analysis of wash water was done in order to determine bacterial survival in wash water after egg washing.

2.2.4. Cuticle analysis

2.2.4.1. Colorimetric Analysis

Eggs were washed in tap water as described previously, and allowed to dry in the Laminar flow hood for 30 min. Dried eggs were then treated in 150 ml of DW, or

sanitizing solutions, by transferring the eggs to a bag containing above mentioned solutions. The bag was kept in a Pyrex® glass beaker and agitated on an orbital shaker for 3 min. Treated eggs were then dried in laminar flow hood for 30 min. They were then allowed to stain for 1 minute in MST Cuticle Blue dye (prepared as described in Section 2.1.3), by submerging in 500 ml of dye, kept in a Pyrex® glass beaker. After staining was completed, the eggs were transferred to a plastic stand and allowed to dry in the laminar flow hood for 30 min.

Stained eggs were analyzed using a Konica Minolta Colorimeter CR-410. Colorimetric readings for lightness or L* and chromaticity values a* and b* were measured in 5 locations around the equatorial region of the egg, as described in Leleu et al. (2011). The difference in color between stained, unsanitized eggs, and sanitized eggs can be depicted by the numerical value of ΔE^*_{ab}, which is given by -

$$\Delta E^*_{ab} = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad \text{.....eq1}$$

The average values for lightness and chromaticity were calculated for eggs prior to sanitization and post sanitization. ΔE^*_{ab} Values for each treatment were calculated against unsanitized eggs, assuming that unsanitized, farm fresh eggs had an even layer of cuticle on their shells, that is, ΔE^*_{ab} is considered equivalent to 0 for unsanitized eggs. Thus, the lower the ΔE^*_{ab} value, the higher is the cuticle coverage after any given sanitizer treatment.

2.2.4.2. Microscopic Analysis

In order to evaluate (if any) of cuticle layer on egg shells after treatment, stained egg shells were observed under Nikon Wide-field Microscope. Eggs were washed, treated and stained as described above, using various staining solutions such as Tartrazine, Green S and MST Cuticle Blue stain, in order to standardize dye used for this study. A mixture of 7.2 g of Tartarazine and 2.8 g of Green S dye were added to 1000 ml of water, to formulate a cuticle sensitive dye (Leleu et al., 2011; Liu et al., 2016). MST Cuticle Blue dye was prepared as described in the above section. According to images obtained from Nikon Elements ™ Version 4.50 as well as from studies proving efficacy of Cuticle Blue in attaching only to the cuticle layer (Samiullah and Roberts, 2013), MST Cuticle was decided to be used for analysis. Eggs were stained in Cuticle Blue dye and its yolk was removed before separating pieces of shell from the equatorial region. This was done so to ensure obtaining almost flat pieces of shells, making it easier to view under a microscope. Separated egg shells were then stuck on to a glass slide, using super glue and placed on the stage to view and analyze. Photographs of each egg shell was captured and analyzed for the frequency of occurrence against intensity of green, red and blue pixels. The higher the percentage of green colored pixels on an egg shell, the more was cuticle coverage.

2.2.5. Texture Analysis

In order to determine whether the acidic nature of novel sanitizers caused a detrimental impact on calciferous egg shells, egg shell strength was tested by texture analysis. It was thought that acidic nature of sanitizers may leach into the spongy calcium layer, and causing weakening of the shell.

2.2.5.1. Optimization of Probe

In order to determine whether the acidic nature of novel sanitizers caused a detrimental impact on calciferous egg shells, egg shell strength was tested. CT3 Brookfield texture analyzer was used to analyze the force needed to crack the shell of an egg, both before and after treatment with sanitizers.

The Brookfield texture analyzer was used for compression studies and to test peak force in the past, but has never been used in order to measure peak strength needed to crack eggs. To optimize and customize a design of probe and stand for this particular study, 3 probes of different materials and sizes were tested (pictorial representation provided in section 3.6.1) -

- acrylic probe (5 cm diameter)
- metallic probe (3 cm diameter)
- metallic probe (5 cm diameter)

2.2.5.2. Peak Force Measurement

Eggs were stored and washed as described previously. Eggs were then placed under the optimized probe, and allowed to be cracked under the force provided by probe. Peak strength or highest force needed to crack eggs was noted and compared against the highest force needed to crack un-sanitized eggs.

Eggs were allowed to crack in 3 positions (pictorial representation provided in section 3.6.2) -

- Small end up (SEU)
- Small end down (SED)

- Equatorial (EQ)

Since eggs are always subjected to transportation, it was considered essential to ensure that the strength of the egg shell was never compromised. Even though eggs are sold with their small end down (small end facing downwards), eggs may experience contact force during transportation and handling causing them to crack, from any side. Hence, texture analysis was performed with egg placed in three different positions.

2.2.6. Statistical Analysis

One way analysis of variance (ANOVA) in Microsoft® Excel® 2015 was used to perform statistical analysis. Null hypothesis was tested and data were considered significantly different (null hypothesis was rejected) if the level of significance (p-value) was less than 0.05.

3. RESULTS AND DISCUSSIONS

3.1. Preliminary Results- Analysis of PAW and PAEW

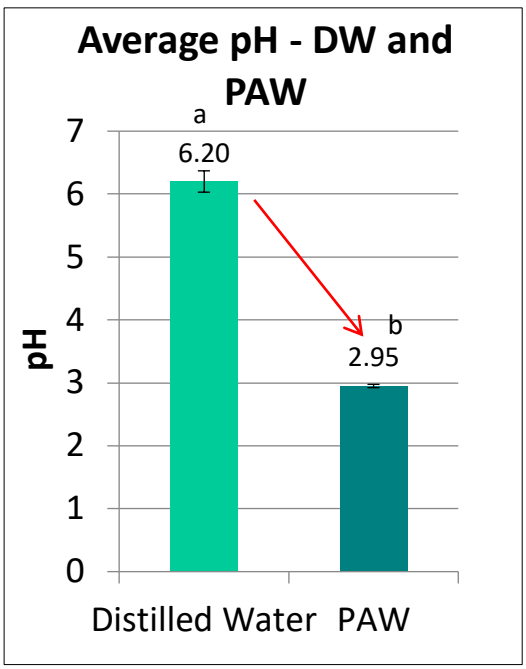
PAW was produced as described in Section 2.2.2, and pH of this sanitizer was measured and compared to the pH of sterile DW prior to activation by plasma jet. The pH of PAW dropped significantly from 6.2 ± 0.2 to 2.95 ± 0.02, upon being subjected to plasma jet exposure (as seen in Fig. 14(a)). This reduction in pH may be attributed to a cumulative effect of nitrites, nitrates, and hydrogen peroxide ions that enter sterile distilled water during activation (Naïtali et al., 2010).

ORP or oxidation reduction potential is a measure commonly used to detect reactive oxidative species in a solution (Tian et al., 2015). The study conducted by Tian et al. (2015) indicated that ORP is a reliable method to detect overall concentration of oxidizers in a solution, and reactive oxygen species in PAW, such that the value of ORP of PAW increased with time of exposure (from 210 mV to 250 mV). The ORP of DW increased significantly from 449.5 ± 11.3 RmV to 575.8 ± 21.5 RmV, due to induction of ROS from plasma jet (as seen in Fig. 4 (b)). This showed that exposure to plasma jet increased the amount of reactive oxygen species and oxidizing agents in distilled water.

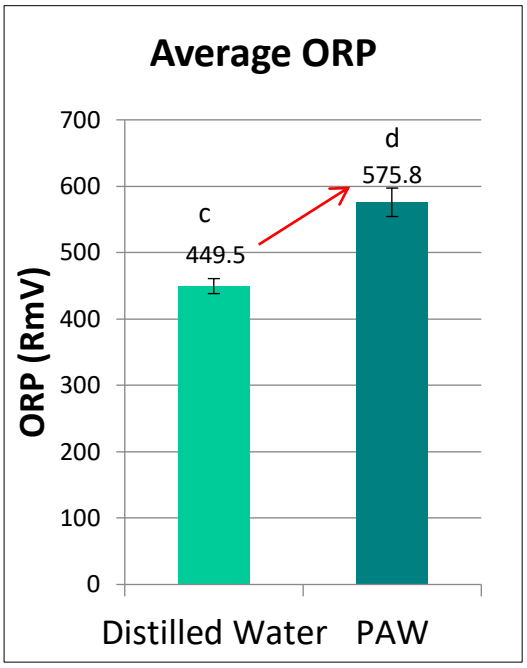
Production of PAW and PAEW followed the methodology described in Section 2.2.2. The values of ORP and pH, of PAW, and PAEW, did not bear any statistically significant difference (P-value > 0.05, at $\alpha = 0.05$). Although the pH of AEW (3.28) and PAEW (2.86) were not statistically different, the ORP of AEW (793.6 ± 16.1 RmV) was higher and significantly different from PAEW (576.1 ± 10.6 RmV). Reduction in ORP of AEW could be attributed to the dynamic nature of AEW. Since AEW has been

recognized to constitute compounds such as HOCl, which when exposed to air revert to water (Bonde et al., 1999). Thus AEW, when subjected to plasma jet, might have reverted to water. Upon continued exposure to plasma jet, AEW might have behaved as water being activated to form a solution with a similar ORP to that of PAW.

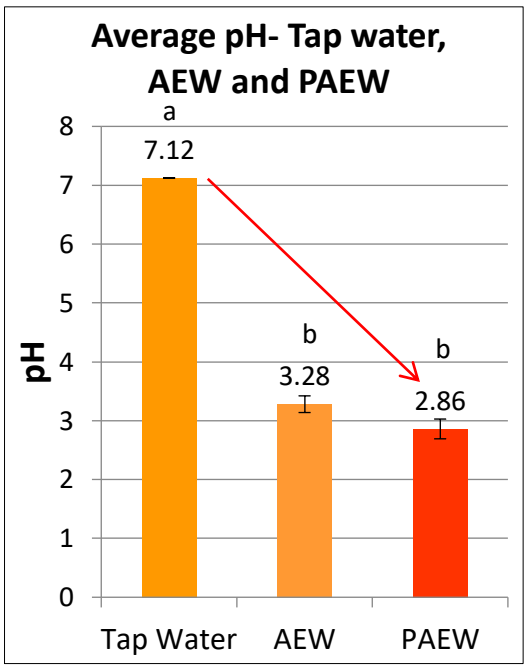
The pH of AEW was 3.30 ± 0.48 and ORP 742.4 ± 111.7 RmV (Fig. 14 c). Although the ORP of AEW was significantly higher than PAW, its antibacterial efficacy was affected by the presence of organic matter (Hricova et al., 2008). Also, in this study, the ORP values of AEW were seen to fluctuate between trials, causing it to be an unreliable sanitizer. Another study showed that AEW effected a reduction of 2.3 log CFU/egg of *S. enteritidis* upon treatment for 5 minutes (typical egg washing time in industry is 2-3 minutes), making AEW not a viable option for egg washing (Park et al., 2005). This is because USDA (2008) regulations require sanitizers to be able to effect a minimum of 5 log CFU reduction of *Salmonella* Enteritidis in eggs (Egg Products Inspection Act, 21 CFR 118.6(f)). Both PAW and PAEW proved to be more desirable options in egg washing, as compared to AEW (explained in Section 3.2).



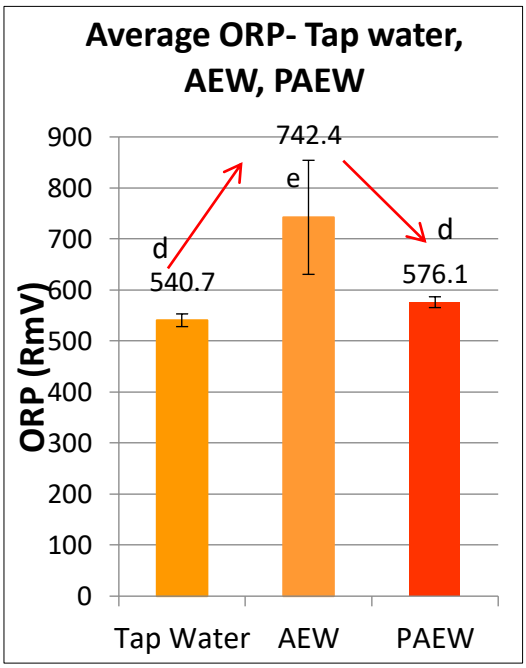
(a)



(b)



c)



d)

Figure 14: (a) Average pH of Sterile DW and PAW; (b) Average ORP of Sterile DW and PAW; (c) Average pH of Tap Water, AEW and PAEW; (d) Average ORP of Tap Water,

AEW and PAEW ((Error bars indicate standard deviations; Data that do not share the same letter, are significantly different from each other (One-way ANOVA, $p<0.05$)).

3.2. Comparison of PAW and PAEW

Sterile DW was used to generate PAW, which was stable between trials at pH of 6.2 ± 0.17and ORP of 449.5 ± 11.3 RmV. However, AEW which was used to generate PAEW, saw more fluctuations in both pH (3.3 ± 0.5) as well as ORP (742.4 ± 111.7 RmV) values, as compared to DW. This instability in AEW caused results obtained from treatment with PAEW to be less reliable as compared to PAW.

Inactivation using planktonic cell system was considered for initial analysis of these novel sanitizers. Statistical analysis of results obtained from treatment of *E. aerogenes* in a planktonic cell system or a suspension culture showed no statistically significant difference between PAW and PAEW, such that both values were below detection limit (3.2 log CFU/ml). This showed, that microbial inactivation efficacy of both sanitizers was comparable.

This was followed by colorimetric analysis, to detect differences in extent of damage to cuticle (if any), caused by the two plasma activated sanitizers. Colorimetric analysis of stained eggs showed no statistically significant difference between damage to cuticle by treatment with PAW (ΔE^*value of 4.72±4.73) and PAEW (ΔE^*value 9.00±7.32). Although PAW and PAEW depicted similar properties in egg washing, the number of steps involved in production of PAEW was more than that of PAW. This indicated that the number of variables that would have to be considered in analysis of PAEW were more in comparison to PAW, which is made directly from distilled water. This may also eventually lead to a higher cost being incurred in

production of PAEW. Thus, even though the efficacy of both sanitizers was comparable, PAW was more desirable than PAEW because of its relatively lesser variability and ease of production.

3.3. Planktonic Cell System

Cell suspension was prepared as described in section 2.2.3.1 and enumerated after diluting in peptone water, to determine initial concentration of the cell culture as 9.2 ± 0.1 log CFU/ml. Upon treatment with sterile distilled water, it was observed that the concentration of bacterial did not change significantly (9.1 ± 0.2 log CFU/ml) as compared to initial number of bacteria.

However, upon treatment with PAW, no viable bacterial colony forming units were observed, that is, the concentration of bacteria that survived the treatment was below detection limit of 3.2 log CFU/ml. This was similar to the survival of bacteria upon treatment with QA, which is a reliable industrial sanitizer.

The U.S. Department of Health and Human Services, Food and Drug Administration Center for Food Safety and Applied Nutrition recommends that sanitizers used in egg washing must contribute to a minimum of 5 log reduction of *Salmonella enteritidis* (USDA, 2010). This enabled PAW to be used as an alternate sanitizer to QA, since analysis of results showed no statistical difference between the two (as seen in Fig. 15).

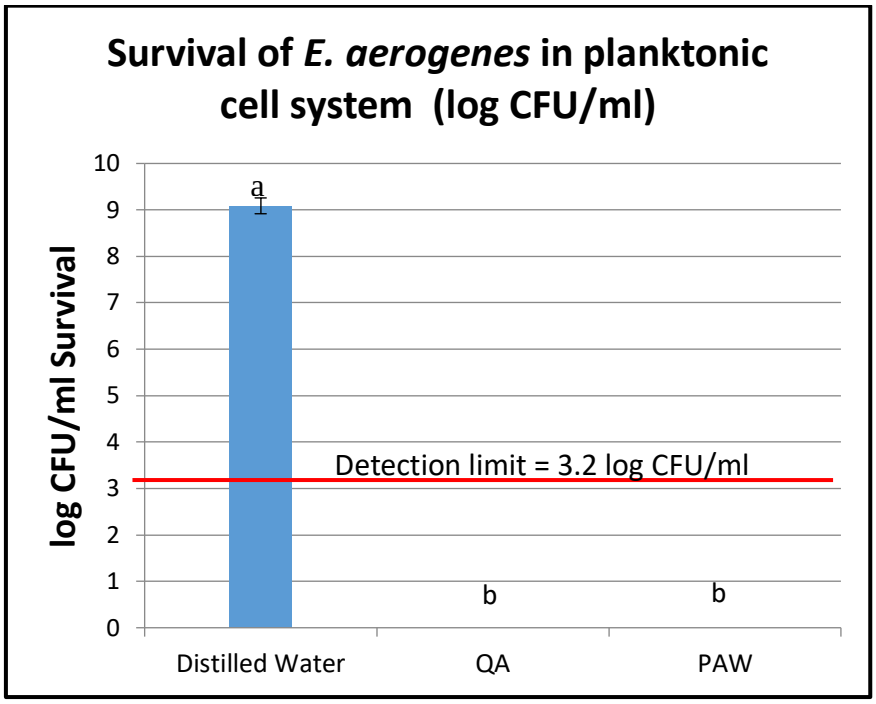


Figure 15: Survival of *E. aerogenes* when treated with DW, QA, and PAW in a planktonic cell system (Error bars indicate standard deviation; Data that do not share the same letter, are significantly different from each other (One-way ANOVA, $p<0.05$)).

3.4. Egg System

3.4.1. Native Background Micro Flora

The native micro flora on egg shell surface was enumerated as described in Section 2.2.3.3.1a). The concentration of microorganisms was measured, both on the surface of dirty eggs (farm fresh eggs) or DE, and visually cleans eggs (after gentle washing to remove debris) or VC. It was observed that DE had 4.4 ±0.4 log CFU/egg of naturally occurring microorganisms, as shown in Fig. 16. However, the concentration of

microorganisms reduced significantly after washing to remove debris from surface of eggs, and was below the detection limit of 1.3 log CFU/egg.

This reduction ensured that the visually clean eggs were comparatively less contaminated than dirty, farm fresh eggs. In the egg washing industry, eggs are usually rid of debris by scrubbing with a soft brush or sponge, to make them appear visually clean, before sanitization.

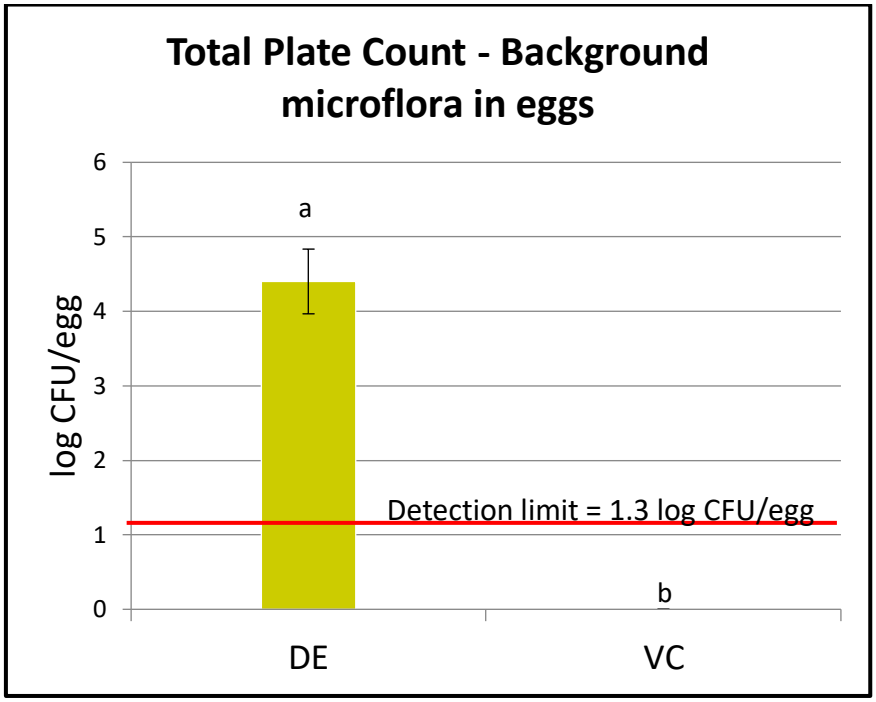


Figure 16: Total aerobic plate count of naturally occurring bacteria on surface of shell eggs; DE refers to dirty egg and VC refers to visually clean eggs (Error bars indicate standard deviation; Data that do not share the same letter are significantly different from each other (One-way ANOVA, $p<0.05$)).

In a study conducted by Rodriguez-Romo and Yousef (2004), the process of inoculation of eggs with *Salmonella* Enteritidis was preceded by washing eggs with tap water as well as with washing in deionized water and soaking in 70% ethanol

(v/v). This ensured that all the native microorganisms were inactivated prior to inoculation. The sanitization of eggs with chlorine-based sanitizers, as a pre-inoculation step has also been used in the past (Russel, 2003). However, the protocol used in this study ensured that complete elimination of natural micro flora from surface of eggs did not occur, since such a scenario would be much less likely in an egg washing plant.

3.4.2. Optimization of Drying Time

Time required for inoculated bacteria to attach to the surface after inoculation was one of the primary criteria used to optimize drying time of spot-inoculated egg. Plating of spot inoculated bacteria, at the end of each hour of drying, indicated that the number of bacteria between hour 0 and hour 1 remained the same, after which, the bacterial population declined steadily. The initial concentration of bacteria after 1 hour of drying was determined by enumeration on TSA with Nalidixic acid, as 7.9±0.3 log CFU/egg (concentration of bacteria in the inoculum was 9.2±0.1 log CFU/ml, of which 0.1 ml was spot inoculated).

It was observed that sanitized eggs dipped in a suspension of Salmonella were able to dry in 1 hour, providing adequate time for bacterial attachment (Ragini et al., 2010). Cao et al. (2013) followed a similar protocol to allow attachment of *Salmonella* Enteritidis to shell egg surface, allowing eggs to dry for 1 h after inoculation. The protocol used in this study was consistent with past studies, in optimizing drying time or time of attachment of bacteria as 1 h.

3.4.3. Microbiological Analysis of Shell Egg Surface

Inoculation of bacteria, followed by 1 h of drying ensured that bacteria remained attached to the surface. Inoculated and dried eggs were treated with DW, PAW, and QA, and enumerated as described in section 2.2.3.3.2. The starting concentration was 7.9±0.3 log CFU/egg.

Eggs treated by submerging in DW and PAW, did not show any statistically significant difference, with survival after treatment in DW at 4.7±0.2 log CFU/egg and PAW at 4.9±0.4 log CFU/egg, as seen in Fig. 17.

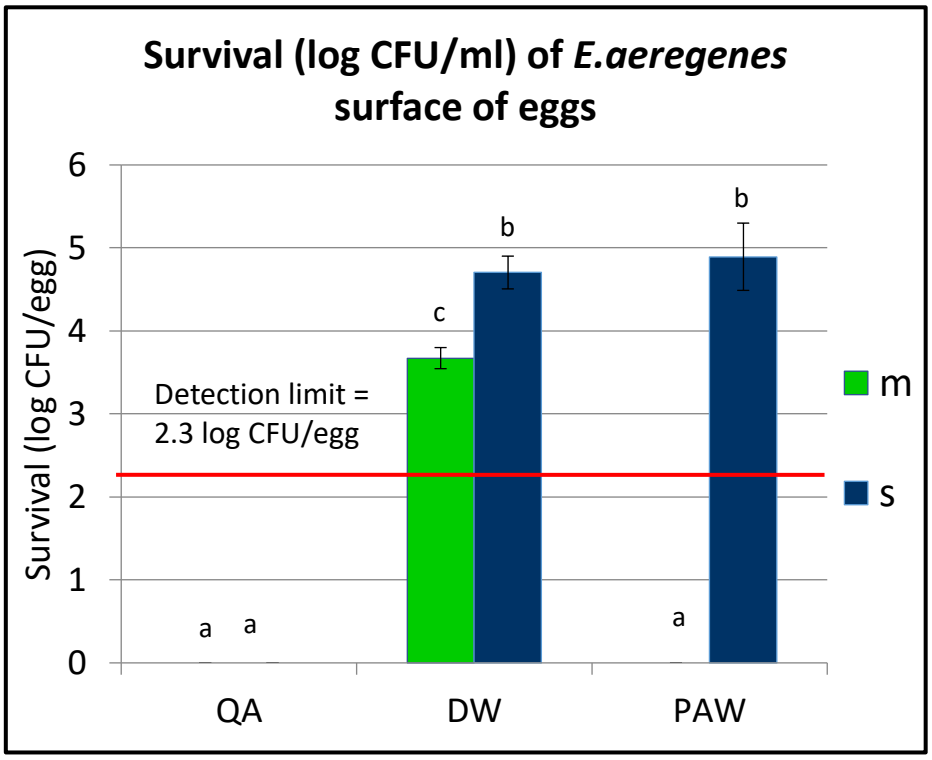


Figure 17: Survival of *E. aerogenes* on surface of egg; 'm' denotes massaging treatment and 's' denotes submerging of eggs in solution (Error bars indicate standard deviation; Data that do not share the same letter, are significantly different from each other (One-way ANOVA, $p<0.05$)).

Bacterial inactivation with PAW was pursued with a more vigorous treatment. Massaging of egg surface was used as a rigorous treatment to determine whether bacterial inactivation would improve upon treatment with PAW, with an increase in rigor of treatment. Upon massaging, it was noted that bacterial survival on egg surface after treatment both PAW and QA was below detection limit (2.3 log CFU/egg). On the other hand, treatment with DW showed survival of 3.7±0.1 log CFU/egg on the surface of egg. This highlights the proposition that PAW shows promise to be used in the industry, as a replacement for QA, with standard egg washing equipment. The rigor of treatment of standard egg washing equipment would be in-between the two extremes covered in this study - submerging and massaging. Thus, it was hypothesized, that when used in an industrial-grade equipment, PAW might cause comparable microbial reduction to QA.

Studies in the past have focused on a similar objective, of replacing chlorine based sanitizers with non-chlorine based sanitizers. A commonly studied sanitizer was AEW (Acid electrolyzed water), that caused a reduction in bacterial population of *S. enteritidis* of up to 2.1 log CFU/g of egg shell, upon 10 minutes of treatment (Bialka et al., 2004). However, AEW was found to have disadvantages such as causing high phytotoxicity in plants and health hazards to industry workers (Guentzel et a., 2008; Cao et al., 2009).

Electrolyzed oxidative water or EO water (commonly known as AEW) was also electrostatically sprayed on to the surface of eggs, resulting in complete elimination (~ 4 log CFU/egg reduction) of *Salmonella typhimurium* from shell egg surface (Russell, 2003). But this method was not suited to be used industrially, since the time required for complete inactivation (EO water was sprayed on to surface of eggs for

15 s every hour, for the length of an entire day) was higher than the time used for sanitization of eggs in the industry. Another commonly studied method involves utilization of NEW (neutral electrolyzed water), also known as SAEW (slightly acidic electrolyzed water), which had shown similar or better reduction of *S. enteritidis* on surface of eggs, as AEW (Cao et al., 2009). Although SAEW or NEW showed promise in eliminating Sodium hypochlorite (NaOCl) and other sanitizers based heavily on chlorine, it has not been shown to entirely inactivate foodborne pathogens, including microorganisms present naturally on surface of foods (Abadias et al., 2008). Plasma activated water was observed to cause inactivation of 6 log CFU/ml of *E. Coli* in 5 minutes (Oehmigen et al., 2010) when planktonic cell system was exposed directly to DBD plasma (to produce PAW in the planktonic cell system). PAW generated by direct exposure to plasma jet, has been attributed to cause 5 log CFU/ml reduction of *S. aereus* in a planktonic cell system, incubated in PAW for 20 min (Tian et al., 2015). Thus, inactivation efficacy of PAW in this study was found to be comparable to past studies, as well as to chlorine-based sanitizers. However, unlike chlorine-based sanitizers, PAW inactivation efficiency depends largely on nitrates, nitrites, and presence of hydrogen peroxide (Naïtali et al., 2010). This allows PAW to be used without apprehensions of health hazards to industry workers by emission of chlorine, as well as with expectation to meet similar antibacterial efficacy as conventional sanitizers.

3.4.4. Microbiological Analysis of Wash Water

Bacterial enumeration of wash water showed that the concentration of bacteria was below detection limit of 0.7 log CFU/ml in PAW and QA. However, analysis of

distilled water used to treat eggs showed that DW wash water had survival of 3.6 ± 0.3 log CFU/ml and 3.6 ± 0.7 log CFU/ml, from massaged and submerged eggs, respectively. Some bacteria in eggs washed with DW, had merely detached from the surface and survived in wash water. As seen in Fig. 18, there was no statistically significant difference between QA and PAW, but there was a difference between QA/PAW and DW. Thus, detachment without inactivation created a possibility for cross-contamination. In the case of PAW and QA, bacteria that had been detached were inactivated in wash water, causing the survival of bacteria in wash water to be below detection limit.

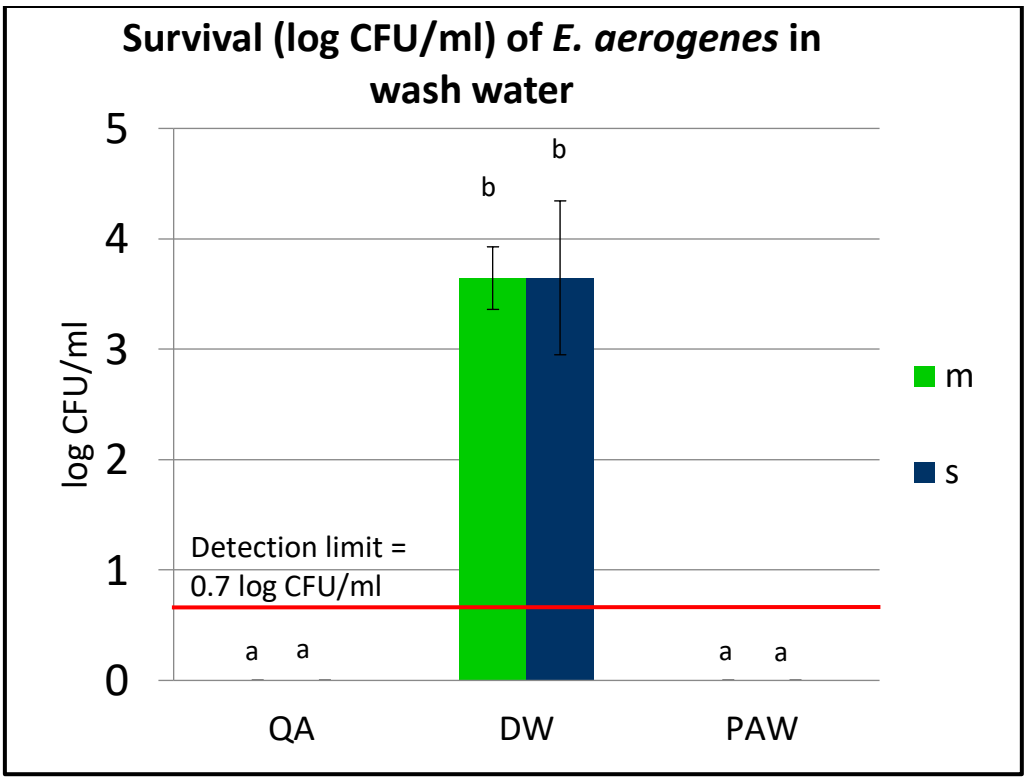


Figure 18: Survival of *E. aerogenes* in wash water used for treatment of artificially contaminated shell egg surfaces 'm' denotes massaging treatment and 's' denotes submerging of eggs in solution (Error bars indicate standard deviation; Data that do

not share the same letter, are significantly different from each other (One-way ANOVA, $p<0.05$)).

Inactivation of bacteria in PAW could be attributed to accumulation of reactive oxygen species or ROS in within bacterial cells. On the other hand, inactivation may also be due to production of oxidation by-products in bacterial cells, caused by external oxidative stress by PAW (Tian et al., 2015). It is essential that bacteria detached from the surface of eggs are inactivated by PAW, since it is a common practice to reuse sanitizer used for washing eggs. Research suggests that bacteria detached from one egg surface, may be transmitted to another egg via cross-contamination, such that the latter egg would take up detached bacteria via pores on egg shell surface (Sparks and Board, 1983).

Analysis of wash water for bacterial survival was of significance because, porosity of egg shells and motility of bacteria enabled penetration of bacteria into eggs (Reu et al., 2006). When eggs were exposed to contaminated environment, horizontal transfer of bacteria would take place through pores of egg shell, leading to entry of bacteria inside eggs (Barrow and Lovell et al., 1991). However, PAW ensures inactivation of bacteria, in wash water with both treatment methods, and could be considered as a suitable alternative for QA. PAW also prevents cross-contamination from occurring, and may be as effective as QA in reduction of detached bacteria in wash water.

3.5. Cuticle Analysis

3.5.1. Colorimetric Analysis

Colorimetric analysis of shell eggs was performed to present any evidence of the presence or absence of cuticle after treatment with sanitizers. Unsanitized eggs were used as control in this experiment, since they were assumed to have full cuticle coverage. As shown in Fig. 19, eggs treated with QA and store bought eggs were visually paler (less green) than unsanitized eggs. Visual inspection also showed that the color of stain on eggs washed with PAW and DW were comparable to unsanitized eggs.

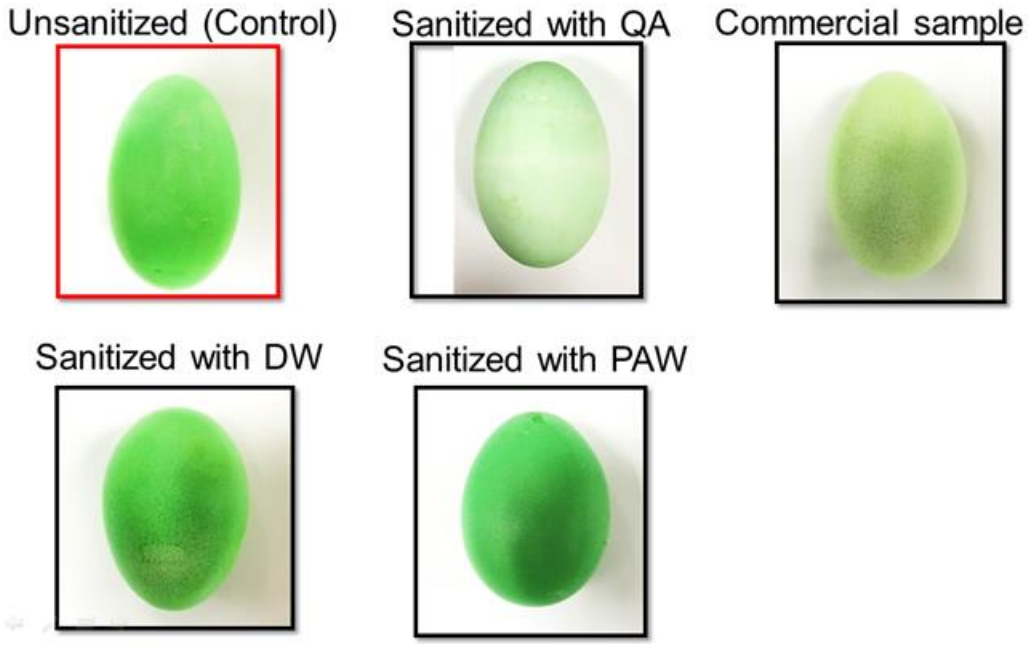


Figure 19: Pictures of eggs stained with MST cuticle blue dye with and without (control) treatment with sanitizers

L*, a* and b* values of the egg shells were analyzed (Fig. 20) to show that there was no statistically significant difference between eggs washed in DW, PAW and unsanitized eggs. However, the values obtained from QA washed eggs, were

significantly lower than the above-mentioned treatments. This may indicate loss of cuticle in whole or partially in eggs washed with QA.

In order to consolidate values of L*, a* and b* obtained from colorimetric analysis, the value of ΔE* was determined by the formula described in Section 2.2.4.1. As seen in Fig. 21, ΔE*values for unsanitized eggs was 0(±4.62), since it was the control and the unsanitized or untreated egg was considered to have maximum cuticle coverage. The ΔE* value of unsanitized eggs was not significantly different from DW (2.37±4.57) and PAW (4.72±4.73). ΔE*value of eggs washed with QA (22.65±2.99) was significantly higher, indicating that it is farthest away from maximum cuticle coverage (ΔE* value =0).

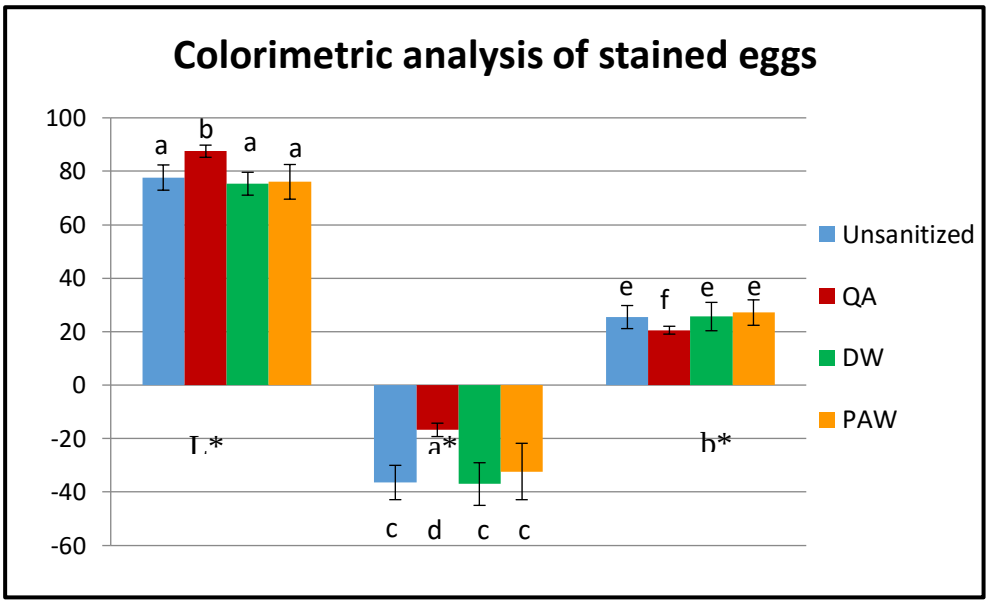


Figure 20: Colorimetric analysis to detect lightness (l*) and color components a* and b* of stained shell eggs (Error bars indicate standard deviation; Data that does not share the same letter, are significantly different from each other (One-way ANOVA, $p<0.05$)).

The importance of cuticle layer in shell eggs, to abate microbial contamination and prevent entry of microbes through pores on the eggs shell had been studied (Board and Halls, 1973). The effectiveness of cuticle layer had been confirmed through artificial contamination studies and observation of bacterial penetration through luminescence techniques (Messens et al., 2005). Also, the amount of water and contaminants entering an avian egg shell had been shown to be higher in cuticle-less eggs, than in eggs with cuticle coverage (Sparks and Board, 1984; Kim and Slavik, 1996). The removal of cuticle from an egg shell was seen to be prevalent primarily due to harsh washing treatments (Samiullah and Roberts, 2013), and high levels of chlorine in sanitizers (Bialka et al., 2004). Thus, it was essential to study the presence of cuticle layer post-treatment with PAW.

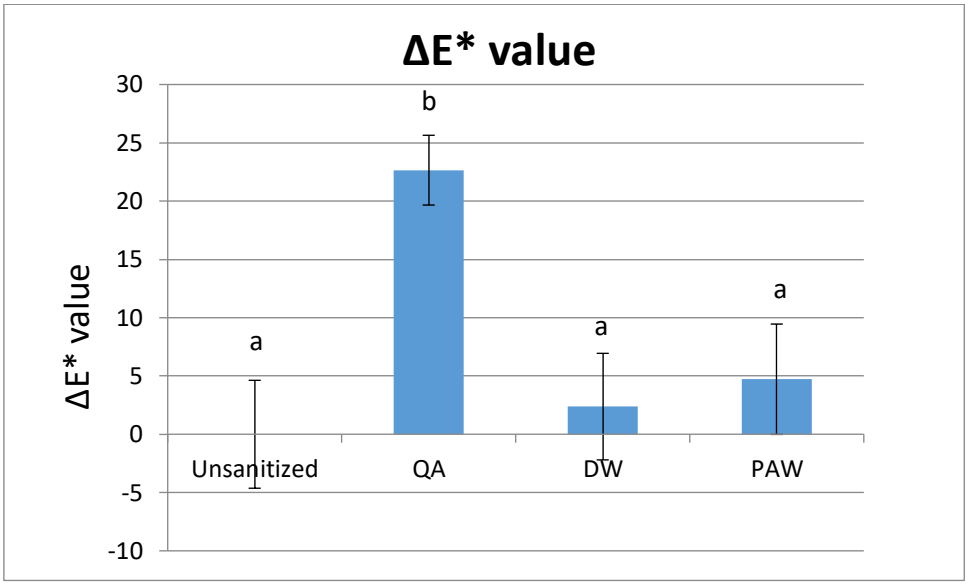


Figure 21: ΔE* value calculated against unsanitized egg (control), to compare the impact of sanitizers on cuticle of eggs (Error bars indicate standard deviation; Data that does not share same letter, are significantly different from each other (One-way ANOVA, $p<0.05$)).

Some of the methods traditionally used to estimate cuticle coverage include staining eggs with Edicol supra Pea-green (Board and Halls, 1973) and Scanning electron microscopy analysis of egg shells (Samiullah, 2012). Samiullah and Roberts (2013) studied and verified a third method of cuticle coverage analysis, involving staining of egg shell with MST Cuticle blue dye. It was observed that, when cuticle layer was chemically removed with EDTA, MST cuticle blue stain was not retained by egg shell (Samiullah and Roberts, 2013).

Liu et al. (2016) used Edicol supra Pea-green dye to study cuticle coverage, and concluded that the cuticle coverage of unwashed eggs was significantly higher than commercially washed eggs. This result was consistent with this study as well as with studies conducted by Kim and Slavik (1996). Kim and Slavik studied the effect of washing farm eggs with Cetylpyridinium chloride (CPC), a quaternary ammonium compound as well as trisodium phosphate, detergent approved for use in poultry processing by United States Department of Agriculture (Kim and Slavik, 1996). Another study conducted to compare the effectiveness of Electrolyzed oxidative (EO) water and commercial detergents, showed that there was a statistically significant difference between eggs washed with commercial sanitizers and unsanitized eggs. The study also showed that eggs washed with EO water and unwashed eggs were statistically different, such that cuticle coverage was affected by EO water treatment, however, significant differences between commercial egg washing and EO treatment did not exist (Bialka et al., 2004). Thus, unlike some of the other novel alternatives to commercial sanitizers like EO water, PAW could be exercised as a viable alternative, since it causes minimal, statistically insignificant cuticle damage.

3.5.2. Microscopic Analysis

3.5.2.1. Selection of Dye

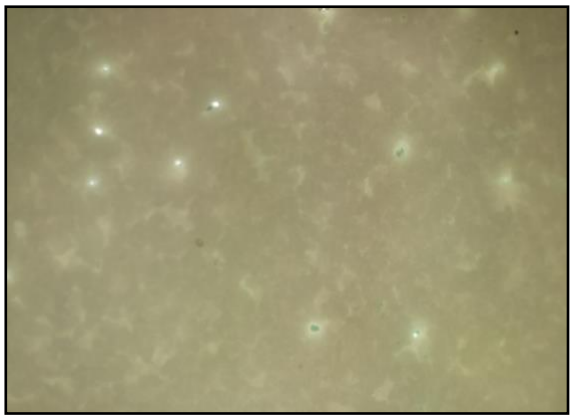
Microscopic analysis was conducted on small pieces of egg shells, from the equatorial region of the egg. Eggs were stained in the following stains:

- Tartrazine
- Green S
- Tartrazine and Green S
- MST Cuticle Blue

The images obtained from analysis of these egg shells was compared to unstained eggs, in order to obtain a perspective on the amount of color change brought about by stain. Visual and image analysis of pictures obtained from microscopy and imaging was performed to determine that MST Cuticle Blue dye was the most suitable for further analysis.

Upon observing unstained egg shells under the microscope, it was concluded that a stain would be desirable to detect changes that might occur in the cuticle post washing of eggs. Although some studies in the past point to utilization of Green S (Edicol pea green) and Tartrazine (Messens et al., 2006; DeReu et al., 2004), the more recent studies have shown MST Cuticle Blue to possess effective technology to attach singularly to the cuticle (Samiullah and Roberts., 2013). In this study, it was observed (Fig. 22) under the wide-field microscope that difference between washed and unwashed eggs was more apparent when stained with MST cuticle blue dye. On the other hand, when stained with Edicol pea green or Tartrazine, the two images depicting differences between washed and unwashed eggs were observed to be

more similar than different. Thus, the use of Green S and Tartrazine made the difference less apparent, compared to MST Cuticle Blue dye.



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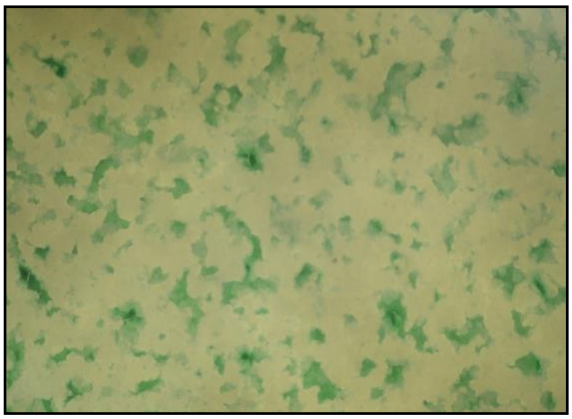

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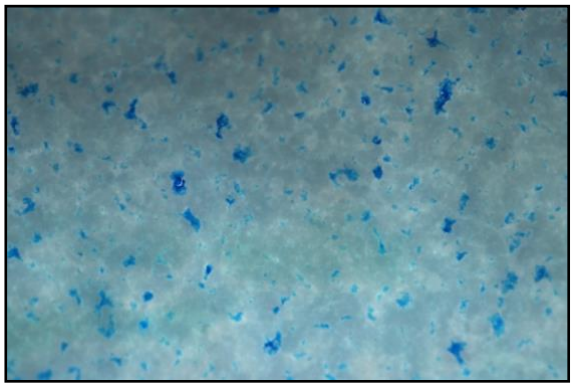

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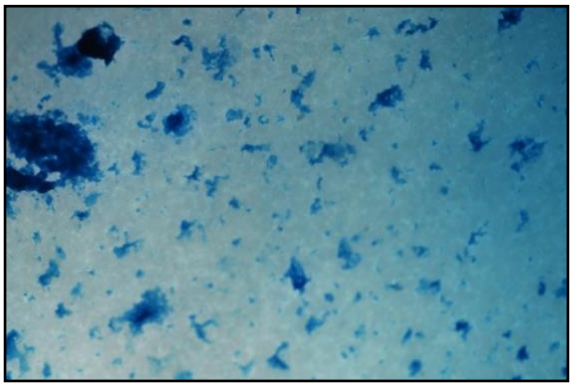

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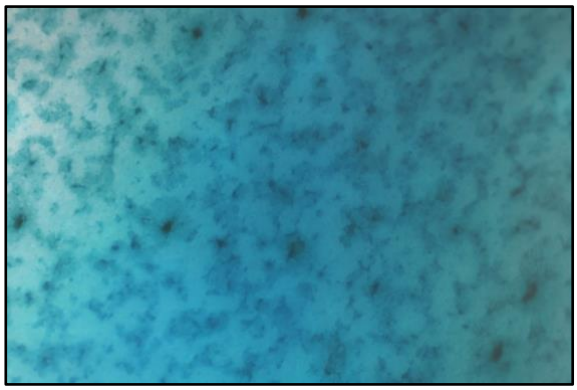

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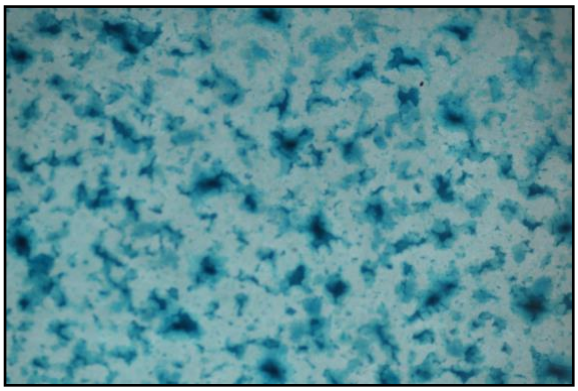
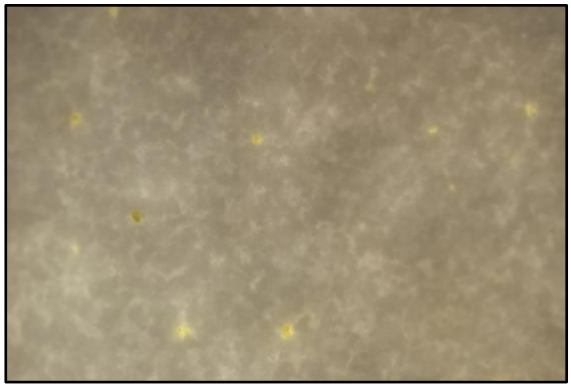
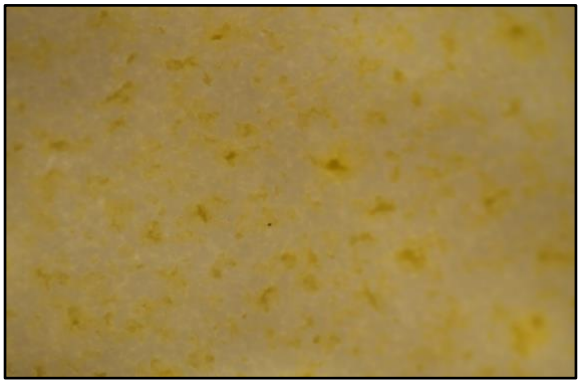


(f)





(g) (h)

Figure 22: Egg shells stained with MST cuticle blue dye, combination of Tartrazine and green S, only green S and only Tartrazine, such that a, c, e, g represents washed eggs and b, d, f, h, show unwashed egg shells.

3.5.2.2. Analysis of Egg Shell

Eggs shells stained in MST Cuticle blue dye were analyzed both before and after treatment with sanitizers, to observe the difference in the presence of cuticle. When placed under the Nikon wide-field microscope, an image of the size 3582.84 µm by 2382.72 µm at 0.73 µm/pixel, was captured by the Nikon Elements (Version 4.50) software. At 10X resolution, each pixel in the picture was analyzed for red, blue, and green color intensities. Finally, data pertaining to intensity against frequency of occurrence of a certain color (red, blue, or green) at that given intensity were obtained. These data were obtained for egg washed with each sanitizer, and the values were used to calculate percentage of red, green, and blue color (as a factor of both intensity and number of pixels) in each image. This was done by calculating the number of pixels of each color, at a given intensity. The sum of these values was then divided by the sum of frequency of occurrence each color, in order to obtain an average, depicting the average number of pixels of that color, in the image. Average

term was used in calculating fraction of occurrence of colors in an image, and the percentage was averaged across each replicate. These values were then plotted against each sanitizer, and compared statistically by one-way ANOVA to determine whether color differences between each treatment were significant.

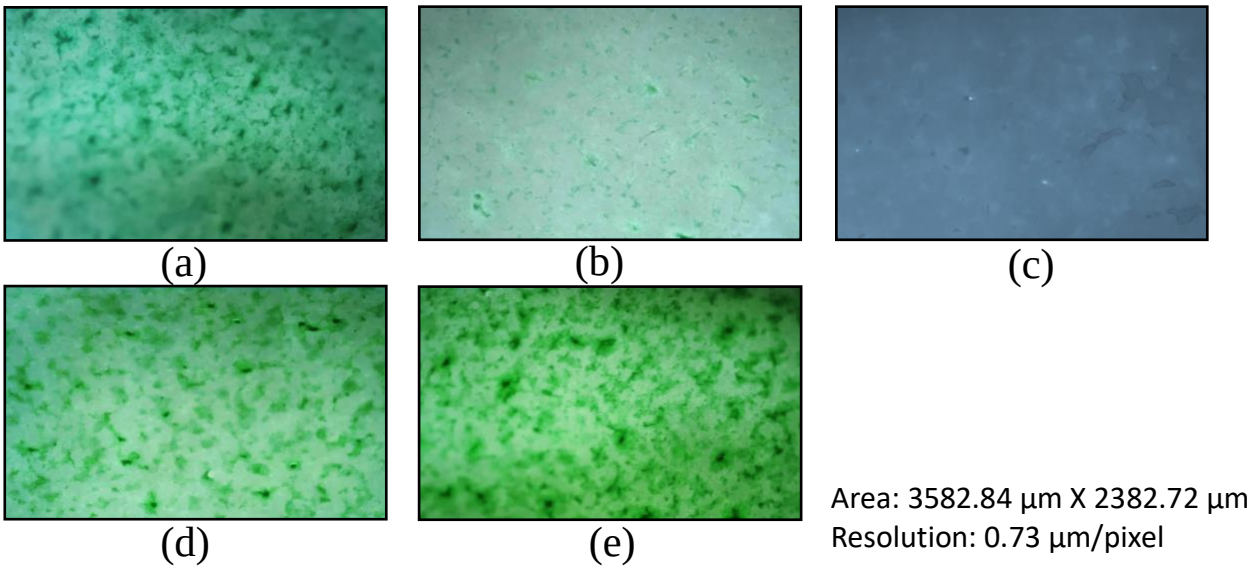


Figure 23: MST cuticle blue dye stained egg shells as seen under the microscope for (a) Unsanitized (b) Commercial sample (c) QA (d) DW (e) PAW

As shown in Fig. 23, a statistically significant difference was observed between the fraction of green color stained on unsanitized egg shells (assumed to have maximum cuticle coverage) and eggs washed with QA (0.37 ± 0.02). Additionally, the difference between green color absorbed by unsanitized eggs (0.42 ± 0.01) and eggs washed with DW (0.42 ± 0.01) and PAW (0.41 ± 0.01), was insignificant, such that both these treatment methods had a significantly higher cuticle coverage than QA treated eggs. The value for fraction of green in shell of stained eggs was most relevant to analysis in presence of cuticle, since MST Cuticle Blue dye stained the egg green, in case

cuticle was present (Samiullah and Roberts, 2013). This method aided in obtaining a closer look at the egg shell, and at the same time helped compare the degree of greenness of egg shell compared to other colors. As a consequence, this method verified the results obtained through colorimetric analysis while proving a magnified image of the sanitizer treated shell. This method of analysis of egg shell cuticle has never been studied in the past, and thus was considered to be an innovative technique. However, this approach might have certain shortcomings, since the images are based singularly on the portion or egg shell bit selected for analysis. In addition to this, in order to reduce the margin of error, the number of replicates for each treatment method would have to be higher than the number of replicates that may be required for colorimetry. This was because, it was more challenging to obtain flat, almost equally sized, and un-cracked bits of egg shell for microscopy than obtain 5 spots on an intact egg shell surface for colorimetry.

As mentioned earlier, wide field microscopy cuticle had not been studied in the past, although other forms of microscopy like scanning electron microscopy or SEM have been commonly used to investigate egg shell cuticle layer. Wang and Slavik (1998) used SEM to determine both presence of cuticle and penetration of *S. enterititis* through egg shells as a consequence of treatment with quaternary ammonium compounds (QAC), sodium hypochlorite, and sodium carbonate. Liquid nitrogen was used to snap-freeze egg shells fixed by Karnovsy's fixative. This allowed them to obtain well-defined cross-sections for analysis with SEM. Analysis proved that sodium carbonate treated eggs shells lacked cuticle layer, while QAC washed eggs somewhat maintained cuticle layer under a heavy deposition of QAC on the shell.

This chemical deposition might explain the reason behind MST Cuticle blue dye not adhering to QA washed eggs in our study.

Research study conducted by Leleu et al. (2011) was based on scores assigned by an experimental assessor, to determine cuticle coverage before and after washing of eggs. SEM images were assigned scores, to reveal that washed eggs had a slightly lower, but insignificant cuticle coverage post washing with Swedish industrial egg washing equipment. The study also revealed that unwashed eggs had low cuticle coverage to begin with. Authors have also studied the presence of cuticle after washing and compared cuticle presence to bacterial penetration with the help of blue lake die. SEM was used in analyzing dye penetration and detecting the amount of cuticle coverage on egg shell (Favier et al., 2000b). Thus, scanning electron microscopy, although more expensive than traditional microscopy provides additional information compared to the former, owing it to be a more popular and reliable technique for cuticle analysis than traditional microscopy.

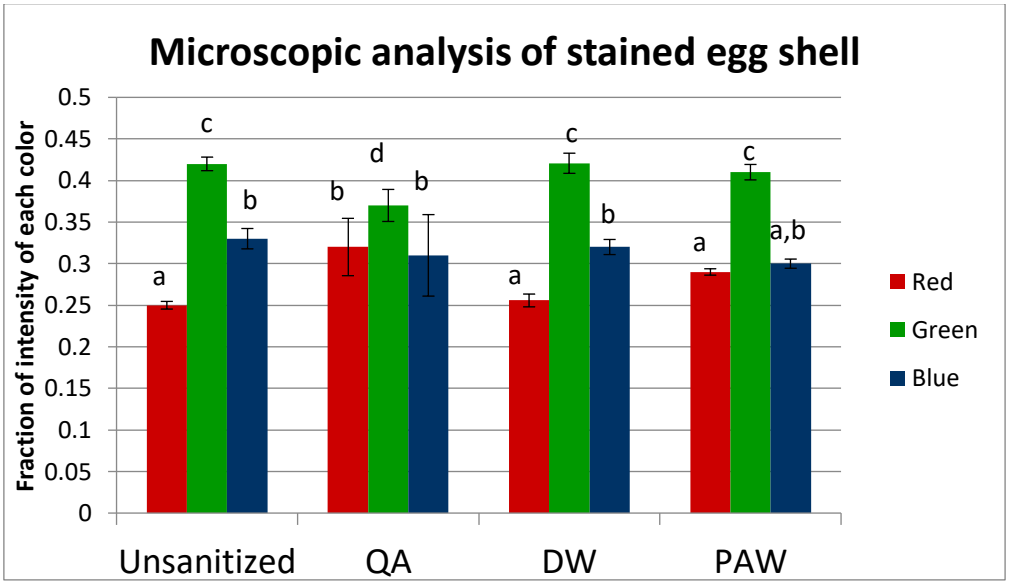


Figure 24: Comparison of microscopic analysis of stained egg shells based on intensity of red, green and blue color pixels of images captured (Error bars indicate

standard deviation; Data that do not share the same letter, are significantly different from each other (One-way ANOVA, $p<0.05$)).

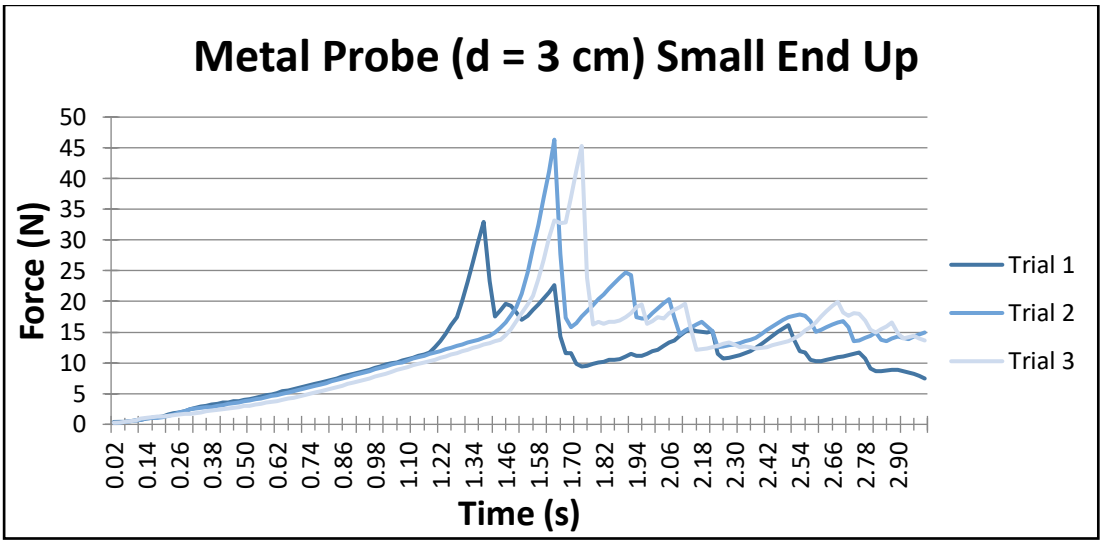
3.6. Texture Analysis

3.6.1. Optimization of Probe

According to the methodology discussed in Section 2.2.5.1, three probes were tested, prior to the study (Fig. 26) which were

- acrylic Probe (5 cm diameter)
- metallic probe (3 cm diameter)
- metallic probe (5 cm diameter)

These probes were tested in order to analyze consistency of results between trials, and also ease of use. Trials were conducted with market eggs, since it was known that industrially processed eggs withstand travel between farm, processing plant, shop and the consumer, without physical damage. Also, market bought eggs had somewhat uniform shapes and sizes, which made optimization studies convenient.



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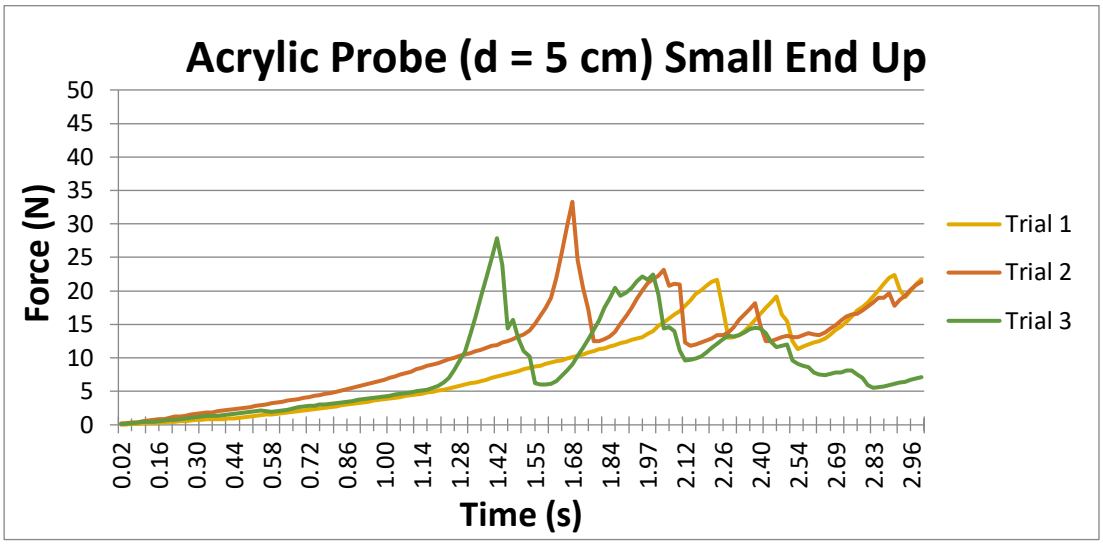

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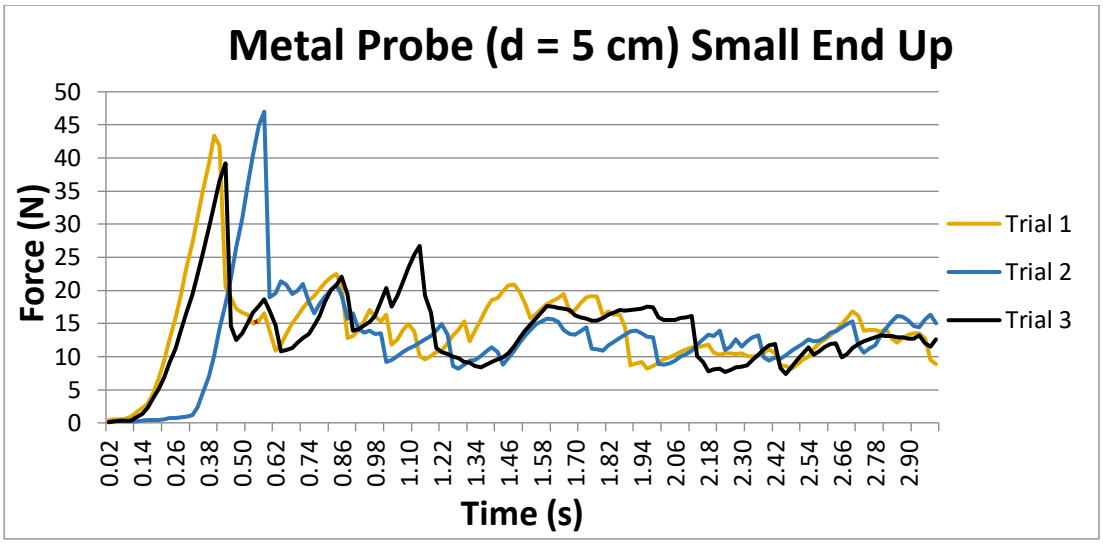


c)

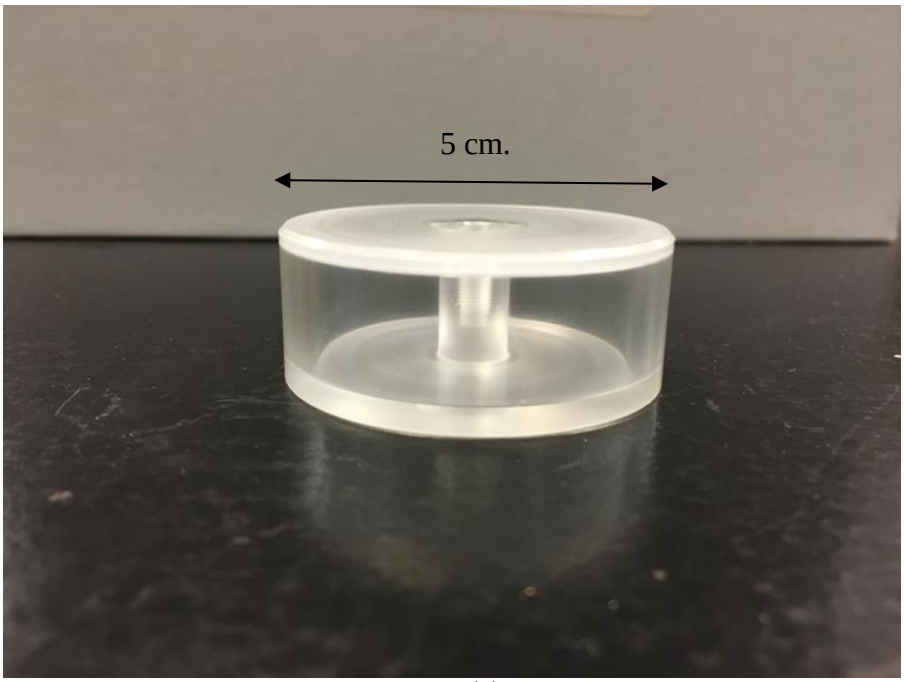
Figure 25: Plot between 3 trials of Force (N) needed to crack eggs and the time required (s) with 3 different probes a) Metal probe (d=3 cm) b) Acrylic probe (d=5 cm) c) Metal probe (d= 5cm)

Figure 25 shows plots between Force (N) needed to crack eggs and the time needed to do so. The probe was optimized to maintain consistency in the peak force, signified by the highest force or the peak force needed to crack eggs. Coherent peak

force and the time at which this force was experienced, with least noise in data, were criteria used in probe optimization. Figure 25 a) shows data for 3 trials conducted on store bought eggs, with similar dimensions, but the peak force was significantly different between each trial. The plot also demonstrates that the data post peak force had noise, but the noise was significantly lower than what was seen in Fig. 25 b). Figure 25 b) also indicated peaks that did not overlap, even though eggs were of similar dimensions. This showed that the results obtained from both probes were inconsistent and the high noise in the data may be due to uneven and unbalanced pressure experienced by the egg surface.

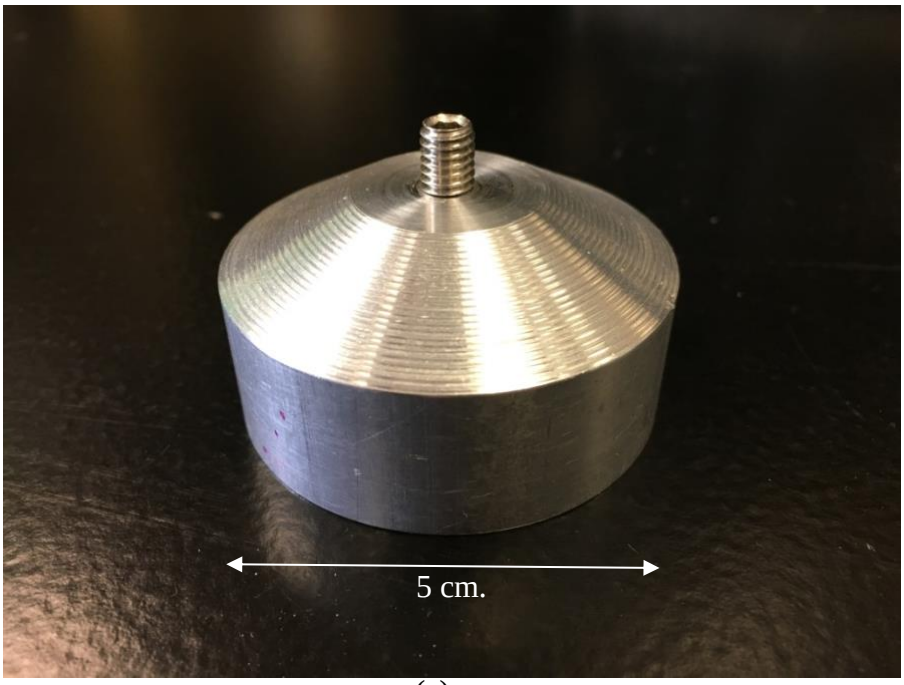
In order to resist inconsistency in results, a metallic (aluminum) probe with a diameter 5 cm and a base (7 cm length, 5 cm width, and 1 cm depth) that acted as an egg stand were crafted (as shown in Fig. 26 a, b). The base (Fig. 26 d) had a hollow indentation at the center, which allowed eggs to be positioned without external support, i.e., it rolling off.

This devise (probe and base) was constructed at Department of Food Science, Rutgers University. The probe was subject to tests similar to the test described above and the data obtained from these assessments is shown in Fig. 25 c. The almost overlapping peak forces proved even force distribution provided by the probe. The slight differences in time of detection of peak force could be attributed to the slight differences in dimensions that may be present between trials. These slight dimensional differences were unavoidable, since no two eggs would have precisely the same height, although the difference in height it may be considered statistically insignificant. Thus, it was concluded that the custom-made probe would be utilized in further analysis of farm fresh eggs treated with sanitizers.



(a)

(b)



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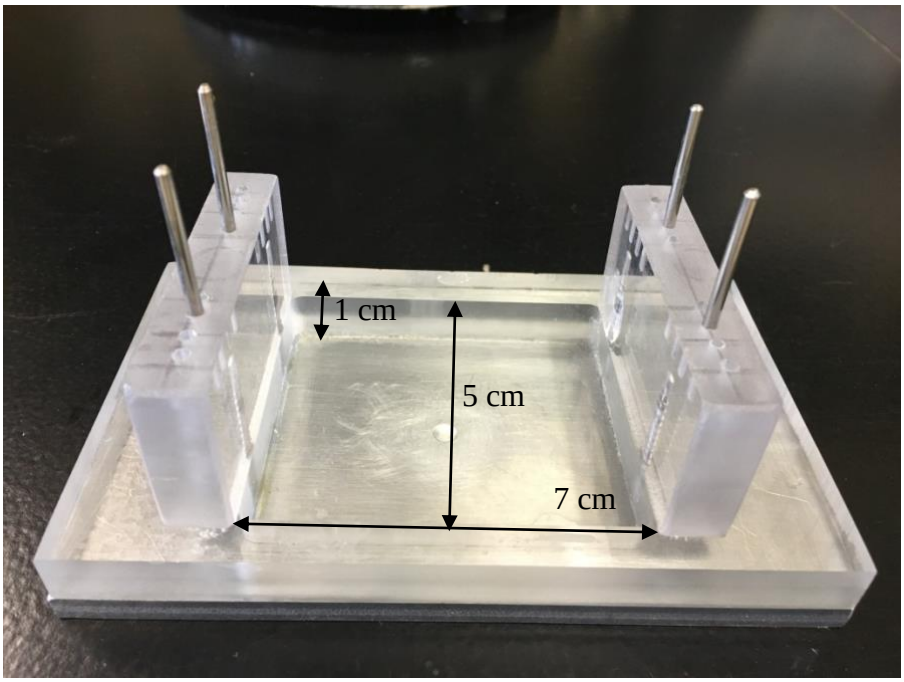
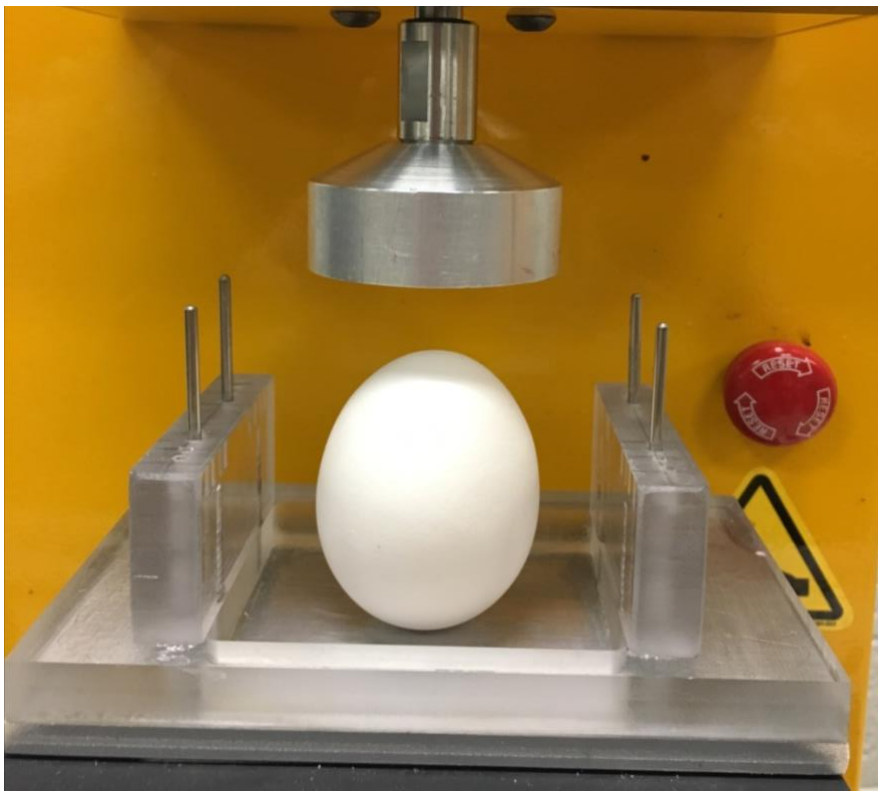


(d)

Figure 26: Probes tested for texture analysis a) Acrylic probe (d=5 cm), b) Metal probe (d=3 cm), c) Customized metal probe (d=5 cm), and d) Customized egg-holder base

3.6.2. Analysis of Peak Force Required to Crack Eggs

Shell eggs were analyzed for shell strength, both prior to and post treatment with sanitizers as described previously in Section 2.2.5.2. This test was performed in order to ensure that the acidic nature of PAW would not contribute to weakening of eggs shell, thereby promoting easy cracking of eggs during transportation. The chart between (egg shell strength) peak force needed to crack eggs and position of eggs used in cracking eggs was plotted in order to evaluate differences, if any, in shell strength. Figure 27 shows the 3 position used to crack eggs, with custom designed probe and egg holder base.



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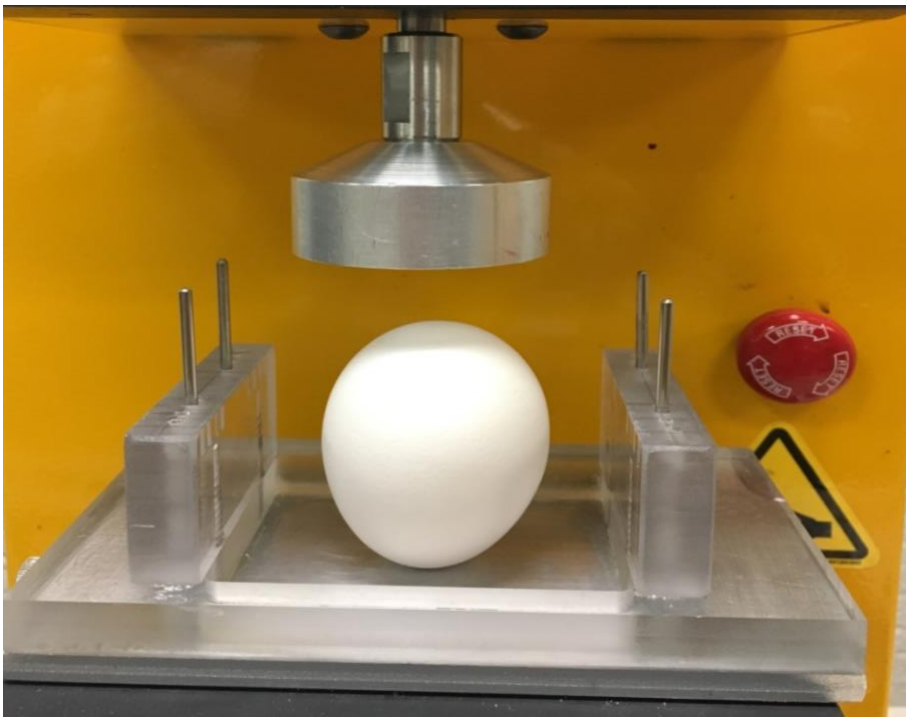

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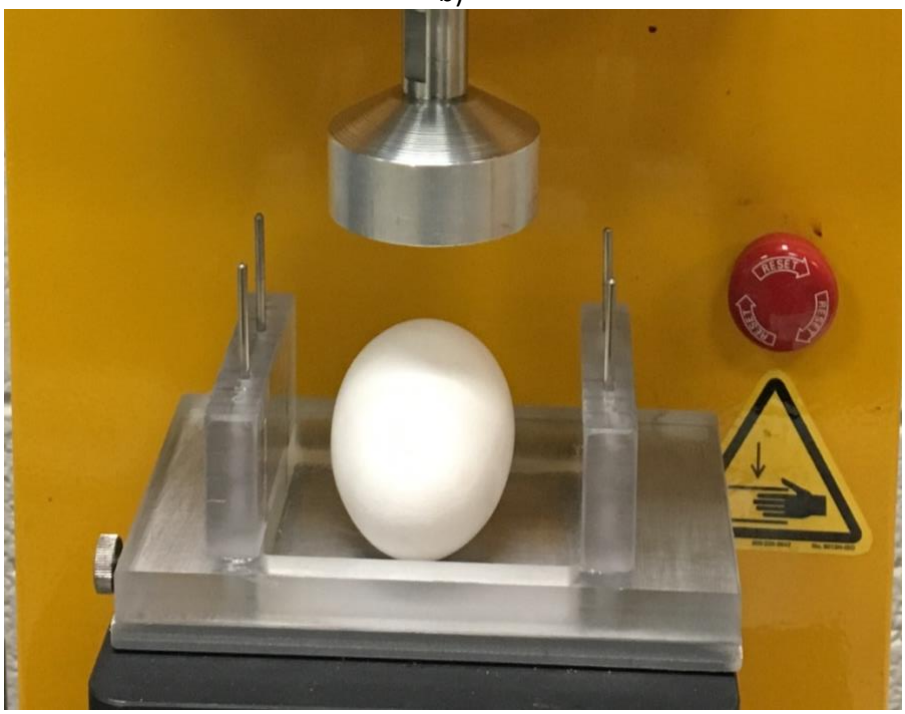

c)

Figure 27: Peak force of eggs tested in 3 positions a) Small end up (SEU), b) Small end down (SED), and c) Equatorial (EQ)

Egg shell strength was seen to be similar across each position between all treatments used in this study (Fig. 28). Peak force needed to crack eggs at the SEU position was not significantly different between eggs treated with PAW, DW, QA, and, unsanitized eggs. A similar trend was noted in the SED position used for shell strength tests, such that the average peak strength needed to crack eggs treated with PAW was 50.35±7.02 N, for DW treated eggs was 45.62±3.76 N, QA sanitized eggs was 48.48±10.34 N and unsanitized egg (control) was 45.89±20.89 N. As shown in Table 3, eggs in equatorial position had a significantly lower tolerance to external force, but this low tolerance was mirrored across all treatments. Eggs in the SED and SEU positions also had significantly different peak forces, such that eggs at SEU position depicted higher egg shell strength.

Table 3: Values of peak force (N) (± standard deviation) at SEU, SED, and EQ position

	SEU (Peak force in N)	SED (Peak force in N)	EQ (Peak force in N)
Unsanitized	74.52±9.11[a]	45.88±20.58[b]	42.35±5.84[c]
QA	54.05±27.02[a]	48.48±10.34[b]	40.20±11.86[c]
DW	64.57±5.10[a]	45.62±3.76[b]	35.34±16.57[c]
PAW	57.27±22.23[a]	50.35±7.02[b]	38.93±13.53[c]

Instron Universal Testing machine, with 2 flat plates as probes had been a popular choice to measure egg shell strength in the past. Bialka et al. (2004) studied eggs shell strength of eggs treated with electrolyzed oxidized (EO) water against detergent sanitizer. This study was conducted with eggs placed in polar and equatorial positions, similar to our study. It was noticed that although eggs washed

in EO water had a lower shell strength in comparison to industrially used detergents, this difference was not statistically significant. These results seem consistent with the results obtained for PAW in our study.

Allo-Kramer shear press (Kennesaw, GA, USA), which works on the principle of determining peak force needed to crack egg shells, similar to the texture analyzer used in this study, has also been used in past studies to detect shell strength of eggs prior to and post washing, and, oiling (Ball et al., 1975). This study revealed that egg washed with industrial egg washing equipment (Model super 80, Kuhl International Corp.), had a significantly lower egg shell strength in both polar (with the small end down) and equatorial positions. This might be due to the fact that washing with industrial detergents may cause depreciation of cuticle layer, which might contribute to lowering of egg shell strength (Tyler and Geake, 1964; Ball et al, 1975).

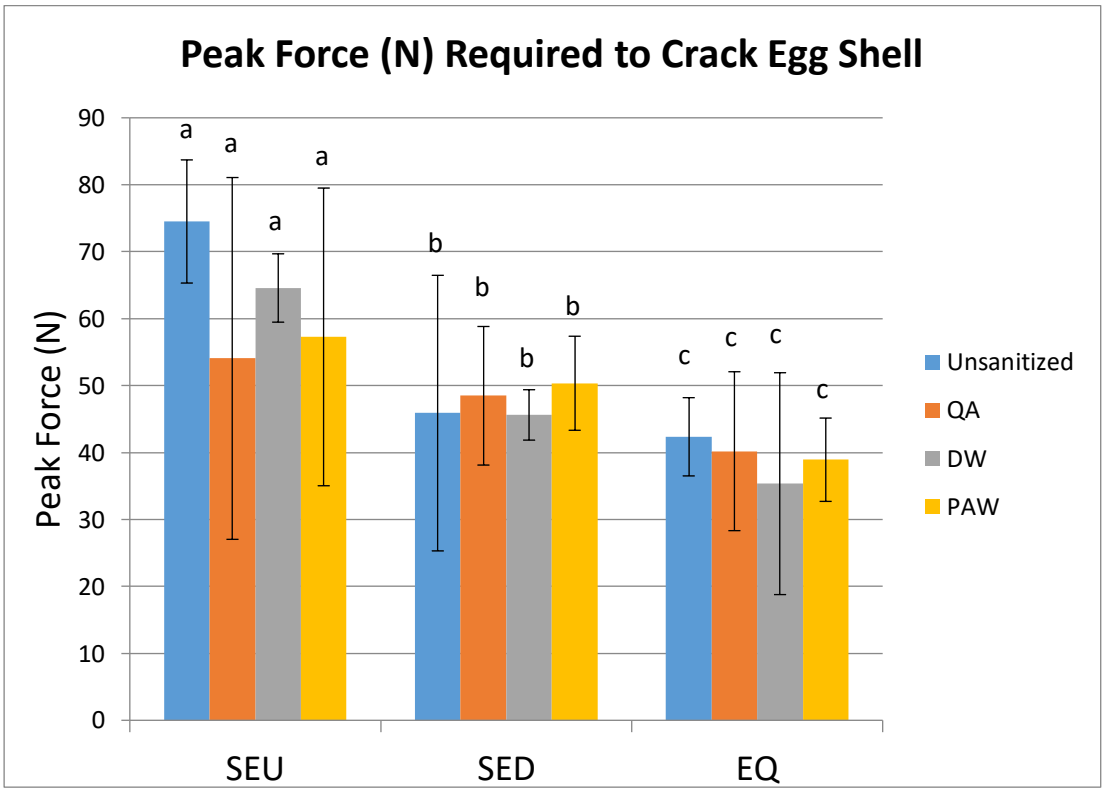

Figure 28: Peak force (N) required to crack shell eggs after treatment with sanitizers, at 3 positions – Small end up, Small end down and Equatorial. (Error bars indicate standard deviation; Data that does not share same letter, are significantly different from each other (One-way ANOVA, $p<0.05$)).

4. CONCLUSIONS

This study showed that PAW possessed comparable anti-microbial efficacy to industrially used sanitizer, QA, under rigorous treatment conditions (bacterial survival was below detection limit of 2.30 log CFU/egg). PAW was also comparable to QA in wash water microbial analysis, in that the microbial enumeration amounted to values below detection limit of 0.7 log CFU/ml. This showed that PAW too, like QA, was capable of microbial inactivation and could prevent cross-contamination. Additionally, PAW was comparatively more sensitive to the presence of cuticle on eggs, than QA. QA (ΔE* value of 22.66) was observed to erode the cuticle layer, while similar treatment with PAW (ΔE* value of 4.72) showed that the cuticle coverage was comparable to unsanitized eggs (ΔE* value of 0). This was confirmed through both colorimetric and microscopic analysis.

Texture analysis was performed to ensure that shell strength of eggs remained uncompromised. PAW (57.28 N at SEU position) washed eggs proved to have similar egg shell strength as QA (54.06 N at SEU position) washed eggs. This further proved that PAW was comparable or in some cases better in performance as a sanitizer in egg washing. Thus, PAW showed promise to be adopted by the egg washing industry.

This study suggested that PAW could be used as a viable option in the egg washing industry, as a replacement to quaternary ammonia. Microbiological analysis in both planktonic and egg system showed that PAW provided comparable reduction to QA, of *E. aerogenes*. However, a more industrial adept egg washing machine would provide better clarity in terms of scale-up of the process to the industrial scale.

5. FUTURE WORK

This study was performed utilizing a fresh batch of PAW at each step, but it would be interesting to observe whether PAW could be re-used with comparable efficacy. This could be achieved by studying efficacy of ions in PAW with the help of a fluorescent spectroscopy.

The process of egg washing could be matched up with industrial standards, or scaled-up to a small-farm based egg washing devise, to note whether PAW would compare in efficacy to chemical sanitizers currently used. This could be initiated with the use of a lab-scale, industrial grade egg washing equipment. This would enable analysis of PAW by industrially approved treatment methods, and would help better compare PAW to industrially used sanitizers, like QA.

The amount of PAW used per cycle could also be optimized in the future. Larger batches of PAW could be generated and tested for pH and ORP as indicators for efficacy, along with microbial inactivation studies in planktonic and egg surface systems.

Lastly, PAW could be tested upon eggs artificially contaminated with pathogens like *Salmonella* Enteritidis. Since, this study has shown promise for microbial reduction by treatment with PAW, on egg surfaces, eggs inoculated with pathogenic organisms could be tested under similar as well as industrial washing conditions to analyze microbial inactivation efficacy.

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